METHODS IN MOLECULAR MEDICINE™

Pediatric Hematology

Methods and Protocols

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HUMANA PRESS

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Molecular Diagnosis of Fanconi Anemia and Dyskeratosis Congenita

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1. Introduction

The inherited bone marrow (BM) failure syndromes Fanconi anemia ***(1)*** and dyskeratosis congenita ***(2)*** are genetic disorders in which patients develop BM failure at a high frequency, usually in association with a number of somatic abnormalities. They are the best characterized and the most common of this group of disorders.

Fanconi anemia (FA) is an autosomal recessive disorder in which progressive BM failure occurs in the majority of patients and in which there is an increased predisposition to malignancy, particularly acute myeloid leukemia. Although many FA patients will have associated somatic abnormalities, approx 30% will not. This makes diagnosis based on clinical criteria alone difficult and unreliable. FA cells characteristically show an abnormally high frequency of spontaneous chromosomal breakage and hypersensitivity to the clastogenic effect of DNA crosslinking agents such as diepoxybutane (DEB) and mitomycin C (MMC). This property of the FA cell has been exploited in the "DEB/MMC stress test" for FA and has been critical in defining the FA complementation groups/subtypes (FA-A, FA-B, FA-C, FA-D1, FA-D2, FA-E, FA-F, and FA-G) and in identification of the FA genes (*FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*) ***(3–9)***. The DEB/MMC test remains the front-line diagnostic test for FA. However, the DEB/MMC test is not able to distinguish FA carriers from normals, antenatal diagnoses based on this are possible only later in pregnancy, and it is unable to classify a patient into an FA subgroup (complementation subtype). Because of these limitations there are circumstances when a molecular diagnosis is desirable. Furthermore molecular analy-

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

Table 1
FA Complementation Groups/Subtypes

Complementation group/subtype	Percentage incidence[a]	Chromosomal location	Size of protein product (kDa)	Mutations identified
A	65–75	16q24.3	163	> 100
B	<1	?	?	?
C	5–10	9q22.3	63	10
D1	<1	?	?	?
D2	<1	3p25.3	155/166	5
E	<5	6p21.3	?	3
F	<5	11p15	42	6
G	10–15	9p13	68	18

[a]The approximate percentage incidences of the different subgroups refer to the EUFAR (European Fanconi Anemia Registry) data.

sis is essential if a FA patient is to be entered into the experimental gene therapy protocols for FA-A and FA-C subtypes, and genotype–phenotype correlations of prognostic significance are emerging. As can be seen from **Table 1**, the six FA genes identified to date collectively represent >90% of FA patients, with FA-A subtype accounting for approx 70% of FA patients. However, several different mutations have been identified in each different FA gene, with more than 100 mutations in the *FANCA* gene alone. This means that molecular diagnosis for FA is very complex.

Given the number of genes mutated in FA, the choice of which gene to begin screening for mutations is obviously critical. In the absence of any information from techniques such as cell fusion or retroviral transduction experiments, or geographical clustering of a particular complementation group, statistically there is a approx 70% chance that the patient carries mutations in *FANCA*. For this reason we present a quantitative fluorescent multiplex genomic polymerase chain reaction (PCR) technique that was shown to detect a high frequency of *FANCA* mutations in a previous study ***(10)***. Another technique (solid-phase fluorescent chemical cleavage of mismatch [FCCM]) formed the balance of our *FANCA* screening, but lack of space prevents its detailed description here. The multiplex PCR technique detects but does not delimit deletions in *FANCA*, which account for a high proportion (40%) of mutations in FA-A patients who are largely compound heterozygotes. Small deletions of less than a whole exon or point mutations were detected with FCCM from reverse transcriptase-PCR (RT-PCR) generated products. Consanguinity in the kindred (and hence pre-

dicted homozygosity) suggests caution when using single techniques for *FANCA* mutation screening, owing to the risk of missing mutations of one type or the other. Used together, we found that the two techniques missed only 17% of *FANCA* mutations.

The multiplex PCR technique is adaptable for other genes in which deletions are present in either a homozygous or heterozygous state, with the simple selection of primer sets that amplify exons known to be deleted in the pathology of the disease. For *FANCA* screening we utilized the fifth and sixth exons of *FANCC,* not known to be deleted in FA-C patients ***(11–12)***, or alternatively exon 1 of myelin protein zero. Use of genomic DNA in short PCRs allows comparison of the intensity of fluorescence contributed by each exon relative to a known diploid exon, as the reactions are still stoichiometric in the early (pre-plateau) phase of the PCR ***(13)***. Fluorescence intensity measurement and size discrimination (for small deletions within an exon) are achieved by the use of fluorescently labeled primers and an ABI 373 DNA sequencer.

Dyskeratosis congenita (DC) is an inherited disorder characterized by the triad of abnormal skin pigmentation, nail dystrophy, and mucosal leucoplakia. Since its first description by Zinsser in 1906 it has become recognized that, as in FA, the clinical phenotype is highly variable, with a variety of noncutaneous (dental, gastrointestinal, genitourinary, neurological, ophthalmic, pulmonary, and skeletal) abnormalities having been observed. X-linked recessive, autosomal dominant, and autosomal recessive forms of the disease are recognized. In the DC registry at the Hammersmith Hospital there are 154 families (comprising 199 males and 56 females) from 33 countries. The clinical phenotype is highly variable both in the age at onset and severity of a particular abnormality and in the combination of such abnormalities in a given patient. This makes diagnosis based on clinical criteria alone difficult and unreliable particularly where non-cutaneous abnormalities (such as hematological abnormalities) precede the classical diagnostic features. A laboratory diagnostic test was therefore very desirable. Unlike the situation for FA, there is no reliable functional phenotypic test for DC. However, the identification of the *DKC1* gene ***(14)*** (which is mutated in X-linked DC) and the *hTR* gene ***(15)*** (mutated in autosomal dominant DC) now makes it possible to undertake molecular analysis in a large subset of DC families. The data from the DCR shows that approx 40–50% of the DC patients have mutations in *DKC1*, and approx <10% of the families have mutations in *hTR* (**Table 2**). This means that for the present it is not possible to substantiate a molecular diagnosis in approx 40–50% of DC patients and highlights the need to identify other DC causing genes. As for FA, once a mutation has been identified, as well as confirming the diagnosis in the

Table 2
DC Subtypes

DC subtype	Percentage incidence[a]	Chromosome location	RNA/protein product	Mutations identified
X-linked recessive	40–50	Xq28	Dyskerin	>25
Autosomal dominant	<10	3q21–3q28	*hTR*	5
Autosomal recessive	40–50	?	?	?

[a]The approximate percentage incidences of the different subtypes are based on the Dyskeratosis Congenita Registry (DCR) at the Hammersmith Hospital.

patient, it is possible to offer carrier detection and antenatal diagnosis in at risk families.

The *DKC1* mutations are almost always missense mutations, and the preferred strategy for their identification has been single-strand conformational polymorphism (SSCP) analysis ***(16)***. The procedure is detailed in this chapter. More recently we have been screening for point mutation on the Transgenomic Wave DNA fragment analysis system. In this procedure, the patient PCR products are mixed with equivalent wild-type products, denatured, and cooled slowly to allow for possible heteroduplex formation and then analysed by reverse-phase ion-pair high-performance liquid chromatography (HPLC). When this procedure is carried out at a partially denaturing temperature, heteroduplex DNA elutes from the column earlier in a gradient of acetonitrile than the fully base-paired homoduplex DNA. Peaks of DNA elution are recorded and disturbances to the highly reproducible normal pattern obtained are indicative of the presence of mutation. This method is simple in that it does not require the use of radiolabeling and gel electrophoresis and may become a more widespread as the equipment becomes available.

2. Materials

2.1. Genomic DNA Purification

1. 1X SET buffer: 10 m*M* Tris-HCl, pH 7.5, 10 m*M* NaCl, 1 m*M* EDTA.
2. 10% (w/v) sodium dodecyl sulfate (SDS).
3. 10 mg/mL of proteinase K in water.
4. 6 *M* NaCl.
5. Isoamyl alcohol:chloroform 1:24.
6. Cold (–20°C) absolute ethanol.
7. TE: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA.

2.2. PCR

1. *Taq* polymerase and oligonucleotide primers: These can be purchased from a variety of different companies. The oligos are usually 18–22 bases in length. For the FA multiplex PCRs the forward primer for each exon must be fluorescently labeled.
2. PCR buffers: These are usually supplied along with the *Taq* polymerase. For FA multiplex PCR the buffer composition is 67 m*M* Tris-HCl, pH 8.8, 16.6 m*M* $(NH_4)_2SO_4$, 1.5 m*M* $MgCl_2$, 0.17 mg/mL of bovine serum albumin (BSA). For the *DKC1* and *hTR* genes the 10X buffer (from Advanced Biotechnologies) is: 750 m*M* Tris-HCl, pH 8.8, 200 m*M* $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20. A solution of 25 m*M* $MgCl_2$ is also provided and added separately to the PCR reaction.
3. 2 m*M* and 10 m*M* dNTP.
4. Dimethyl sulfoxide (DMSO).

2.3. Multiplex Electrophoresis and Fluorescent Detection

1. 5% Denaturing polyacrylamide gel (poured according to recommendations for use with ABI Genescan).
2. 10X TBE running buffer (*see* **Subheading 2.4.**).
3. Formamide loading buffer: 95% formamide in 1X TBE with 5 mg/mL of dextran blue.
4. Internal size standard: Genescan-500 ROX (PE Biosystems).
5. PCR machine.
6. ABI 373 DNA sequencer with workstation running Genescan and Genotyper software.

2.4. SSCP Gel Electrophoresis

1. A vertical gel electrophoresis tank with appropriate plates, clips, combs, and spacers.
2. A slab gel dryer with vacuum pump.
3. 10X Tris borate EDTA (TBE) buffer : Add 216 g of Trizma base, 18.6 g of EDTA, and 110 g of orthoboric acid to 1600 mL of water, dissolve and top up to 2 L; dilute 1:10 for use as 1X TBE buffer.
4. Routine SSCP gel mix: For an 80-mL gel, take 53.6 mL of H_2O, 8 mL 10X TBE, 4 mL of glycerol, 12 mL 40% (w/v) acrylamide solution (**Caution:** acrylamide is a potent neurotoxin), and 2.4 mL of 2% (w/v) *bis*-acrylamide solution.
5. 10% (w/v) ammonium persulfate. This reagent is not stable at room temperature. It can be kept for only a few weeks on the bench, and should be stored at –20°C.
6. TEMED: *N*,*N*,*N*',*N*'-Tetramethylethylenediamine.
7. Formamide dye: to 10 mL of deionized formamide, add 10 mg of xylene cyanol FF, 10 mg of bromophenol blue, and 200 µL of 0.5 mol/L of EDTA.

3. Methods

3.1. DNA Preparation

Prepare genomic DNA from lymphoblastoid cell lines (*see* **Note 1**) by salt–chloroform extraction, essentially as described elsewhere ***(17)***: In brief:

1. Resuspend the cell pellet in 4.5 mL of 1X SET.
2. Add 250 µL of 10% SDS and 100 µL of 10 mg/mL of proteinase K, mix, and leave at 37°C overnight.
3. If clear, proceed. If not, add a further 100 µL of proteinase K and continue incubation for 2–3 h
4. Add prewarmed (37°C) 6 *M* NaCl to a final concentration of 1.5 *M* (i.e., for 4.5 mL, add 1.5 mL 6 *M* of NaCl).
5. Add an equal volume of isoamyl alcohol–chloroform, and place on a rolling mixer for 30–60 min.
6. Centrifuge at 2000 rpm for 10 min at room temperature
7. Remove the upper aqueous layer and add two volumes of cold absolute ethanol. Mix by inversion two or three times. Place at –20°C for 1 h or longer.
8. Centrifuge at 2000 rpm for 10 min at 4°C. Remove the supernatant and wash the pellet twice with 70% ethanol.
9. Briefly air-dry pellet and resuspend in TE (*see* **Note 2**).

3.2. Fluorescent Multiplex PCR for the FANCA Gene

1. Incubate DNA samples at 55°C for 1 h to redissolve fully the DNA (allowing accurate measurement of DNA concentration). Take an aliquot of this sample to determine the concentration by A_{260} measurement, and dilute the remainder in TE to 25 ng/µL for the PCR.
2. Set up the multiplex PCRs as required. Include four control DNAs from normal individuals for use in the later data analysis. The primer sets, and the exons amplified by them, are shown in **Table 3**. All forward primers must be labeled with either the fluorescent phosphoramidite 6' carboxyfluoroscein (6-FAM) or 4,7,2',4',5',7',-hexachloro-6-carboxyfluoroscein (HEX) dyes (PE Biosystems). PCR amplifications are performed in 25-mL reactions with 125 ng of DNA, 1X *Taq* DNA polymerase buffer, and a 200 µ*M* concentration of each dNTP. Of each of the primer pairs, 0.2 µ*M* worked well for each of the multiplexes, apart from 0.4 µ*M* for *FANCA* exons 5, 11, 12, and 31.

After an initial denaturation at 94°C for 3 min, "hot-start" the reaction with the addition of 1.5 U of *Taq* DNA polymerase (Promega), and perform 18 PCR cycles of: 93°C for 1 min, annealing for 1 min at either 60°C (multiplexes 1, 3, and 4) or 58°C (multiplex 2), and extension for 2 min at 72°C, followed by a final extension for 5 min at 72°C at the end of the 18 cycles.

3.3. ABI Gel Electrophoresis and Data Analysis

1. Add an aliquot of the PCR product (4 µL) to 3.5 mL of formamide loading buffer (95% formamide in 1X TBE and 5 mg of dextran blue/mL) and 0.5 of mL internal lane size standard (Genescan-500 Rox; PE Biosystems).
2. Denature the samples for 5 min at 94°C and electrophorese on a 5% denaturing polyacrylamide gel at 45 W for 6 h on an ABI 373 fluorescent DNA sequencer (according to the manufacturer's instructions, omitted here for economy of space). Up to 24 samples can be run on each gel, remembering to include four control DNAs known to be undeleted in any of the exons under test.
3. Data are analyzed by means of Genescan and Genotyper software, to obtain electrophoretograms for each sample. The position of the peaks indicates the size (in basepairs) of the exons amplified, and the areas under the peaks indicate the amount of fluorescence from the product.
4. The copy number of each exon amplified is established by importing the peak area values into an Excel spreadsheet and calculating a dosage quotient for each exon relative to all the other amplified exons in patients and controls (for an example *see* **Table 4**).

Choose peak areas from the best two control samples (with approximately equal values), and calculate dosage quotient values from them as below; values are typically within the range 0.77–1.25 ***(13)*** (*see* **Note 3**).

Essentially the calculation takes the average peak area of an exon from these controls and compares this with the peak area of the same exon from the patient samples. As an example (*see* **Table 4**), in patient X the dosage quotient for *FANCA* exon 10 and *FANCC* exon 5 is given by DQ *FANCA* exon 10/*FANCC* exon 5 and is calculated by:

[sample *FANCA* exon 10 peak area/sample *FANCC* exon 5 peak area] / [control *FANCA* exon 10 peak area/control *FANCC* exon 5 peak area] = [1857/5301]/[6034/8180] = 0.47.

The threshold for classification as heterozygous for an exon of interest is generally a DQ < 0.77. Good quality data are often significantly closer to 0.5 for heterozygously deleted, and 1.0 for homozygously intact (*see* **Table 4** for sample data). Clearly, homozygous deletions are easily confirmed by conventional PCR.

The results for each patient are generally collated and examined periodically to determine the next step of the investigation. It is also wise to take an overview of detection rates for each multiplex to determine whether a simpler, achievable multiplex reaction could expedite rapid screening of a large number of samples (*see* **Notes 4** and **5**).

Table 3
PCR Primer Sets for Multiplex Dosage Assays of the FA Genes

Exon	Primers	Size (bp)
Multiplex 1		
FANCA		
10	Forward, GAT TGT AGA AGT CTT GAT GGA TGT G Reverse, ATT TGG CAG ACA CCT CCC TGC TGC	259
11	Forward, GAT GAG CCT GAG CCA CAG TTT GTG Reverse, AGA ATT CCT GGC ATC TCC AGT CAG	301
12	Forward, CCA CAA CTT TTT GAT CTC TGA CTT G Reverse, GTG CCG TCC ACG GCA GGC AGC ATG	224
31	Forward, CAC ACT GTC AGA GAA GCA CAG CCA Reverse, CAC GCG GCT TAA ATG AAG TGA ATG C	205
32	Forward, CTT GCC CTG TCC ACT GTG GAG TCC Reverse, CTC ACT ACA AAG AAC CTC TAG GAC	369
FANCC		
Exon 5	Forward, CTG ATG TAA TCC TGT TTG CAG CGT G Reverse, TCC TCT CAT AAC CAA ACT GAT ACA	186
Exon 6	Forward, GTC CTT AAT TAT GCA TGG CTC TTA G Reverse, CCA ACA CAC CAC AGC CTT CTA AG	293
Multiplex 2		
FANCA		
Exon 5	Forward, ACC TGC CCG TTG TTA CTT TTA Reverse, AGA ACA TTG CCT GGA ACA CTG	250
Exon 17	Forward, CCC TCC ATG CCC ACT CCT CAC ACC Reverse, AAA AGA AAC TGG ACC TTT GCA T	207
Exon 35	Forward, GAT CCT CCT GTC AGC TTC CTG TGA G Reverse, GCA TTT TCC CTG AGA TGG TAA CAC C	315
Exon 43	Forward, GCC TGG CTG GCA ATA CAA CTC GAC Reverse, GGC AGG TCC CGT CAG AAG AGA TGA G	223

FANCC		
Exon 5	Forward, CTG ATG TAA TCC TGT TTG CAG CGT G Reverse, TCC TCT CAT AAC CAA ACT GAT ACA	186
Exon 6	Forward, GTC CTT AAT TAT GCA TGG CTC TTA G Reverse, CCA ACA CAC CAC AGC CTT CTA AG	293
Multiplex 3		
FANCA		
Exon 21	Forward, CAG GCT CAT ACT GTA CAC AG Reverse, CAC CGG CTT GAG CTG GCA CAG	335
Exon 27	Forward, CAG GCC ATC CAG TTC GGA ATG Reverse, CCT TCC GGT CCG AAA GCT GC	285
FANCC		
Exon 5	Forward, CTG ATG TAA TCC TGT TTG CAG CGT G Reverse, TCC TCT CAT AAC CAA ACT GAT ACA	186
Exon 6	Forward, GTC CTT AAT TAT GCA TGG CTC TTA G Reverse, CCA ACA CAC CAC AGC CTT CTA AG	293
Multiplex 4		
FANCA		
Exon 5	Forward, ACC TGC CCG TTG TTA CTT TTA Reverse, AGA ACA TTG CCT GGA ACA CTG	250
Exon 11	Forward, GAT GAG CCT GAG CCA CAG TTT GTG Reverse, AGA ATT CCT GGC ATC TCC AGT CAG	301
Exon 17	Forward, CCC TCC ATG CCC ACT CCT CAC ACC Reverse, AAA AGA AAC TGG ACC TTT GCA T	207
Exon 21	Forward, CAG GCT CAT ACT GTA CAC AG Reverse, CAC CGG CTT GAG CTG GCA CAG	335
Exon 31	Forward, CAC ACT GTC AGA GAA GCA CAG CCA Reverse, CCC AAA GTT CTG GGA TTA CAG GCG TG	308
Myelin protein zero		
Exon 1	Forward, CAG TGG ACA CAA AGC CCT CTG TGT A Reverse, GAC ACC TGA GTC CCA AGA CTC CCA G	389

Table 4
Statistical Profile of *FANCA* Dosage Multiplex 1

Exon	Peak area in		Dosage Quotient in	
	Control	Patient X[a]	*FANCC* Exon 5	*FANCC* Exon 6
Control				
FANCC exon 5	8180	5301	—	0.99
FANCC exon 6	4490	2949	1.01	—
Test				
FANCA exon 10	6034	1857	0.47	0.47
FANCA exon 11	16967	5392	0.49	0.48
FANCA exon 12	9068	3256	0.55	0.55
FANCA exon 31	5466	3110	0.88	0.87
FANCA exon 32	5838	3887	1.03	1.01

[a]Heterozygous for a deletion of exons 10–12.

3.4. PCR Amplification of the DKC1 *and* hTR *Genes*

Oligonucleotide sequences and annealing temperatures for the PCR amplification of the 15 exons of the *DKC1* gene and the *hTR* gene are given in **Table 5**. The standard composition of the PCR mix for varying numbers of 25 µL reactions are given in **Table 6**. This composition works for all primers in **Table 5** except for the *hTR* reaction, to which 10% DMSO must be added and the volume of H_2O reduced accordingly. Cycling conditions used are 95°C for 5 min, followed by 30 cycles of 58°C for 45 s, 72°C for 1 min, and 94°C for 45 s, followed by 58°C for 45 s and final extension at 72°C for 5 min.

Restriction enzyme digestion of the PCR products can be performed as detailed in **Table 5**. These are performed overnight at the appropriate temperature using the buffers supplied with the enzymes.

3.5. SSCP Gel Electrophoresis

The following procedure describes the preparation of a large, thin (34 cm × 40 × 0.4 mm) 6% nondenaturing polyacrylamide gel.

1. Clean the glass plates thoroughly with detergent and a scourer. Rinse well and dry. Swab the larger plate with 100% ethanol. Treat one surface of the smaller plate with a siliconizing solution or a nontoxic gel coating solution (e.g., Gel Slick from FMC), by applying a small amount, a few milliliters, and buffing dry with a paper towel. Assemble the gel using spacers, bulldog clips, and electrical

Table 5
Primer Pairs used in the Amplification of the *DKC1* Exons and the *hTR* Gene

Oligo	Sequence	Exon	Temperature	Size	RE enzyme	Cut sizes(bp)
PH7	CCGAGCCAGCAAATCGCATT	1	60	318	*Sty*I	189, 145
Ex1R	CGGGAACCAGAGGGAGGCGTG					
AAF1	AATCCATTTCCTACCTGCCC	2	60	159		
AAR1	CAATGCTGGCCCATTCCTTG					
BBF1	AAAGGCATACATTTCCATGG	3	58	268	*Hin*fI	147, 121
BBR1	CAAGGATGCCAGCAGTAAG					
CCF1	GCCACATAGTGGTACTGACTC	4	56	243	*Mbo*II	146, 97
CCR2	CCTGAATAGCTGATGTGAAAG					
DDF1	GATTTGTTGTTTCACTGGAGC	5	58	288	*Bam*HI	174, 114
DDR1	TTCACTCTAGCCAGTCCTTC					
EEF1	GGAGTGACTGAGCATATAAG	6	58	219	*Hpa*II	117, 102
EER1	AACCCATCTCCAGATGTTTAG					
FFF1	GCTGCAGCCAGCCTGGACC	7	60	293	*Pst*I	167, 125
FFR1	AGTCTTCAACTTCAAGGGCATC					
GGF1	ATAACTGCATTTCTCAACC	8	60	277	*Sfa*NI	157, 120
GGR1	AAGCAAGTGGAGTGCCATC					
HHF1	GGTCTGATGGGCTGAGATAC	9	60	264	*Fok*I	145, 119
HHR1	GAGCAAGCGTCATCTTTGGAG					
IIF1	CACTCCCTTGTTGTCCTCC	10	56	271	*Taq*I	136, 135
IIR1	TATATACACCTAGTATGTAACC					
VVF1	TAAAGTGGCATACAACAGTAG	11	58	242	*Nco*I	131, 111
VVR1	ACCTGGCAGGGCACGCAAC					
SSF1	ATTCTTTGTAGTCACCATGCC	12	58	227	*Hae*III	124, 103
SSR1	AGCAAGTGTGCCGTCTCTACC					
TTF1	CTACATAACATCAGTACTGCC	13	56	220	*Bsp*MI	114, 106
TTR1	TAAGACGAATGCCAGTGCC					
XXF1	TACCTTTTGACTCACTGAACC	14	56	288	*Bcl*I	156, 132
XXR1	GGTACCACCTGGGTAATTC					
WWF1	GAACTTTGTGTCACATGCAGC	15	56	278	*Fok*I	160, 118
NAP3R	AACATGTTTTCTCAATAAGGC					
*hTR*F	TCATGGCCGGAAATGGAACT		58	653	*Bst*NI	229, 183, 167, 74
*hTR*R	GGGTGACGGATGCGCACGAT					

tape around the bottom of the gel. Ensure that the gaskets closely abut the smaller plate (*see* **Note 6**)

2. Take 80 mL of the SSCP gel mix. Add 560 µL of 10% ammonium persulfate and 28 µL of TEMED, mix, and pour the solution slowly between the glass plates using a 50-mL syringe (*see* **Note 7**). When full, insert an inverted sharks tooth comb (smooth surface downward) no more than 6 mm into the gel. Leave to polymerize.
3. Remove the electrical tape and bulldog clips and place the gel in the electrophoresis tank. Fill the top and bottom chambers with 1X TBE. Remove the comb

Table 6
PCR Reagent Mixes Used in Amplification of *DKC1* Exons

Reagent	Volume of reagent (μL) per number of reactions (*N*)				
	N = 6	*N* = 8	*N* = 10	*N* = 12	*N* = 16
10X PCR buffer	15	20	25	30	40
25 m*M* $MgCl_2$	12	16	20	24	32
100 ng/μL of primer 1	6	8	10	12	16
100 ng/μL of primer 2	6	8	10	12	16
10 m*M* dNTP	4	6	8	8	12
dd H_2O	106	140	175	210	280
[α^{32}P]dCTP	0.5	0.75	1	1	1.5
5 U/μL of *Taq* polymerase	0.5	0.75	1	1	1.5

(*see* **Note 8**) and flush the surface of the gel with TBE buffer using a syringe and bent needle. Clean and invert the comb and insert it between the plates until the teeth just indent the surface of the gel.

4. Mix 1–4 μL of the radiolabeled PCR product with 6 μL of formamide dye. Heat at 95°C for 5 min. Snap chill on wet ice. Flush out each well using TBE buffer and load 5 μL of each sample between the teeth. Run the gel overnight at 8–12 mA in a cool laboratory (*see* **Note 9**). As a guide, the bromophenol blue and xylene cyanol will comigrate with approx 60 bp and 220 bp DNA fragments respectively in a 6% polyacrylamide gel. The single-stranded DNA fragments will migrate considerably slower.
5. Disconnect the power supply, and remove the plates and place them on a flat surface. Pull one of the spacers out from between the plates. Insert a metal spatula or a fine plastic wedge horizontally into the gap between the plates at the bottom corner where the spacer had been. Lift the smaller siliconized plate off the gel. Cut a piece of 3MM Whatman paper so that it is slightly larger than the gel area, and lay it down onto the gel. Return the smaller plate over the Whatman paper, apply gentle pressure, and invert the plates. Carefully pull up the larger plate, ensuring that the gel sticks to the Whatman paper. Cover the gel with cling film (e.g., Saran Wrap) and trim all the edges.
6. Dry the gel under vacuum at 80°C for approx 1 h. Peel off the Saran Wrap and expose the gel to X-ray film overnight at –80°C to obtain an autoradiograph.

A shift in migration of the single-stranded fragment is indicative of a mutation in the relevant exon. Variations in the gel composition can be introduced to increase the chances of observing aberrant mobilities (or shifts) of mutant DNA strands. These include altering the content of the gel such as the percent of glycerol, the percent of the acrylamide and the acrylamide/*bis*-acrylamide ratio used.

3.6. DNA Sequencing

1. Reamplify the appropriate exon using the conditions described in **Subheading 3.4.**, but scaling up the volume of the reaction to 100 µL and the cycle number to 35.
2. Load the entire product onto a 1.5% agarose gel; after sufficient electrophoresis, cut out the fragment and elute the DNA using a QIAquick Gel Extraction column.
3. Direct sequence analysis of the fragment is now performed by a specialized service.

4. Notes

1. Large-scale DNA extraction from peripheral blood may be performed using the Puregene DNA isolation kit from Gentra Systems, Minneapolis, MN, USA.
2. DNA quality is critical when performing these multiplex PCRs. If the DNA quality is poor, and cannot be improved by phenol–chloroform extraction, it may be necessary to use more template in the PCR, paying attention to **Note 3**.
3. When analyzing the data with Genotyper, the peak heights in the electropherograms should ideally be between 100 and 1000 units. If significantly greater than this, the PCR has probably advanced beyond the exponential phase, potentially skewing any analysis of these results.
4. After assessment of which exons were most frequently deleted from *FANCA*, multiplex 4 was developed, with exon 1 of the myelin protein zero gene as the external control. It is left to the reader to determine which multiplex is best suited to his or her needs, or to develop his or her own multiplex. Key in this development is the ability to demonstrate robust amplification of all exons of interest in a variety of samples, and for all amplified exons to be of different sizes to allow single-color detection during the gel run.
5. If the analysis shows a single exon deletion, this result must be repeated to confirm that this exon did not "drop out" (fail to amplify) during the PCR. If possible look for deletion of other exons upstream and downstream to delimit the extent of the deletion. A well-designed combination of multiplex PCRs will allow rapid scanning of the gene for large deletions, although it should be clearly noted that unless an exon has been examined directly, it may be deleted. Such deletions are present in *FANCA* in some patients, and may be missed in a scanning approach.
6. The interface between small plate and the gasket is the most likely location of a leak down from the upper chamber of the gel. When the gel is set and assembled, it is possible to dab a small amount of molten agarose into this junction to ensure a good seal.
7. The formation of bubbles as the gel is poured is a recurrent problem. Concentrate on ensuring a steady constant flow of the acrylamide solution between the plates. If they persist, hold the plates vertically and bang the bubbles to the surface. If the problem occurs repeatedly, soak the plates in 2 *M* NaOH for 1 h, and rinse well with water before reassembling.
8. The combs can sometimes be difficult to remove. Very carefully, insert a flat-edged razor blade between the comb and the plate and gently prise the comb away from the plate before trying to pull it out.

9. The migration of the single-stranded fragments varies considerably with the temperature of the gel. Our standard procedure is to air condition the lab at 18–20°C. Running the gel in a cold room (at 4°C) would represent a different set of conditions.

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2

Molecular Diagnosis of Diamond–Blackfan Anemia

Sarah Ball and Karen Orfali

Introduction

1.1. Clinical Features of Diamond–Blackfan Anemia (DBA)

Diamond–Blackfan anemia (DBA) is a rare congenital pure red cell aplasia, with an incidence of 4–7 per million live births ***(1–5)***. Typically, affected children present in the second or third month of life with profound anemia, often in association with craniofacial ***(6)*** or thumb anomalies ***(2,7)***, and small stature ***(2)***. In 15–20% there is a positive family history, characterized by an autosomal dominant pattern of inheritance ***(2–4)***. In the majority of cases, the anemia is responsive to steroids, but eventually up to 40% of affected individuals are dependent on a life-long transfusion program, unless they undergo successful stem cell transplantation ***(2,3,8)***. Spontaneous remission may occur, although this is unpredictable. In the longer term, there is an increased risk of myelodysplasia and myeloid leukemia ***(9)***, and probably also of other malignancies ***(10)***.

1.2. Hematological Parameters of DBA

The blood cell count at presentation is characterized by anemia and reticulocytopenia, with isolated marrow erythroid hypoplasia. The mean cell volume is usually within the normal range for infants, but is generally raised in children presenting at an older age ***(9)***. During steroid-induced or spontaneous remission, there is usually a persistent mild macrocytic anemia ***(2,11,12)***, often with raised fetal hemoglobin (HbF), and persistent strong expression of the blood group antigen i ***(12,13)***, which is usually only weakly expressed beyond infancy.

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

1.3. Differential Diagnosis of DBA: Potential Applications for a Molecular Diagnostic Approach

The differential diagnosis of red cell aplasia presenting in infancy is primarily between an early presentation of transient erythroblastopenia of childhood (TEC) ***(14–16)*** and chronic parvovirus infection ***(17–19)***. The diagnosis of an acquired immune-mediated pure red cell aplasia should also be considered in older children.

In individuals with "classical" DBA as outlined, the diagnosis may be unequivocal. However, for children with less typical features, especially those presenting at an older age, the diagnosis may be less clear cut. As chronic parvoviremia is associated with a failure to mount an appropriate immune response ***(17–19)***, there may not be serological evidence of parvovirus, and parvovirus infection should be excluded by direct detection of parvovirus DNA ***(20)***.

Misdiagnosing other causes of childhood red cell aplasia as DBA may not only result in inappropriate treatment, but can also induce unnecessary familial anxiety, as the diagnosis of DBA carries genetic and other longer term implications ***(9,10)***. A molecular diagnostic approach is therefore of potential value in the differential diagnosis of red cell aplasia in childhood. It also has other important applications; in genetic counseling, in the exclusion of subclinical DBA in sibling donors when planning stem cells transplantation (BMT) ***(21)***, and in the detailed characterization of probands and other family members for genetic linkage studies ***(13,22)***.

1.4. Erythrocyte Adenosine Deaminase Activity

Currently the most useful investigation in the molecular diagnosis of DBA is the measurement of erythrocyte adenosine deaminase (eADA) activity, which is raised in the great majority of patients with DBA ***(11–13,23–27)***. Its value as a diagnostic tool is limited in patients who are transfusion dependent. Although measurement as late as possible posttransfusion may reveal eADA activity above the normal range (*unpublished observations*), a normal level is uninterpretable. Cord blood eADA activity falls within the normal reference range ***(23,25,28)***, making eADA a potentially useful tool to screen for DBA in the newborn siblings of affected children.

1.4.1. Interpretation of Results: Differential Diagnosis of Raised eADA Activity

Raised eADA activity is not specific for DBA, although it is still useful in discriminating between DBA and TEC ***(25)***. It has also been described in paroxysmal nocturnal hemoglobinuria (PNH) ***(29)***, myelodysplastic syndromes

(MDS) ***(29,11,23)***, and Down's syndrome ***(30)***; interestingly, like DBA, all of these disorders are associated with macrocytosis.

1.4.2. eADA Measurement in Family Studies

Several studies have shown that eADA activity may also be raised in some first degree relatives of patients with DBA ***(13,23,26,27,31)***. In families with *RPS19* mutations (*see* below), raised eADA activity usually, but not always, cosegregates with the family *RPS19* mutation. Isolated raised eADA in first-degree relatives has also been demonstrated in families without *RPS19* mutations. Although 15–20% have an unequivocal family history of DBA ***(2–4)***, detailed family studies, including measurement of eADA activity, have revealed a wider range of phenotypic expression of DBA ***(13,27)***. The cosegregation of *RPS19* mutations with increased eADA activity ***(13)*** has led to the realization that family members with high eADA activity, with or without mild anemia or macrocytosis, should be considered as having a subclinical or silent form of DBA ***(13,27)***. It is often possible in such families to elicit a history of unexplained self-remitting anemia in early childhood or during pregnancy ***(13,23,27)***. However, the broadening of the accepted DBA phenotype to include isolated high eADA activity does challenge the accepted classical diagnostic criteria for DBA ***(32)***. The other conclusion to be drawn from these family studies is that fewer cases of DBA are truly sporadic than originally believed; the proportion of these is possibly as low as 50%.

1.4.3. Principle of Erythrocyte Adenosine Deaminase Assay

ADA catalyzes the hydrolytic deamination of adenosine to produce inosine and ammonia. The method described here is a continuous spectrophotometric assay, based on the difference in the extinction coefficients of adenosine and inosine at 265 nm ***(33,34)***. It is convenient, inexpensive, and entails no exposure to radioactivity. However, it must be stressed that it is not designed for the diagnosis of ADA deficiency. Radioisotopic ***(28)*** or radioimmunoassay ***(35)*** methods are also available, while coupled enzyme assays can be used to measure the production of ammonia ***(36)***, or the conversion of inosine to xanthine and uric acid ***(37)***.

1.5. Genetics of DBA: Direct Detection of Mutation

1.5.1. Gene Encoding Small Ribosomal Protein 19 (RPS19)

Most patients with DBA have normal cytogenetics, but the serendipitous finding of a child with sporadic DBA and a balanced chromosomal translocation t(X;19) ***(38)*** led to the identification of *RPS19* as the first "DBA gene" ***(39)***. Twenty-five percent of individuals with DBA have been found to have

mutations affecting a single allele of *RPS19* ***(40)***, consistent with the observed autosomal dominant pattern of inheritance. Direct detection of a mutation affecting *RPS19* thus leads to a definitive diagnosis of DBA, although the wide variation in phenotype observed between individuals with identical mutations, even within the same family ***(41,42)***, should lead to caution in genetic counseling. There may also be difficulty in differentiating between polymorphisms and functionally significant mutations affecting noncoding sequences, especially if the control population and the family with the putative mutation are from divergent ethnic backgrounds. The method described here is that of direct sequencing of PCR products to include all coding regions, intron–exon boundaries, and upstream noncoding regions ***(39–41,43)***. This approach may of course miss total allele loss or large deletions, which might be detected by Southern blot or loss of heterozygosity of linked polymorphisms. In the original 19q13 linkage study ***(44)***, one sporadic case (of 13 studied) had evidence of loss of heterozygosity of 19q13 markers, the result of a large deletion that included one *RPS19* allele. Large deletions encompassing *RPS19* may be associated with more severe skeletal anomalies and mental retardation, suggesting a contiguous gene syndrome ***(45)***. However, such total allele deletions do not appear to be common, making it unlikely that many *RPS19* deletions will be missed by using polymerase chain reaction (PCR) and direct sequencing. In addition, the demonstration of heterozygosity for intragenic polymorphisms rules out total loss of one allele, while the clear lack of cosegregation between DBA and 19q in the majority of families ***(22,46)***, despite the high logarithm of odds (LOD) score for 19q13 in the original linkage study ***(44)***, is consistent with *RPS19* mutations accounting for only 25% of cases ***(40)***.

1.5.2. Other DBA Genes

Significant linkage has been established to chromosome 8p in 47% of cases of familial DBA, but the gene responsible has not yet been identified ***(22)***. In addition, the observed failure of linkage to both 19q and 8p in up to 20% of families with a clear pattern of inheritance demonstrates the existence of at least a third DBA gene ***(22)***. Thus there is currently no way to diagnose DBA at the genetic level in the 75% of patients without an *RPS19* mutation.

2. Materials

2.1. Erythrocyte Adenosine Deaminase Assay

*2.1.1. Preparation of Lysate (*see ***Notes 1*** *and* ***2****)*

1. Blood (in EDTA).
2. Normal saline: 0.9% NaCl in water.
3. Lysate stabilizer ***(47)***: 2.7 m*M* EDTA, pH 7.0, 0.7 m*M* β-mercaptoethanol. Store at 4°C.

Table 1
Primers for RPS19 PCR

Set	Amplifies	PCR product	Primers	T_m°C (%GC)
1	5' UTR/exon 1	722 bp	F 5'-TCCCCTCCTCCAGCGCCTCA-3'	54.9
			R 5'-GCACGCGCTCTGAGGCTTC-3'	52.4
2	Exons 2 and 3	642 bp	F 5'-GCCAGGCCTGTGTTCACATG -3'	50.8
			R 5'-GGAGTACCAAGTTATCGAATG-3'	45.3
3	Exons 4 and 5	850 bp	F 5'-CAAGGAATTGTTTACCTGAGAC-3'	46.0
			R 5'-TGGAAATGCTTGGGCAGCG-3'	48.1
4	Exon 6/3' UTR	354 bp	F 5'-CCTTGAGACCCAGTTTCCAC-3'	48.7
			R 5'-CATTTGAACCCAGAAGGCGG-3'	48.7

2.1.2. ADA Asay

1. Distilled water.
2. ADA assay buffer: 1 *M* Tris-HCl, 5 m*M* EDTA, pH 8.0, store at 4°C.
3. 4 m*M* Adenosine (Sigma): Make up in lysate stabilizer and store at –20°C in small aliquots.
4. Drabkin's solution (Sigma).
5. Cuvets suitable for wavelength (e.g., silica from Merck).
6. Ultraviolet (UV)/visible spectrophotometer with heated carousel (30°C) (e.g., Ultrospec range from Amersham–Pharmacia) (*see* **Note 3**).
7. Enzyme kinetics application software for spectrophotometer (*see* **Note 4**).

2.2. RPS19 *PCR for Sequencing*

1. Genomic DNA to be sequenced, 200 ng/PCR reaction.
2. Nuclease-free water.
3. Primers at a working concentration of 10 pmol/µL (*see* **Table 1**)
4. Hot start DNA polymerase (AmpliTaq Gold; Applied Biosystems).
5. 10X PCR buffer containing 15 m*M* $MgCl_2$ (supplied with AmpliTaq Gold; Applied Biosystems).
6. dNTP mix: 2.5 m*M* of each deoxynucleoside triphosphate.
7. 5X Q-Solution (Qiagen).
8. Mineral oil (Sigma) (*see* **Note 5**).
9. Thermocycler.
10. Equipment for agarose gel electrophoresis.
11. 10X TAE electrophoresis buffer: 0.4 *M* Tris-acetate, pH 8.0, 10 m*M* EDTA.
12. UV transilluminator (305 nm).
13. QIAquick Gel Extraction Kit (Qiagen).

3. Method

3.1. Preparation of Lysate (47)

1. Wash 100 µL of whole blood twice with normal saline (*see* **Notes 6** and **7**):
   a. Suspend cells in saline in a 15-mL polypropylene tube.
   b. Centrifuge at 1000*g* for 5 min.
   c. Remove as much supernatant as possible.
   d. Repeat.
2. Add 500 µL of lysate stabilizer to cell pellet and vortex mix.
3. Rapid-freeze to ensure complete lysis, using one of the following methods:
   a. In a –70°C freezer for 1–2 h (*see* **Note 8**).
   b. In methanol prechilled to –20°C.
   c. In dry-ice–acetone bath.
4. Thaw in a 25°C water bath.
5. Transfer to ice as soon as thawed (*see* **Note 9**).

3.2. ADA Assay

1. Take an aliquot of 4 m*M* adenosine from the freezer and thaw on ice.
2. Switch on the spectophotometer and water bath.
3. Pipet into silica cuvets and mix by inversion or stirring.
   a. 860 µL of distilled water.
   b. 100 µL of ADA assay buffer.
   c. 20 µL of hawed lysate.
4. Place cuvets in a heated carousel at 30°C.
5. Incubate for 10 min before starting the assay.
6. Blank against first test cuvet before adding adenosine (*see* **Note 10**).
7. Start the assay by adding 20 µL of 4 m*M* adenosine and mix by inversion.
8. Follow reaction for 19 min, recording A_{265} every 30 s.
6. Record the slope (ΔA_{265}) and linearity for the last 5 min (*see* **Note 11**).

3.3. Measurement of Hb Concentration of Lysate

1. Add 20 µL of lysate to 980 µL of Drabkin's solution.
2. Allow to stand for 5 min before reading absorbance.
3. Read absorbance at 540 nm against Drabkin's blank.
4. Calculate the hemoglobin concentration of the lysate using the formula:

$$\text{Hb (g/dL)} = A_{540} \times 7.47$$

3.4. Calculation of ADA Activity (see Notes 12–15)

$$\text{eADA (U/gHb)} = \frac{\Delta A_{265} \times 5000}{8.1 \times \text{Hb (g/dL)}}$$

3.5. RPS19 PCR Reactions

3.5.1. Set 1

In a 500-µL PCR tube, combine:

a. Nuclease-free water to a total volume of 50 µL.
b. 200 ng of template DNA (*see* **Note 16**).
c. 5 µL of 10X PCR buffer.
d. 10 µL of Q solution (*see* **Note 17**).
e. 2 µL of dNTP mix.
f. 2.5 µL of forward primer.
g. 2.5 µL of reverse primer
h. 1.25 U of hot start *Taq* polymerase.
i. Overlay with a few drops of mineral oil (*see* **Note 18**).

3.5.2. Sets 2–4

As for *Set 1*, but with the omission of Q solution, with relevant primers for the appropriate set.

3.5.3. Cycling Reactions

1. Preincubate at 95°C for 5–10 min (*see* **Note 19**)
2. 35–40 cycles of:

   94°C 45 s
   62°C 45 s
   72°C 60 s
   (*see* **Note 20**)
3. Extend at 72°C for 10 min.
4. Purify immediately or store at –20°C until needed.

3.6. Gel Purification of PCR Products

1. Separate PCR products on 1% TAE-agarose gel (*see* **Note 21**).
2. Visualize ethidium bromide stained gel on UV transilluminator.
3. Excise bands (*see* **Notes 22–24**).
4. Purify bands with Qiagen QIAquick Gel Extraction Kit according to the manufacturer's instructions.
5. Elute purified DNA in the minimum recommended volume to avoid overdilution for sequencing (*see* **Notes 25–27**).

4. Notes

1. This is a whole blood method; there is no need for a white blood cell depletion step.
2. Stable for up to 72 h at room temperature ***(11)***, and up to 1 wk at 4°C ***(25)***.
3. We use Ultrospec III, which is adequate for the purpose but now newer models are available.
4. Not essential, as manual recording of a fall in absorbance is an alternative (albeit laborious) option.

5. Not needed if using a heated lid.
6. If very anemic increase the volume of whole blood to give a minimum estimated packed red blood cell volume of 30 µL.
7. Include a normal control in each batch.
8. Maximum overnight.
9. Activity is stable on ice for several hours but not overnight.
10. As reaction causes a fall in OD, blanking after adding adenosine will cause the OD to drop into the negative range.
11. Often nonlinear at start.
12. Based on 8.1 = difference in millimolar extinction coefficient of adenosine and inosine. One unit is defined as the activity that catalyzes deamination of 1 mmol adenosine per minute under the assay conditions.
13. ΔA_{265} is usually in the range –0.002 to 0.004/min for normals.
14. Established normal range in our laboratory ($n = 33$): mean ± SD = 0.605 ± 0.198 ***(27)***. Upper limit as defined by normal mean + 2 SD = 1.00 U/g Hb. In a study of 28 transfusion-independent patients with DBA, the mean eADA was 2.631 ± 1.873 ($p < 0.0001$) ***(27)***.
15. Collaborative European studies, to allow for differences in methods between centers, generally express patient eADA in terms of number of SD from mean for that method.
16. Include negative control tube with no DNA for each set of primers in each batch of reactions.
17. Needed because of high GC content and secondary structure.
18. Unless using a cycler with a heated lid.
19. This is essential if using a hot start polymerase, as it both denatures mispriming that may have occurred during setup and activates the enzyme.
20. These conditions are good in our laboratory for all the primer sets, allowing all to be amplified using the same conditions, but modification may be necessary to optimize the PCR product for different thermocyclers.
21. Run 3 µL if simply confirming success of reaction. For purification, run the entire contents of the PCR reaction.
22. *See* **Table 1** for band sizes.
23. Primer set 2 often generates a second minor band, migrating more slowly.
24. Cut as close to the bands as possible to minimize the presence of excess agarose for the gel extraction procedure.
25. Methods for sequencing are not included here. For a laboratory that does not routinely undertake sequencing, it is usually more cost effective to use a commercial sequencing service, using their recommended primer and template concentrations.
26. The PCR primers are also suitable for sequencing. Further primer sequences, for example, for confirmation of a mutation, can be derived from the genomic sequence of *RPS19* (GenBank accession numbers AF092906 and AF092907) ***(39)***.
27. Primer set 2 encompasses an insertion/deletion polymorphism in intron 2, which generates a multiple sequencing reaction downstream of the polymorphism in

heterozygotes. The heterozygosity rate is approx 50% (*unpublished observations*). There is also a linked single nucleotide polymorphism in intron 2, and another in intron 4.

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3

Antenatal Diagnosis of Hemoglobinopathies

John M. Old

1. Introduction

The hemoglobinopathies are a diverse group of inherited recessive disorders that include the thalassemias and sickle-cell disease. They were the first genetic diseases to be characterized at the molecular level and consequently have been used as a prototype for the development of new techniques of mutation detection. There are now many different polymerase chain reaction (PCR)-based techniques that can be used to diagnose the globin gene mutations, including dot blot analysis, reverse dot blot analysis, the amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis (DGGE), mutagenically separated PCR, gap-PCR, and restriction endonuclease (RE) analysis ***(1,2)***. Each method has its advantages and disadvantages, and the particular one chosen by a laboratory to diagnose point mutations depends not only on the technical expertise available in the diagnostic laboratory but also on the type and variety of the mutations likely to be encountered in the individuals being screened.

Prenatal diagnosis of β-thalassemia was first accomplished in 1974, and since then many countries have developed an extremely successful program for controlling the disorder based on population screening and fetal diagnosis. Initially this was performed by the measurement of globin chain synthesis in fetal blood, obtained by fetal blood sampling at 18–20 wk of gestation. However DNA analysis techniques soon began to replace the globin chain synthesis approach, first by the indirect technique of restriction fragment length polymorphism (RFLP) analysis, then by direct detection of mutations by restriction enzyme digestion and later by oligonucleotide hybridization to DNA fragments on a Southern blot. All of these Southern blot based technique were complex and expensive and prenatal diagnosis remained inaccessible for developing

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

countries until the discovery of PCR, which led to the development of simpler, quicker, and less expensive nonradioactive methods of mutation detection.

Fetal DNA was obtained from cultured amniotic fluid cells until 1982, when chorionic villus sampling (CVS) in the first trimester of pregnancy was developed *(3)*. Currently, prenatal diagnosis by CVS DNA analysis is the method of choice because it is carried out at the 10th to 12th wk of gestation, the risk of fetal mortality associated with the method is acceptably low at 1%, and sufficient DNA is obtained for analysis without cell culture.

1.1. Identification of At-Risk Couples

Couples at risk for severe hemoglobin disorders are first identified by hematological screening tests as directed by published guidelines and flow charts *(4)*. The basic tests are the measurement of the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values, the levels of HbA_2 and HbF, and the detection of abnormal hemoglobins by electrophoresis methods or high-performance liquid chromatography (HPLC). An individual with a reduced MCV and MCH with a normal HbA_2 level has α-thalassemia; an individual with a raised HbA_2 level has β-thalassemia, and one with a raised HbF level of 5–15% has δβ-thalassemia. An individual with normal red cell indices and a HbF level of 15–30% has hereditary persistence of fetal hemoglobin (HPFH). This approach has many pitfalls, however, that may lead to a wrong carrier identification. These include: the presence of iron deficiency, which also reduces the MCV and MCH; mild β-thalassemia mutations, which are associated with borderline raised HbA_2 levels; and the coinheritance of a δ-thalassemia mutation, which reduces the HbA_2 level in an individual with β-thalassemia trait to a normal value *(5)*. Certain carrier combinations can give rise to a hidden risk (i.e., a risk not easily discernible by simple hematological analysis) of having a fetus affected with homozygous α^0-thalassemia. This occurs because the carrier state for various β-thalassemia disorders can mask coexisting α^0-thalassemia trait in regions where both disorders are found (e.g., in Southeast Asia). Thus for couples in which one partner is diagnosed by hematological screening as a carrier of β-thalassemia and the other a possible carrier of α^0-thalassemia, the individual with β-thalassemia should also be screened for α^0-thalassemia mutations by DNA analysis.

1.2. Diagnostic Approaches to Mutation Screening

The main diagnostic approaches for the PCR diagnosis of the hemoglobinopathies are listed in **Table 1**. The ones well used in my laboratory are gap-PCR, ARMS-PCR, and RE analysis of amplified product. Detailed protocols for each of these techniques are presented in this chapter. The alternative well

Table 1
DNA Diagnosis of the Hemoglobinopathies

Disorder and mutation Type	Diagnostic method
α^0-Thalassemia	Gap-PCR, Southern blotting
α^+-Thalassemia	
Deletion	Gap-PCR, Southern blotting
Nondeletion	ASO, RE, DGGE
β-Thalassemia	
Deletion	Gap-PCR
Nondeletion	ASO, ARMS, DGGE
δβ-Thalassemia	Gap-PCR, Southern blotting
HPFH	
Deletion	Gap-PCR, Southern blotting
Nondeletion	ASO, ARMS, RE, DGGE
Hb Lepore	Gap-PCR
HbS	ASO, ARMS, RE
HbC	ASO, ARMS
HbE	ASO, ARMS, RE
HbD Punjab	ASO, ARMS, RE
HbO Arab	ASO, ARMS, RE
Hb variants	RT-PCR and DNA sequencing

ASO, allele-specific oligonucleotide hybridization; ARMS, amplification refractory mutation system; DGGE, denaturing gradient gel electroporesis; Gap-PCR, nutagenically separated polymerase chain reaction; Hb, hemoglobin; HPFH, hereditary persistence of fetal hemoglobin; RE, restriction endonuclease analysis.

used method for the diagnosis of hemoglobin mutations, that of allele-specific oligonucleotide (ASO) hybridization by dot blotting or reverse dot blotting, is not covered in this chapter but detailed protocols may be found elsewhere ***(5)***.

1.2.1. α-Thalassemia

Gap-PCR provides a quick diagnostic test for α^+-thalassemia and α^0-thalassemia deletion mutations but requires careful application for prenatal diagnosis. Most of the common α-thalassemia alleles that result from gene deletions can be diagnosed by gap-PCR. Primer sequences have now been published for the diagnosis of five α^0-thalassemia deletions and two α^+-thalassemia dele-

Table 2
Halassemia Deletion Mutations That Have Been Diagnosed by Gap-PCR

Disorder	Deletion mutation	Reference
α^0-Thalassemia	$--^{SEA}$	***(6)***
	$--^{MED}$	***(6)***
	$-(\alpha)^{20.5}$	***(6)***
	$--^{FIL}$	***(8,9)***
	$--^{THAI}$	***(8,9)***
α^+-Thalassemia	$-\alpha^{3.7}$	***(7)***
	$-\alpha^{4.2}$	***(7)***
β^0-Thalassemia	290-bp deletion	***(38)***
	532-bp deletion	***(39)***
	619-bp deletion	***(40)***
	1393-bp deletion	***(41)***
	1605-bp deletion	***(42)***
	3.5-kb deletion	***(43)***
	10.3-kb deletion	***(44)***
	45-kb deletion	***(45)***
$(\delta\beta)^0$-Thalassemia	Hb Lepore	***(20)***
	Spanish	***(20)***
	Sicilian	***(20)***
	Vietnamese	***(20)***
	Macedonian/Turkish	***(20)***
$(^A\gamma\delta\beta)^0$-Thalassemia	Indian	***(20)***
	Chinese	***(20)***
HPFH	HPFH1 (African)	***(20)***
	HPFH2 (Ghanaian)	***(20)***
	HPFH3 (Indian)	***(20)***

tions ***(6–9)***, as listed in **Table 2**. The α^0-thalassemia deletions diagnosable by PCR are: the $--^{SEA}$ allele, found in Southeast Asian individuals; the $--^{MED}$ and $-(\alpha)^{20.5}$ alleles found in Mediterranean individuals; the $--^{FIL}$ allele, found in Fillipino individuals and finally the $--^{THAI}$ allele, found in Thai individuals. The two α^+-thalassemia deletion mutations are 3.7-kb and the 4.2-kb single α-gene deletion mutations, designated $-\alpha^{3.7}$ and $-\alpha^{4.2}$.

Amplification of sequences in the α-globin gene cluster is technically more difficult than that of the β-globin gene cluster, requiring more stringent conditions for success owing to the higher GC content of the breakpoint sequences

and the considerable sequence homology within the α-globin gene cluster. Experience in many laboratories has shown some primer pairs to be unreliable, resulting occasionally in unpredictable reaction failure and the problem of allele dropout. The more recently published primers ***(8,9)***, however, seem to be much more robust at amplifying than the earlier published sequences, possibly owing to the addition of betaine to the reaction mixture. They are also designed for a multiplex screening test, although in my laboratory they are still used in pairs to test for individual mutations.

The other α^0- and α^+-thalassemia mutations cannot be diagnosed by PCR because their breakpoint sequences have not been determined. These deletion mutations are diagnosed by the Southern blot technique using ζ-gene and α-gene probes. This approach is still very useful, as it permits the diagnosis of α-thalassemia deletions and α-gene rearrangements (the triple and quadruple α-gene alleles) in a single test ***(10)***. α^+-Thalassemia is also caused by point mutations in one of the two α-globin genes. These nondeletion alleles can be detected by PCR using a technique of selective amplification of each α-globin gene followed by a general method of mutation analysis such as DGGE ***(11)*** or DNA sequence analysis ***(12)***. Several of the nondeletion mutations alter a restriction enzyme site and may be diagnosed by selective amplification and restriction endonuclease analysis in a manner similar to that reported for the mutation that gives rise to the unstable α-globin chain variant Hb Constant Spring ***(13)***.

1.2.2. β-Thalassemia

The β-thalassemia disorders area very heterogeneous group of defects with more than 170 different mutations characterized to date ***(14)***. The majority of the defects are single nucleotide substitutions, insertions, or deletions. Only 13 large gene deletions have been identified, and eight of these can be diagnosed by gap-PCR, as listed in **Table 2**. Different methods are required for the detection of other mutations although the basic principles are the same. That is to say that mutation is region specific and each at-risk population has a few common mutations together with a larger variable number of rare ones. Thus for any given ethnic region a PCR method designed to detect the common specific mutations simultaneously is employed initially. Such an approach will identify the mutation in more than 80% of cases for most ethnic groups. Further screening of the known rare mutations will identify the defect in another 10–15% of cases if necessary. Mutations remaining unidentified at this stage are characterized by DNA sequencing.

The first PCR-based method to gain widespread use was the hybridization of ASO to amplified DNA bound to nylon membrane by dotblotting ***(15)***.

Although still in use, the method is limited by the need for separate hybridization steps to test for multiple mutations. This was overcome by the development of the reverse dotblotting technique, in which amplified DNA is hybridized to a panel of mutation-specific probes fixed to a nylon strip. This technique is compatible with the optimum strategy for screening β-thalassemia mutations, using a panel of the commonly found mutations for the first screening and a panel of rare ones for the second screen ***(16)***.

My laboratory uses the ARMS ***(10)***. This fulfils the main requirements of a PCR technology—speed, cost, convenience, and the ability to test for multiple mutations simultaneously. No labeling of primers or amplified DNA is required. The simplest approach is to screen for mutations with simultaneous PCR assays although the multiplexing of ARMS primers in a single PCR assay is possible ***(17)***.

DGGE ***(18)*** is the most widely used indirect method to characterize β-thalassemia mutations. This detects at least 90% of β-thalassemia mutations by a shifted band pattern to normal and provides an alternative approach to ASO probes or ARMS in countries where a very large spectrum of β-thalassemia mutations occur ***(19)***.

1.2.3. δβ-Thalassemia and HPFH

δβ-Thalassemia and the HPFH disorders result from large gene deletions affecting both the β- and δ-globin genes. Restriction enzyme mapping has enabled the characterization of more than 50 different deletions starting at different points between the $^{G}\gamma$-gene and the δ-gene and extending up to 100 kb downstream of the β-globin gene. In two cases, the Macedonian/Turkish $(\delta\beta)^0$-thalassemia gene and the Indian $(A\gamma\delta\beta)^0$-thalassemia gene, the mutation is a complex rearrangement consisting of an inverted DNA sequence flanked by two deletions. The breakpoint sequences have been characterized in small number of these deletions and these can be diagnosed by gap-PCR ***(20)***. Gap-PCR can also be used for the diagnosis of the Hb Lepore, created by a deletion of the DNA sequence between the δ- and β-globin genes. Hb Lepore is the product of the δβ fusion gene, and is associated with a severe β-thalassemia phenotype. All the deletion mutations currently diagnosable by gap-PCR are listed in **Table 2**; the others can be diagnosed only by the identification of characteristic breakpoint fragments with Southern blot analysis.

1.2.4. Hb Variants

More than 700 hemoglobin variants have been described to date, most of which were identified by protein analysis and have never been characterized at the DNA level. Positive identification at the DNA level is achieved by selec-

tive globin gene amplification and DNA sequence analysis. However, the clinically important variants, HbS, HbC, HbE, HbD Punjab and HbO Arab, can be diagnosed by simpler DNA analysis techniques. All these variants can be diagnosed by ASO hybridization, the ARMS technique, or, except HbC, by restriction endonuclease digestion of the PCR product ***(10)***. The sickle-cell gene mutation abolishes a *Dde*I recognition site at codon 6 and diagnosis by *Dde*I digestion of amplified product remains the simplest method of DNA analysis or sickle-cell disease. Similarly, the mutations giving rise to HbD Punjab and HbO Arab abolish an *Eco*RI site and at codon 121. However, the HbC mutation at codon 6 does not abolish the *Dde*I site and is diagnosed by other methods. HbE interacts with β-thalassemia trait to produce a clinical disorder of varying severity ranging from thalassemia intermedia to transfusion-dependent thalassemia major. The HbE mutation can be diagnosed by ASO hybridization, ARMS, or RE analysis as the mutation abolishes a *Mnl*I site in the β-globin gene sequence.

1.3. Indications for Antenatal Diagnosis

Antenatal diagnosis is indicated for homozygous α^0-thalassemia, homozygous β-thalassemia, HbSS disease, and various compound heterozygous states for globin gene mutations that interact to result in a severe clinical disorder as detailed in **Table 3**. A brief description of the main globin gene disorders for which prenatal diagnosis should be offered is given below.

1.3.1. α-Thalassemia

The most severe form of α-thalassemia results from the homozygous state for α^0-thalassemia, known as HbBart's hydrops fetalis syndrome ***(21)***. This condition results from a deletion of all four α-globin genes, and an affected fetus cannot synthesize any α-globin to make HbF or HbA. The examination of fetal blood by HPLC or isoelectric focussing reveals only the abnormal hemoglobin Bart's (γ_4) and a small amount of Hb Portland ($\zeta_2\gamma_2$). The resulting severe fetal anemia leads to asphyxia, hydrops fetalis, and stillbirth or neonatal death. Prenatal diagnosis is always indicated to avoid the severe toxemic complications that occur frequently in pregnancies with hydropic fetuses.

HbH disease results from the compound heterozygous state of α^0-and α^+-thalassemia (--/-α), or more rarely, from the homozygous state of nondeletion α^+-thalassemia mutations affecting the more dominant α_2 gene ($\alpha^T\alpha/\alpha^T\alpha$) ***(22)***. Individuals with HbH disease have a moderately severe hypochromic microcytic anemia and produce large amounts of HbH (β_4) as a result of the excess β-chains in the reticulocyte. Patients may suffer from

Table 3
Genotype/Phenotype Relationships of the Thalassemias, Sickle-Cell Disease, and the Various Thalassemia Interactions With Hb Variants

Type	Phenotype	DNA diagnosis
1. Homozygous state		
α^0-Thalassemia (--/--)	Hb Bart's hydrops fetalis	S Blot/PCR
α^+-Thalassamia (-α/-α)	No clinical problems	S Blot/PCR
α^+-Thalassemia ($\alpha^T\alpha/\alpha^T\alpha$)	HbH disease	S Blot/PCR
β-Thalassemia		
β^0 or severe β^+ mutation	Thalassemia major	PCR
Mild β^+ mutation	Thalassemia intermedia	PCR
$\delta\beta^0$-Thalassemia	Thalassemia intermedia	S Blot/PCR
HPFH	No clinical problems	S Blot/PCR
Hb Lepore	Variable: intermedia to major	PCR
HbS	Sickle-cell disease	PCR
HbC	No clinical problems	PCR
HbD	No clinical problems	PCR
HbE	No clinical problems	PCR
2. Compound-heterozygous state		
α^0-Thalassemia/α^+-thalassemia (--/-α)	HbH disease	S Blot/PCR
α^0-Thalassemia/α^+-thalassemia (--/$\alpha^T\alpha$)	Severe HbH disease	S Blot/PCR

β^0/severe β^+	Thalassemia major	PCR
Mild β^+/β^0 or severe β^+	Variable: intermedia to major	PCR
$\delta\beta^0/\beta^0$ or severe β^+	Variable: intermedia to major	S Blot/PCR
$\delta\beta^0$/mild β^+	Mild thalassemia intermedia	S Blot/PCR
$\delta\beta^0$/Hb Lepore	Thalassemia intermedia	S Blot/PCR
$\alpha\alpha\alpha/\beta^0$ or severe β^+	Mild thalassemia intermedia	S Blot/PCR
Hb Lepore/β^0 or severe β^+	Thalassemia major	PCR
HbC/β^0 or severe β^+	Variable: β-thalassemia trait to intermedia	PCR
HbC/mild β+	No clinical problems	PCR
HbD/β^0 or severe b^+	No clinical problems	PCR
HbE/β^0 or severe β^+	Variable: intermedia to major	PCR
HbO Arab/β^0	Severe thalassemia intermedia	PCR
HbS/b^0 or severe β^+	Sickle-cell disease	PCR
HbS/mild β^+	Mild sickle-cell disease	PCR
HbS/HbC	Sickle-cell disease, variable severity	PCR
HBS/HbD Punjab	Sickle-cell disease	PCR
HbS/HbO Arab	Sickle-cell disease	PCR
HbS/HPFH	No clinical problems	PCR

Hb, Hemoglobin; S Blot, Southern blotting; PCR, PCR; HPFH, hereditary persistence of fetal hemoglobin.

fatigue, general discomfort, and splenomegaly, but they rarely require hospitalization and lead relatively normal lives. Therefore prenatal diagnosis is not normally performed for HbH disease. There is also a more severe form of HbH disease, however, arising from the compound heterozygous state of α^0-thalassemia and nondeletion α^+-thalassemia (--/$\alpha^T\alpha$). Such patients seem to exhibit more severe symptoms with a possible requirement of recurrent blood transfusions and splenectomy. In some situations couples at risk for this more severe form of HbH disease have opted for prenatal diagnosis and termination of an affected fetus ***(21)***.

1.3.2. β*-Thalassemia*

The β-thalassemias are a heterogeneous group of disorders characterized by either an absence of β-globin chain synthesis (β^0 type) or a severely reduced rate of synthesis (b^+ type) ***(21)***. The majority of the b^0 and b^+ type mutations are called severe mutations because either in the homozygous or compound heterozygous state they give rise to the phenotype of β-thalassemia major, a transfusion-dependent anemia occurring early in life. Some β-thalassemia mutations (the mild β^+ type, sometimes designated β^{++} type) in the homozygous state are associated with a milder clinical condition called thalassemia intermedia. Thalassemia intermedia is caused by a wide variety of genotypes including mild β-thalassemia genes, δβ-thalassemia, and Hb Lepore. The coinheritance of α-thalassemia or one of the many determinants resulting in a raised HbF level in adult life may cause thalassemia intermedia in patients who are homozygous for a severe β-thalassemia gene. Patients with thalassemia intermedia present later in life and are capable of maintaining a hemoglobin level above 6 g without transfusion. In contrast, the phenotype of compound heterozygotes when one of these mild mutations is inherited with a severe mutation is less clear and less predictable. This remains a diagnostic and counseling problem.

1.3.3. Interaction of Thalassemia With Hb Variants

The β-thalassemia mutations and various Hb variants can interact to produce number of thalassemia and sickle-cell disorders for which genetic counseling and prenatal diagnosis should be offered. These interactions are listed in **Table 3**. HbE, HbO Arab, and Hb Lepore interact with β-thalassemia trait to result in a potentially severe thalassemia disorder. Sickle-cell disease is caused by not only homozygosity for HbS, but also, in varying degrees of severity, from the interaction of HbS with HbC, HbD Punjab, HbO Arab, and β-thalassemia trait.

2. Materials

1. Kits for DNA extraction as preferred (*see* **Subheading 3.**).
2. dNTPs: Add together 50 µL of a 100 m*M* solution of each dNTP (as purchased) and 3.8 mL of distilled water. The 1.25 m*M* dNTP stock solution should be stored in frozen aliquots.
3. 10X Gap-PCR reaction buffer (composition varies according to primers used) *see* **Subheading 3.** and **Table 2**.
4. Betaine (Sigma-Aldrich Chemical Co. Ltd., England).
5. Mineral oil to overlay PCR reactions.
6. PCR primers as per **Tables 2**, **4**, **6–8**. Dilute aliquots of primer stock solutions to make a working solution of 1 OD U/mL and store frozen.
7. ARMS PCR buffer: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3 at room temperature; 1.5 m*M* $MgCl_2$; 100 mg/mL of gelatin. A 10X stock buffer can be prepared by adding together 0.5 mL of 1 *M* Tris-HCl, pH 8.3 at room temperature; 1.25 mL of 2 *M* KCl, 75 µL of 1 *M* $MgCl_2$; 5 mg of gelatin; and 3.275 mL of distilled water. The stock buffer is heated at 37°C until the gelatin dissolves and then frozen in aliquots.
8. Ammonium sulfate buffer for IghJH PCR: 75 m*M* Tris-HCl, pH 9.0, 20 m*M* $(NH_4)_2SO_4$, 2.0 m*M* $MgCl_2$, 0.01% Tween, 10% dimethyl sulfoxide (DMSO), 10 m*M* β-mercaptoethanol (all final concentrations).
9. *Taq* polymerases and 10X *Taq* buffers: In my laboratory, AmpliTaq Gold (PE Biosystems) works best for ARMS-PCR/RE digestion assays and Platinum *Taq* (Gibco Life Technologies) for gap-PCR (*see* **Subheading 3.**).
10. Tris–borate–EDTA (TBE) buffer: 89 m*M* Tris, 89 m*M* boric acid,10 m*M* EDTA, pH 8.0.
11. Agarose.
12. Blue running dye:15% Ficoll/0.05% bromophenol blue.
13. Ultraviolet (UV) transilluminator and Polaroid land camera.
14. 0.5 µg/µL Ethidium Bromide.

3. Methods

3.1. DNA Extraction

3.1.1. Blood DNA

DNA is normally prepared from blood that is anticoagulated with heparin, or preferably, EDTA. The DNA can be isolated by the standard method of phenol–chloroform extraction and ethanol precipitation, or by using one of the several kits on the market based on salt extraction, protein precipitation, and so forth. Sufficient DNA is obtained from 5–10 mL of peripheral blood for molecular analysis and subsequent storage in a DNA bank at –20°C. If this is not required, a much smaller quantity of blood may be used for PCR diagnosis

of the globin gene disorders. Mutation analysis may be carried out by simply adding 1 µL of boiled whole blood to the PCR reaction mixture ***(8)***.

3.1.2. Amniotic Fluid DNA

DNA can be prepared from amniotic fluid cells directly or after culturing. It is prudent to prepare DNA directly from half the sample and to set up the other half of the sample for cell culturing as a backup source of DNA. It takes 2–3 wk to grow amniocytes to confluence in a 25-mL flask, but culturing has the advantage that a large amount of DNA is obtained (in our experience, the yield from such a flask has varied from 15 to 45 µg, enough DNA for all types of analyses). A diagnosis can be made using DNA from noncultivated cells in most cases. Approximately 5 µg of DNA is obtained from 15 mL of amniotic fluid and this is sufficient for any PCR-based method of analysis. For genotype analysis by Southern blotting, however, this is enough only for one attempt and thus a back up culture is essential in case of failure. The method of DNA preparation for both cultured and noncultivated cells is essentially the same as that for chorionic villi ***(23)***.

3.1.3. Chorionic Villi DNA

The two main approaches to chorionic villus sampling, ultrasound-guided transcervical aspiration and ultrasound-guided transabdominal sampling, both provide good quality samples of chorionic villi for fetal DNA diagnosis. Sufficient DNA is normally obtained for both PCR and Southern blot analysis of the globin genes. For our first 200 CVS DNA diagnoses the average yield of DNA was 46 µg and only in one instance was < 5 µg obtained ***(23)***.

The main technical problem with this source of fetal DNA is the risk of contamination with maternal DNA, which arises from the maternal decidua that is sometimes obtained along with the chorionic villi. However, by careful dissection and removal of the maternal decidua with the aid of a phase-contrast microscope, pure fetal DNA samples can be obtained, as shown by Rosatelli et al. ***(24)*** who reported no misdiagnoses in a total of 457 first-trimester diagnoses for β-thalassemia in the Italian population. Maternal contamination can be ruled out in most cases by the presence of one maternal and one paternal allele following the amplification of highly polymorphic repeat markers ***(25)***. The risk of misdiagnosis through maternal DNA contamination can be reduced further by the preparation of DNA from a single villus frond.

3.1.4. Fetal Cells in Maternal Blood

Fetal cells have long been known to be present in the maternal circulation and they provide an attractive noninvasive approach to prenatal diagnosis. However, attempts to isolate the fetal cells using immunological methods and

cell sorters have had little success in providing a population of cells pure enough for fetal DNA analysis. Until recently analysis of fetal cells in maternal blood could be applied only for the prenatal diagnosis of β-thalassemia in women whose partners carried a different mutation, as reported for the diagnosis of Hb Lepore ***(26)***. However, the development of the technique of isolation of single nucleated fetal erythrocytes by micromanipulation under microscopic observation ***(27)*** has permitted the analysis of both fetal genes in single cells from maternal blood. This approach was shown to be possible for prenatal diagnosis by the report of two successful cases of sickle-cell anemia and β-thalassemia ***(28)***, but it remains technically very difficult and the technique has not been widely adopted.

3.1.5. Preimplantation Genetic Diagnosis Samples

Preimplantation genetic diagnosis (PGD) for the globin gene disorders is now possible by either DNA analysis of single cells biopsied from cleaving embryos or by the analysis of polar body DNA obtained from the two polar bodies extruded during the maturation of the oocyte ***(29)***. Both approaches use a nested PCR technique and appear to be subject to the problem of allele drop-out. This problem, like that of maternal contamination, is overcome by the simultaneous analysis of other maternal and paternal markers. Only the eggs without the defect are fertilized and implanted in the mother. This approach is appealing to couples whose religious beliefs will not permit the termination of a pregnancy and for those who have already had several therapeutic abortions. However, the PGD approach is limited in its applicability by the degree of technical difficulty of the amplification procedure and the high costs of the obstetric procedure.

3.2. Detection of Mutations

The methods of DNA analysis used for prenatal diagnosis are identical to those used for mutation screening. In addition to the mutation-specific primers, however, normal sequence-specific primers are also required for the determination of a fetal genotype when both parents carry the same mutation. Further tests are also necessary: a second method of diagnosis is applied whenever possible to confirm the result; and the analysis of the inheritance of fetal DNA polymorphisms is necessary for checking maternal DNA contamination. The analysis of the linkage of the β-globin gene haplotype polymorphisms is a useful second approach for confirmation of results in some cases in which a family study is possible. However, they are not so useful for checking the presence of maternal DNA contamination and the occasional case of false paternity; a more general approach is obtained by the use of highly polymorphic markers. These can be short tandem repeat (STR) polymorphisms or variable

number tandem repeat (VNTR) polymorphisms ***(25)***, the latter approach having the advantage simplicity, using the same gel electrophoresis protocol as the ARMS procedure.

The main concern with prenatal diagnosis is the possibility of diagnostic error leading to misdiagnosis. An audit of the accuracy of prenatal diagnosis for the hemoglobin disorders in the United Kindom from 1974 to 1999 revealed a diagnostic error rate of 0.41%. Diagnostic errors were recorded to have occurred from the very high sensitivity of PCR to maternal DNA contamination, the failure to amplify the target sequence, false paternity, sample exchange, and various nonlaboratory errors such as incorrect referral or diagnosis by hematological screening of parental phenotypes ***(30)***. The fetal DNA diagnosis report should detail the types of DNA analysis used and clearly state the risk of misdiagnosis due to technical errors based on current data. A code of practice that aims to minimize diagnostic error is provided in **Notes 1–5**.

3.2.1. Gap-PCR

Gene deletion mutations in the β-globin gene cluster may be detected by PCR using two primers complementary to the sense and antisense strand in the DNA regions that flank the deletion. For small deletions of less than 1 kb, the primer pair will generate two products, the smaller fragment arising from the deletion allele. For large deletions, the distance between the two flanking primers is too great to amplify the normal allele and the product is obtained only from the deletion allele. In these cases the normal allele is detected by amplifying across one of the breakpoints, using a primer complementary to the deleted sequence and one complementary to the flanking DNA. An example of a prenatal diagnosis of α^0-thalassemia by gap-PCR is shown in **Fig. 1**.

1. Set up the reaction mixture to a final volume of 22 µL into a 0.5-mL tube with the following components as required: 1 µL of genomic DNA (100 ng/µL), 1 µL of forward primer—flanking sequence (10 pmol/µL), 1 µL reverse primer—flanking sequence (10 pmol/µL), 1 µL of primer—deleted sequence (10 pmol/µL), 1 µL of primer—inverted sequence (10 pmol/µL), 2.5 µL of 1.25 m*M* dNTP mixture, 2.3 µL of 10X gap-PCR buffer as recommended for the primers. The buffer for the α-thalassemia primers should also contains 0.5 *M* betaine, which can be achieved by adding 2.5 µL of 5 *M* betaine. All reactions should be made up to a final volume of 22 µL with the addition of sterile distilled water (dH_2O) (*see* **Note 5**).
2. Overlay with 25 µL of mineral oil.
3. Prepare the enzyme mixture: 0.2 µL of reaction buffer (10X), 0.1 µL Ampli Taq (5 U/mL) (PE Biosystems) for the β-gene primers, 0.1 mL of Platinum Taq (5 U/mL) (Life Technologies) for the α-gene primers, and 2.7 µL of sterile dH_2O to a final volume of 3 µL.
4. Mix enzyme mixture and hold on ice.

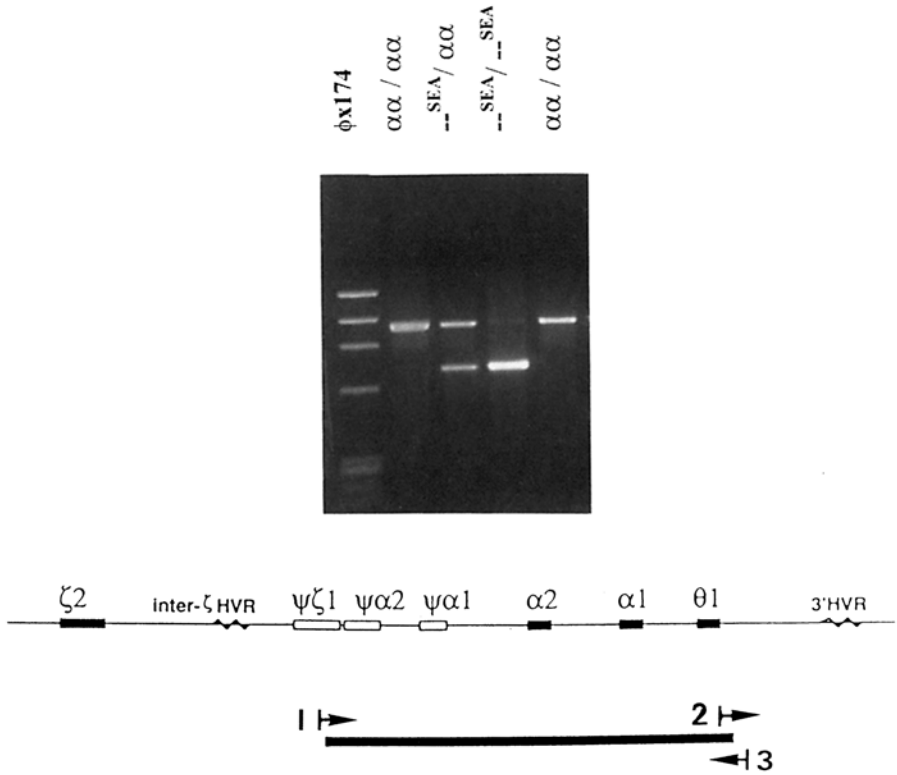


Fig. 1. Prenatal diagnosis of α^0-thalassemia using gap-PCR to detect the --^{SEA} allele. The amplification products after agarose gel electrophoresis and ethidium bromide staining are shown as follows: *track 1*, DNA marker fragments; *track 2*, normal DNA; *track 3*, maternal DNA; *track 4*, homozygous control DNA; *track 5*, chorionic villus DNA. A diagram shows the location of the --^{SEA} deletion (solid black line) with respect to the α-globin gene cluster, together with the positions of primers 1 and 3, which amplify --^{SEA} DNA to give a 660-bp product and primers 2 and 3, which amplify only the normal allele to give a 1009-bp product. The results show the fetus is normal.

5. Place reaction mixtures in thermal cycler and perform one cycle as follows, adding 3 μL of the enzyme mix after 2 min of the 94°C denaturation step: 4 min at 94°C/1 min at 55–65°C (as recommended)/1.5 min at 72°C.
6. Continue for 33 cycles with the following steps per cycle: 1 min at 94°C/1 min at 55–65°C (as recommended according to primer sequence)/1.5 min at 72°C.
7. Finish with one cycle as follows: 1 min at 94°C/1 min at 55–65°C (as recommended)/10 min at 72°C.
8. Hold at 15°C until gel electrophoresis.

9. Remove tubes from the thermal cycler, add 5 µL of blue dye, mix, and centrifuge.
10. Load a 20-µL aliquot onto a 1–3% agarose gel (depending on expected fragment sizes) and run at 100V for 45 min in 1X TBE. Stain the gel in 0.5 µg/mL of ethidium bromide solution for 15–30 min, visualize bands on a UV light box (312 nm) and photograph with an electronic camera system or a Polaroid CU-5 camera fitted with an orange filter (e.g., Wratten 22A). For guidance on interpretation *see* **Notes 6–8**.

3.2.2. ARMS PCR

The ARMS technique for detecting known point mutations was first described by Newton et al ***(31)***. It has been developed for the diagnosis of all the common β-thalassemia mutations found in all the main ethnic groups ***(10)***. The technique is based on the principle of allele-specific priming of the PCR process, that is, a specific primer will permit amplification to take place only when its 3' terminal nucleotide matches with its target sequence. Thus to detect the β-thalassemia mutation IVSI-5 (G→C), the 3' nucleotide of the ARMS primer is G in order to basepair with the substituted C in the mutant DNA. The primer forms a G–G mismatch with normal DNA, but this is a weak mismatch and will not prohibit extension of the primer by itself. Only strong mismatches (C–C, G–A, and A–A) reduce priming efficiency to zero or below 5% ***(32)***. To prevent amplification, a further mismatch with the target sequence is introduced at the second, third, or fourth nucleotide from the 3' end of the primer. As a general rule, if the 3' terminal mismatch is a weak one, a strong secondary mismatch is engineered. If it is a strong one, a weak secondary mismatch is introduced.

The mutation-specific ARMS primers used in my laboratory to diagnose the 25 most common β-thalassemia mutations, plus the hemoglobin variants HbS, HbC, and HbE, are listed in **Table 4**. All are 30 bases long so that they can all be used at a single high annealing temperature (65°C). Primers for the diagnosis of the normal alleles for many of these mutations are listed in **Table 5**. These are required when both partners of a couple requesting prenatal diagnosis of β-thalassemia carry the same mutation.

A typical ARMS test for a single mutation consists of two amplifications in the same reaction mixture using the same genomic DNA as substrate. One amplification product results from the specific ARMS primer and its primer pair (when the mutation is present in the genomic DNA) and the other amplification results from two primers that generate a control fragment in all cases. The generation of control product indicates the reaction mixture and thermal cycler are working optimally. An example of a prenatal diagnosis of β-thalassemia by ARMS-PCR is shown in **Fig. 2**.

1. Prepare 4 mL of a reaction mixture consisting of: 0.5 mL of 10X ARMS PCR buffer; 1.25 mL of 1.35 m*M* dNTP mixture; 2.65 mL of sterile distilled water.
2. Pipet 20 µL of PCR reaction mixture into a 0.5-µL tube.
3. Add 1 µL of each primer (1 OD U/mL).
4. Add 0.05 µL of *Taq* DNA polymerase (5 U/µL).
5. When more than one test is being performed, a primer and the enzyme can be mixed together in a separate tube before addition to the reaction mix. This decreases pipeting errors, as larger quantities are used.
6. Add 1 µL of 100 ng/µL of genomic DNA.
7. Overlay with 25 µL of mineral oil.
8. Mix, centrifuge, and place in a thermal cycler.
9. Amplify for 25 cycles as follows: 1 min at 94°C/1 min at 65°C/1.5 min at 72°C with a final extension period of 3 min at 72°C following the 25th cycle.
10. Remove tubes from the thermal cycler and add 5 µL of blue dye. Mix and centrifuge.
11. Load a 20-µL aliquot onto a 3% agarose gel and run at 100 V for approx 45 min in TBE (*see* **Subheading 3.2.1.**).
12. Stain with ethidium bromide and visualize bands as described in **Subheading 3.2.1.** For guidance on interpretation *see* **Note 9**.

3.2.3. Restriction Enzyme Digestion

A small number of the β-thalassemia mutations create or abolish a restriction endonuclease recognition site in the globin gene sequence. Provided that the enzyme is commercially available (not always the case) and that there is not another site too close to the mutation, the loss or creation of a site can be used to diagnose the presence or absence of the mutation. This is useful for the diagnosis of a few of the common β-thalassemia mutations, as listed in **Table 4**, but the main use of this PCR technique is for the diagnosis of the clinically important Hb variants HbS (**Fig. 3**), HbD Punjab and HbO Arab. The primer sequences used in my laboratory for diagnosing these Hb variants are listed in **Table 6**. When possible the amplified product should include a second site for the appropriate restriction enzyme. This site will act as a control for the digestion reaction, as it should be fully cleaved in products from both the normal and mutant DNA alleles. This is possible for the HbS and HbE mutations but not for HbO Arab and HbD Punjab, for which the flanking *Eco*RI sites are too far away from the one in codon 121. An example of a prenatal diagnosis of sickle-cell anemia by restriction enzyme digest PCR is shown in **Fig. 3.**

1. Add the following to a 0.5-mL tube: 20 µL of PCR reaction mixture (as detailed in **Subheading 3.2.**, **step 1**); 1 µL of each primer; 1 µL of genomic DNA (100 ng/µL); 2 µL of sterile dH_2O; 0.05 µL of AmpliTaq DNA polymerase (5 U/µL)
2. Overlay with 25 µL of mineral oil.

Table 4
ARMS-PCR Primer Sequences Used for the Detection of the Common β-Thalassemia Mutations

Mutation	Oligonucleotide sequence	Second primer	Product size (bp)	Altered restriction site
–88 (C→T)	TCACTTAGACCTCACCCTGTGGAGCCTCAT	A	684	+*Fok*I
–87 (C→G)	CACTTAGACCTCACCCTGTGGAGCCACCCG	A	683	–*Avr*II
–30 (T→A)	GCAGGGAGGGCAGGAGCCAGGGCTGGGGAA	A	626	
–29 (A→G)	CAGGGAGGGCAGGAGCCAGGGCTGGGTATG	A	625	+*Nla*III
–28 (A→G)	AGGGAGGGCAGGAGCCAGGGCTGGGCTTAG	A	624	
CAP+1 (A→G)	AAAAGTCAGGGCAGAGCCATCTATTGGTTC	A	597	
CD5 (-CT)	TCAAACAGACACCATGGTGCACCTGAGTCG	A	528	–*Dde*I
CD6 (-A)	CCCACAGGGCAGTAACGGCAGACTTCTGCC	B	207	–*Dde*I
CD8 (-AA)	ACACCATGGTGCACCTGACTCCTGAGCAGG	A	520	
CD8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCACACC	B	225	
CD15 (G→A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	A	500	
CD16 (-C)	TCACCACCAACTTCATCCACGTTCACGTTC	B	238	
CD17 (A→T)	CTCACCACCAACTTCATCCACGTTCAGCTA	B	239	+*Mae*I
CD24 (T→A)	CTTGATACCAACCTGCCCAGGGCCTCTCCT	B	262	
CD39 (C→T)	CAGATCCCCAAAGGACTCAAAGAACCTGTA	B	436	+*Mae*I
CD41/42 (-TCTT)	GAGTGGACAGATCCCCAAAGGACTCAACCT	B	439	
CD71/72 (+A)	CATGGCAAGAAAGTGCTCGGTGCCTTTAAG	C	241	

IVSI-1 (G→A)	TTAAACCTGTCTTGTAACCTTGATACCGAT	B	281	–*Bsp*MI
IVSI-1 (G→T)	TTAAACCTGTCTTGTAACCTTGATACGAAA	B	281	–*Bsp*MI
IVSI-5 (G→C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	B	285	
IVSI-6 (T→C)	TCTCCTTAAACCTGTCTTGTAACCTTCATG	B	286	+*Sfa*NI
IVSI-110 (G→A)	ACCAGCAGCCTAAGGGTGGGAAAATAGAGT	B	419	
IVSII-1 (G→A)	AAGAAAACATCAAGGGTCCCATAGACTGAT	B	634	–*Hph*I
IVSII-654 (C→T)	GAATAACAGTGATAATTTCTGGGTTAACGT*	D	829	
IVSII-745(C→G)	TCATATTGCTAATAGCAGCTACAATCGAGG*	D	738	+*Rsa*I
b^S CD6 (A→T)	CCCACAGGGCAGTAACGGCAGACTTCTGCA	B	207	–*Dde*I
b^C CD6 (G→A)	CCACAGGGCAGTAACGGCAGACTTCTCGTT	B	206	
b^S CD26 (G→A)	TAACCTTGATACCAACCTGCCCAGGGCGTT	B	236	–*Mnl*I

The above primers are coupled as indicated with either primer A: CCCCTTCCTATGACATGAACTTAA, B: ACCTCA CCCTGTGGAGCCAC; C: TTCGTCTGTTTCCCATTCTAAACT; or D: GAGTCAAGGCTGAGAGATGCAGGA. The control primers used were primers D plus E: CAATGTATCATGCCTCTTTGCACC (which yield a 861-bp product as shown in **Fig. 1**) for all the mutation-specific ARMS primers except the two marked *. Control primers listed in the table used with these two are the $^G\gamma$-*Hin*dIII RFLP primers ***(8)***.

Table 5
Primer Sequences Used for the Detection of the Normal DNA Sequence by the Allele Specific Priming Technique

Mutation	Oligonucleotide sequence	Second primer	Product size (kb)
–87 (C→G)	CACTTAGACCTCACCCTGTGGAGCCACCCC	A	683
CD5 (-CT)	CAAACAGACACCATGGTGCACCTGACTCCT	A	528
CD8 (-AA)	ACACCATGGTGCACCTGACTCCTGAGCAGA	A	520
CD8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCACACT	B	225
CD15 (G→A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	A	500
CD39 (C→T)	TTAGGCTGCTGGTGGTCTACCCTTGGTCCC	A	299
CD41/42 (-TCTT)	GAGTGGACAGATCCCCAAAGGACTCAAAGA	B	439
IVSI-1 (G→A)	TTAAACCTGTCTTGTAACCTTGATACCCAC	B	281
IVSI-1 (G→T)	GATGAAGTTGGTGGTGAGGCCCTGGGTAGG	A	455
IVSI-5 (G→C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAC	B	285
IVSI-6 (T→C)	AGTTGGTGGTGAGGCCCTGGGCAGGTTGGT	A	449
IVSI-110 (G→A)	ACCAGCAGCCTAAGGGTGGGAAAATACACC	B	419
IVSII-1 (G→A)	AAGAAAACATCAAGGGTCCCATAGACTGAC	B	634
IVSII-654 (C→T)	GAATAACAGTGATAATTTCTGGGTTAACGC	D	829
IVSII-745 (C→G)	TCATATTGCTAATAGCAGCTACAATCGAGC	D	738
β^S CD6 (A→T)	AACAGACACCATGGTGCACCTGACTCGTGA	A	527
β^E CD26 (G→A)	TAACCTTGATACCAACCTGCCCAGGGCGTC	B	236

See the footnote to **Table 4** for details on primers A–D and control primers.

3. Place in the thermal cycler and perform 30 cycles of: 1 min at 94°C/1 min at 65°C/1.5 min at 72°C with a final period at 72°C for 3 min after the last cycle.
4. Remove tubes and add 5–10 U of the appropriate restriction enzyme, plus 2 µL of the corresponding 10X buffer.
5. Incubate at 37°C for a minimum of 1 h.
6. Add blue dye, mix, and centrifuge as described in **Subheading 3.2.1.**
7. Load a 20-µL aliquot onto a 3% agarose gel consisting of 50% Nusieve GTC agarose and 50% ordinary agarose.
8. Electrophoresis, stain, and photograph as described in **Subheading 3.2.1.**

3.2.4. Haplotype Analysis

Linkage analysis of RFLPs within the β-globin gene cluster can often be used for prenatal diagnosis of β-thalassemia in the rare cases in which one or both of the mutations remain unidentified after screening using a direct detection method such as ARMS. The technique can also enable the prenatal diagnosis of uncharacterized δβ-thalassemia deletion mutations through the apparent non-Mendelian inheritance of RFLPs (owing to the hemizygosity created by the inheritance of deleted sequences on one chromosome). Finally,

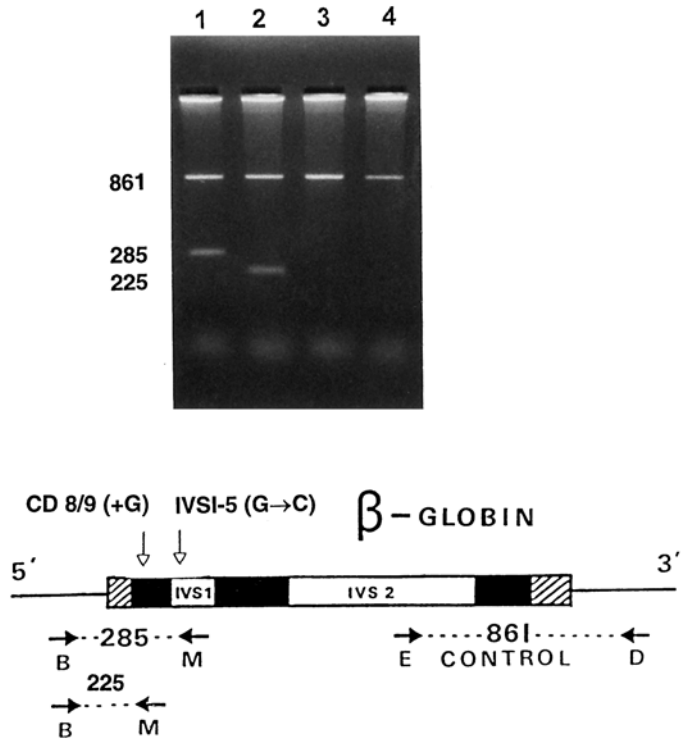


Fig. 2. Prenatal diagnosis for β-thalassemia using ARMS-PCR to detect the mutations IVS1–5 (G→C) and codon 8/9 (+G). *Track 1* shows parental DNA heterozygous for IVS1–5 (G→C), *track 2* shows parental DNA heterozygous for codon 8/9 (+G), *track 3* shows fetal DNA tested for IVS1-5 (G→C), track 4 shows fetal DNA tested for codon 8/9 (+G). The upper band is the 861-bp control product, the lower bands the mutant-specific fragments of 285 bp and 225 bp. The results show the fetus is normal.

haplotype analysis may provide an alternative approach for the confirmation of a prenatal diagnosis result obtained by a direct detection method such as ARMS and in very rare instances has helped to reveal a possible diagnostic error ***(30)***.

At least 18 RFLPs have been characterized within the β-globin gene cluster ***(33)***. However, most of these RFLP sites are nonrandomly associated with each other and thus they combine to produce just a handful of haplotypes ***(34)***. In particular they form a cluster that is 5' to the δ-gene and a 3' cluster that extends downstream from the β-globin gene. The DNA in between the two clusters contains a relative hot spot for meiotic recombination with a rate of approx 1 in 350 meioses ***(35)***. The β-globin gene cluster haplotype normally consists of

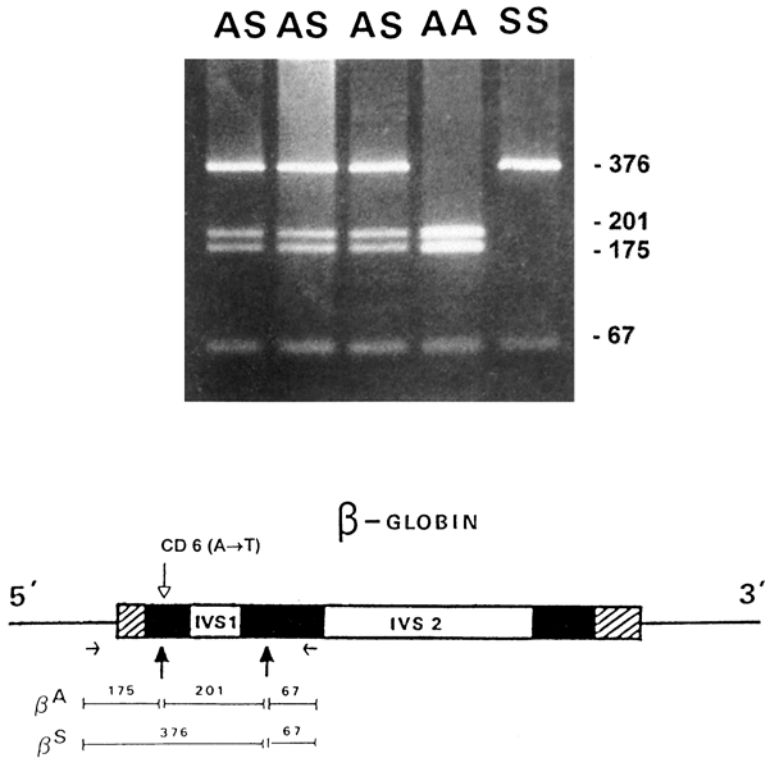


Fig. 3. Diagnosis of sickle-cell genotypes by *Dde*I digestion. The diagram shows a map of the *Dde*I sites at the 5' end of the β-globin gene together with the results of analysis of heterozygous parental DNA samples (AS) in *tracks 1* and *2*, the CVS DNA in *track 3*, a normal DNA (AA) control in *track 4*, and a homozygous (SS) DNA control in *track 5*. Diagnosis of sickle cell genotypes by *Dde*I digestion. The results show the fetus has sickle-cell trait.

five RFLPs located in the 5' cluster (*Hin*dII/ε-gene; *Hin*dIII/^{G}γ-gene; *Hin*dIII/^{A}γ-gene; *Hin*dII/3'ψβ-gene; and *Hin*dII/5'ψβ-gene), and two RFLPs in the 3' cluster (*Ava* II/β-gene; *Bam*HI/β-gene) ***(36)***.

All seven of the RFLPs except *Bam*HI site can be analyzed very simply and quickly by PCR, using the procedure described in **Subheading 3.2.3.** The primer sequences and sizes of the fragments generated are listed in **Table 7**. The *Bam*HI RFLP is located within a L1 repetitive element, creating amplification problems. A *Hin*fI RFLP located just 3' to the β-globin gene is used instead, because these two RFLPs have been found to exist in linkage disequilibrium ***(37)***.

Table 6
Oligonucleotide Primers for the Detection of β^S, β^E, β^D Punjab, and β^0 Arab Mutations as RFLPs

Mutation and affected RE site	Primer sequences (forward and reverse)	Annealing temperature (°C)	Product size (bp)	Absence of site (bp)	Presence of site (bp)
β^S CD6 (A→T)	ACCTCACCCTGTGGAGCCAC	65	443	376	201
(Loses *Dde*I site)	GAGTGGACAGATCCCCAAAGGACTCAAGGA	65		67	175
					67
β^E CD26 (G→A)	ACCTCACCCTGTGGAGCCAC	65	443	231	171
(Loses *Mnl*I site)	GAGTGGACAGATCCCCAAAGGACTCAAGGA			89/56/35/33	89/60/35/33
β^D Punjab CD121 (G→C)	CAATGTATCATGCCTCTTTGCACC	65	861	861	552
(Loses *Eco*RI site)	GAGTCAAGGCTGAGAGATGCAGGA	65			309
β^O Arab CD121 (G→A)	CAATGTATCATGCCTCTTTGCACC	65	861	861	552
(Loses *Eco*RI site	GAGTCAAGGCTGAGAGATGCAGGA	65			309

Table 7
Primers Used for the Analysis of β-Globin Gene Cluster RFLPs

RFLP and Primers 5' Primer 3' Prime	Product size (bp)	Coordinates on GenBank sequence U01317	Absence of site (bp)	Presence of site (bp)	Annealing temperature
*Hin*dII/ε	760		760	315	55°
5'-TCTCTGTTTGATGACAAATTC-3'		18652–18672		445	
5'-AGTCATTGGTCAAGGCTGACC-3'		19391–19411			
*Hin*dIII/Gγ	326		326	235	65°
5'-AGTGCTGCAAGAAGAACAACTACC-3'		35677–35700		91	
5'-CTCTGCATCATGGGCAGTGAGCTC-3'		35981–36004			
*Hin*dIII/Aγ	635		635	327	65°
5'-ATGCTGCTAATGCTTCATTAC-3'		40357–40377		308	
5'-TCATGTGTGATCTCTCAGCAG-3'		40971–40991			
*Hin*dII/5' ψβ	795		795	691	55°
5'-TCCTATCCATTACTGTTCCTTGAA-3'		46686–46709		104	
5'-ATTGTCTTATTCTAGAGACGATTT-3'		47457–47480			
*Hin*dII/3' ψβ	913		913	479	55°
5'-GTACTCATACTTTAAGTCCTAACT-3'		49559–49582		434	
5'-TAAGCAAGATTATTTCTGGTCTCT-3'		50448–50471			
*Ava*II/β	328				
5'-GTGGTCTACCCTTGGACCCAGAGG-3'		62720–62743	328	228	65°
5'-TTCGTCTGTTTCCCATTCTAAACT-3'		62416–62439		100	
*Hin*fI/β	474		320	213	55°
5'-GGAGGTTAAAGTTTTGCTATGCTGTAT-3'		63974–64001	plus constant	107	
5'-GGGCCTATGATAGGGTAAT-3'		64429–64447 & 154	fragment of 154		

3.2.5. VNTR Analysis

A check for maternal contamination in the fetal DNA sample by polymorphism analysis should always be set up at the same time as the globin gene mutation assays. In my laboratory we routinely analyze the fetal DNA and parental DNA samples for two VNTR polymorphisms, the ApoB and IgJh VNTRs ***(25)***. In the very rare cases in which neither of the polymorphic markers provides informative results that exclude the possibility of maternal contamination, other VNTR polymorphisms are tried such as the *Col2A1* gene VNTR locus and the D4S95 marker from the Huntington's disease region of chromosome 4. The primer sequences and size ranges of the PCR products for the four mentioned VNTR markers are listed in **Table 8**. All except the IgJh primers use the standard ARMS PCR buffer. The IgJh primers requires a $(NH_4)_2SO_4$ buffer (*see* **Subheading 2.**).

4. Notes

1. Nonlaboratory errors are minimized by insistence on receiving fresh blood samples for confirmation of the parental phenotypes in every case. In cases when a fresh blood sample from the father is simply not available (in couples at risk for a sickle-cell disorder), extra tests for other possible globin gene mutations are carried out. In particular, when a fetal genotype of sickle-cell trait AS is diagnosed, the fetal DNA is always analyzed for the β^C mutation and the common β-thalassemia mutations observed in the ethnic group of the father.
2. Laboratory errors are minimized by performing duplicate tests, and preparing DNA both from a single frond and from the bulk CVS material whenever possible. Technical errors such as partial digestion or allele dropout are minimized by using two independent diagnostic methods on each sample whenever possible.
3. Polymorphism analysis is used routinely to exclude error due to maternal DNA contamination or nonpaternity. Maternal DNA contamination must be excluded in all cases in which the fetal diagnosis is the same genotype as the mother. The risk of maternal DNA contamination is much lower in cases where the fetus is normal, homozygous, or has inherited a mutation different from that carried by the mother.
4. The aforementioned precautions form a best code of practice for minimizing errors in prenatal genetic testing for any genetic disorder. The guidelines for best practice are:
   a. Ensure that fresh parental blood samples are obtained with the fetal sample in order to check the parental phenotypes and to provide fresh control DNA samples.
   b. Ensure that the chorionic villus sample has undergone careful microscopic dissection to remove any contaminating maternal decidua.

Table 8
Primers Used to Check for Material Contamination by Amplification of VNTR Polymorphisms

VNTR	Primer pair	Annealing temperature	Repeat length (bp)	Size range of products (bp)
Apo B	5'-GAAACGGAGAAATTATGGAGGG-3' 5'-TCCTGAGATCAATAACCTCG-3'	55°	30	541–871
IgJh	5'-GGGCCCTGTCTCAGCTGGGGA-3' 5'-TGGCCTGGCTGCCCTGAGCAG-3'	68°	50	520–1720
Col2A1	5'-CCAGGTTAAGGTTGACAGCT-3' 5'-GTCATGAACTAGCTCTGGTG-3'	55°	34 and 31	584–779
D4S95	5'-GCATAAAATGGGGATAACAGTAC-3' 5'-GACATTGCTTTATAGCTGTGCCTCAGTTT-3'	60°	39	900–1600

c. Always analyze parental and the appropriate control DNAs with the fetal DNA and always repeat the fetal DNA analysis to confirm the result.
d. Whenever possible use an alternative diagnostic method to confirm the diagnosis.
e. Use a limited number of amplification cycles to minimize any coamplification of maternal DNA sequences.
f. Check for maternal DNA contamination in every case.

5. Gap-PCR seems to work reasonably well for amplifying deletion mutations in the β-globin gene cluster. However, amplification of deletions in the α-gene cluster is technically more difficult, possibly owing to the high GC content of the α-globin gene cluster sequence. Experience in my laboratory has shown some of the first primer pairs to be published are unreliable, resulting occasionally in unpredictable reaction failures due to allele dropout, especially when the normal and mutant specific primers are multiplexed together. However, the recently published multiplex primers seem to give more robust and reproducible results. The addition of betaine to the reaction mixture and the use of Platinum Taq DNA polymerase (which has been developed for automatic "hot start" amplification of problematic or GC-rich templates) are key features of their success. The technique is useful for screening for a particular α^0-thalassemia deletion mutation, but because of the problem of allele dropout, a prenatal diagnosis of the result should be confirmed by Southern blot analysis.
6. The relationship between fragment intensities after staining should be constant for all DNA samples. Any deviation from the expected pattern of band intensities in a particular sample should be treated as suspect and the sample retested (e.g., as a result of poor amplification of one of the two alleles, or a partial digestion of the amplified product by the restriction enzyme).
7. Failure of the PCR to produce any product may be the result of the genomic DNA sample being too dilute (remedy: reprecipitate in a smaller volume) or, more often, the DNA being too concentrated (remedy: try a 1:10 dilution). Do not increase the number of cycles to obtain a result, as nonspecific amplification products are likely to appear and there could be a problem of coamplifying any contaminating maternal DNA in a fetal sample. Amplification failure may also be due to an error in the primer sequence. Check the published sequence against the GenBank sequence for typing errors!
8. The unexpected failure of all the PCR tests set up at the same time usually results from a problem with the dNTP mixture. The first troubleshooting step should be to try new dNTP solutions.
9. At times an ARMS primer may produce a faint positive response with a negative DNA control. This is usually less intense than that of the product observed in the positive DNA control and may be a false-positive result. This occurs if there has been a subtle change in the reaction conditions or if the ARMS primer has started to lose its specificity through degradation of the oligonucleotide. Always use small aliquots of primers as a working solution and store the stock solution at –20°C or –70°C if possible.

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4

Prenatal Diagnosis of Hemophilia

David Stirling

1. Introduction

There are widely differing views about the acceptability of prenatal diagnosis and selective abortion for hemophilia both among affected families and healthcare professionals. This debate is beyond the scope of this chapter, but it is essential that the mechanistic approach outlined here is not seen in isolation, but as part of a coordinated package of laboratory tests and "nondirective" counseling.

Prenatal diagnosis provides parents with the opportunity to plan a family without the risk of and responsibility for the birth of a child affected with hemophilia. Historically, fetal sex was determined by amniocentesis and karyotype during mid-trimester. This carried with it the difficult knowledge that there was a 50% chance that a male fetus was not affected by hemophilia. Fetoscopy and ultrasound-guided cordocentesis allowed specific diagnosis, but it was the development of chorionic villus sampling (CVS), and the characterization of the genes for factors VIII and IX that led to the possibility of first-trimester diagnosis ***(1)***. While direct mutational analysis has become the gold standard for prenatal diagnosis, it carries with it the risk of identifying carriers of hemophilia *in utero*. The Nuffield Council for Bioethics recommends that "genetic screening of children which is not of immediate benefit to them should normally be deferred until they can give valid consent" ***(2)***. The initial approach of fetal sexing, followed by other tests only for male fetuses, circumvents this problem.

While fetal sexing is often performed by polymerase chain reaction (PCR) analysis of SRY sequences, specific for the Y chromosome, this relies on the failure of a PCR reaction to identify a female. Our experience is that there are a great many reasons why a PCR reaction can fail, other than absence of spe-

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

cific template. We therefore favor the approach of amplifying a portion of the X-linked amelogenin gene, and its Y chromosome pseudogene which is 5 basepairs (bp) larger ***(3)***.

The basic procedures of linkage analysis, mutation detection, and their use in hemophilia are beyond the scope of this volume. Here we shall not deal with the procedures for obtaining the CVS (generally performed by the obstetric/cytogenetics team) or the extraction of the DNA (any method capable of isolating high molecular weight DNA should give good results). What we shall provide is an example procedure for prenatal diagnosis when the mother is a carrier of the intron 22-inversion mutation. This procedure will be considered in two parts: determination of fetal sex and assay for the presence or absence of the mutation ***(4,5)***.

2. Materials (*see* Table 1)

1. *Taq* DNA polymerase (Promega; Madison, WI).
2. *Taq* DNA polymerase 10X buffer (Mg^{2+}-free)(Promega; Madison, WI).
3. 25 m*M* $MgCl_2$ (Promega; Madison, WI).
4. Expand Long Template PCR System (Roche).
5. Dimethyl sulfoxide (DMSO) (Sigma).
6. dNTPs supplied separately by Roche (dATP, dCTP, dGTP, dTTP) at concentrations of 100 m*M*. Use 10 m*M*. Aliquot 10 µL of stock dNTP into labeled tubes containing 90 µL of sterile distilled water. Store at –20°C.
7. 7-Deaza GTP (Roche).
8. Oligonucleotide primers (from any reputable supplier). Dilute to 100 pmol/µL.
9. 20X TAE: 484 g of Tris, 114 mL of glacial acetic acid, 20 mL of 0.5 *M* EDTA. Dissolve in 3 L of distilled water.
10. Then make up to 5 L with distilled water.
11. 1X TAE: 1:20 dilution of 20X TAE in distilled water.
12. Agarose gel (0.8%). Add 0.8 g of agarose to 100 mL of 1X TAE. Heat in the microwave oven until completely dissolved (1–2 min), then add 20 µL of ethidium bromide. Mix well, allow to cool to approx 65°C, and pour into the prepared electrophoresis chamber. Allow to set for at least 30 min prior to use.
13. 1 Kb Plus DNA Ladder (Invitrogen Ltd.).

3. Methods

3.1. Fetal Sexing

1. One PCR tube should be appropriately labeled for each sample.
2. Into each tube, place the following reagents: 1 µL of DNA, 1 µL of oligonucleotides (mix containing 10 pmol/µL each of AM5' and AM3'), 3 µL of dNTP, 5 µL of $MgCl_2$, 5 µL of buffer, 34.5 µL of distilled H_2O, 0.5 µL of *Taq* polymerase. (*See* **Notes 2–4**.)
3. Tubes should be briefly centrifuged to ensure proper mixing.

Table 1
Primer Sequences Used in This Example

AM 5'	5' TGGGCACCCTGGTTATATCAACT - 3'
AM 3'	5' AGGCCAACCATCAGAGCTTAAACT - 3'
INT22-P	5' GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC - 3'
INT22-Q	5' GGC CCT ACA ACC ATT CTG CCT TTC ACT TTC AGT GCA ATA - 3'
INT22-A	5' CAC AAG GGG GAA GAG TGT GAG GGT GTG GGA TAA GAA - 3'
INT22-B	5' CCC CAA ACT ATA ACC AGC ACC TTG AAC TTC CCC TCT CAT A - 3'

4. Tubes are then placed in the thermal cycler, and subjected to the following conditions. Denaturation at 94°C for 3 min followed by 29 cycles of denaturation at 94°C for 20 s and annealing at 65°C for 45 s and then a final extension step of 72°C for 10 min.
5. When cycles are complete, 10 µL of sample is analyzed on a 1.2% agarose gel, and the results recorded by ethidium bromide staining and ultraviolet (UV) transillumination. The following bands are expected (*see* **Note 1**):

Female	207 bp and 212 bp
Male	207 bp

3.2. Intron 22 Analysis

This and any other subsequent investigations should be carried out only when the fetus has been identified as male.

1. Remove reagents from freezer and allow to thaw fully, then mix thoroughly and briefly centrifuge before pipeting. Reagents to be thawed: Buffer 2 from Expand Long Template PCR Kit, dNTPs, deaza dGTP.
2. Collect appropriate DNA sample(s) from the refrigerator/freezer and allow to thaw if necessary.
3. Label 0.2-mL flat-cap PCR tubes with the DNA number, patient's surname, and primer grouping, if applicable (e.g., APQ).
4. Using sterile pipet tips, pipet 0.5 µL of each DNA sample into the appropriately labeled tube. *Ensure that the DNA is pipeted directly into the bottom the tube*. If the concentration of DNA is very low, 1 µL of DNA should be added. Place tubes on ice. (*See* **Notes 2–4**.*)*
5. Prepare the oligo-mixes as follows: Prepare a 1:50 dilution of A oligo, 1:50 dilution of B oligo, 1:25 dilution of P and Q oligo pairing. The number of samples being tested will determine the actual quantities required.
6. Prepare PCR mastermix 1 and 2 for appropriate number of tests: *See* **Tables 2** and **3**. Place both mastermixes on ice.
7. For each reaction, aliquot 14.9 µL of mastermix 2 into the labeled PCR tubes (which already contain the DNA template). Keep on ice.
8. Immediately prior to amplification, add 9.2 µL of mastermix 1. Centrifuge briefly and proceed to PCR step immediately.

Table 2
Guide to the Composition of Mastermix 1 for Varying Number of Reactions

	1	2	5	10
10X Buffer 2	2.5	5	12.5	25
Taq mix	0.94	1.88	4.7	9.4
Water	5.69	11.4	28.53	57.08

Table 3
Mastermix 2, Per Reaction

	1	2	5	10
10 m*M* aATP	1.25	2.5	6.25	12.5
10 m*M* aTTP	1.25	2.5	6.25	12.5
10 m*M* aCTP	1.25	2.5	6.25	12.5
10 m*M* aGTP	0.625	1.25	3.125	6.25
10 m*M* deaza aGTP	0.625	1.25	3.125	6.25
P/Q primer mix	5	10	25	50
A (or B) primer	3	6	15	30
100% DMSO	1.875	3.75	9.375	18.75

9. Place tubes in thermal cycler subject to the following conditions:

Step	Temperature/Command	Time (min:sec)/repetitions
1	94.0°	2:00
2	94.0°	0:10
3	65.0°	0:30
4	68.0°	12:00
5	Go to **step 2**	9 times
6	94.0°	0:10
7	65.0°	0:30
8	68.0°	12:00 + 20 s/cycle
9	Go to **step 6**	20 times
10	72.0°	5:00
11	End	

10. When the cycle is complete, remove PCR products and keep in the refrigerator prior to electrophoresis.
11. Dilute 4 µL of each PCR product in 6 µL of sterile distilled water.
12. Add 1 µL of diluted PCR product to a tube containing 1 µL of loading buffer and 4 µL of sterile distilled water and mix by repeat pipeting.

13. Prepare a molecular weight marker by adding 1 µL of diluted 1-kb DNA plus ladder to a tube containing 1 µL of loading buffer and 4 µL of sterile distilled water. Mix by repeat pipeting.
14. Load samples (approx 6 µL) into the wells of a 0.8% agarose gel in the horizontal electrophoresis tank and electrophorese at approx 75 V for approx 3 h along with the molecular weight marker.
15. Remove the gel from the tank and record the UV image.
16. Label and check the photograph to ensure all PCR reactions have produced a satisfactory picture.
17. The inversion is associated with a band of 11 kb; the normal allele is associated with a band of 12 kb.
    a. An upper 12-kb band only indicates the inversion is not present.
    b. A lower band of 11-kb only indicates the inversion is present in an affected male.
    c. Bands at both 11 kb and 12 kb indicate a female carrier of the inversion.

4. Notes

1. Where the fetus has been identified as male, there should of course be only one factor VIII alelle present. If the result of mutational analysis reveals a heterozygous phenotype, either the sex assignment was incorrect, or the CVS was contaminated with maternal tissue.
2. Extreme care should be exercised to prevent the possibility of cross-contamination of samples with amplified DNA.
3. PCR should be performed in an area physically separated from that used for product analysis.
4. Samples of known genotype (male, female, heterozygous and homozygous negative) should be analyzed along with unknowns.

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5

Human Platelet Antigen Genotyping by PCR-SSP in Neonatal/Fetal Alloimmune Thrombocytopenia

Colin Hurd and Geoff Lucas

1. Introduction

Neonatal alloimmune thrombocytopenia (NAIT), or fetal maternal alloimmune thrombocytopenia (FMAIT), is a rare disease caused by maternal alloimmunization to inherited paternal human platelet antigens (HPA) expressed on fetal platelets (*see* **Note 1**). Maternal anti-HPA IgG antibodies can cross the placenta and cause the immune destruction of fetal platelets. Severe fetal thrombocytopenia can result in intracerebral haemorrhage (ICH) or death *in utero*. Unlike hemolytic disease of the newborn, NAIT can occur in the first pregnancy ***(1)***. Most cases are diagnosed post-delivery and the incidence of the disorder is about 1 in 2000 live births, which accounts for about 10% of all neonatal thrombocytopenias (platelets $< 100 \times 10^9$/L) ***(2)***. Spontaneous *in utero* intracranial hemorrhage occurs in approx 10% of NAIT cases and can develop as early as 20 wk of gestation. In the absence of routine antenatal screening for maternal anti-HPA antibodies, the first affected baby is usually diagnosed postnatally or *in utero* after intracranial hemorrhage has already occurred. Antenatal management can be planned in advance for women known to have anti-HPA antibodies.

HPA-genotyped, selected, blood donor panels are used to support intrauterine fetal and neonatal platelet transfusions and allow prompt treatment in cases of known or suspected NAIT/FMAIT ***(3)***. HPA-typed blood donor panels can also be used as a source of HPA-compatible red cells, if required by the mother during delivery, to prevent the possibility of posttransfusion purpura (PTP). All of the most clinically significant human platelet antigens, HPA-1, -2, -3, -4, -5, and Gov, are biallelic systems as a result of single nucleotide

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

polymorphisms in the DNA sequence that give rise to single amino acid substitutions in the expressed proteins.

In NAIT, genotyping of the mother supports serological investigations by identifying the possible HPA alloimmunisation(s), while HPA genotyping of the neonate confirms the inheritance of the alloimmunizing antigen. Part of the assessment of risk for subsequent pregnancies involves the determination of paternal zygosity, that is, whether the father is homozygous or heterozygous for the relevant antigen(s). If the father is heterozygous, HPA genotyping of the fetus in subsequent pregnancies, either from chorionic villus, amniotic cell, or fetal blood sampling, can determine whether the fetus is at risk. In this clinical setting, the result of fetal HPA typing profoundly influences the clinical management of the pregnancy. If the fetus has not inherited the offending allele from the father, then the pregnancy can go to term without intervention. However, if the fetus has inherited the antigen, clinical decisions must be made, in consultation with the parents, about the most suitable way to manage the pregnancy or whether a termination is the most appropriate course of action.

The most frequently implicated antibodies in Caucasian women are anti-HPA-1a, which accounts for 80–90% of cases, and anti-HPA-5b, which is responsible for a further 5–15% of cases. A recent report has suggested that the newly described Gov antibodies may be as frequent as HPA-5b antibodies in NAIT ***(4)***. Antibodies to other platelet-specific antigens are found only at very low frequencies. Fewer than 10% of HPA-1a negative women (HPA-1b1b phenotype) who are exposed to the HPA-1a antigen will become alloimmunized to this antigen. The ability to make anti-HPA-1a is strongly associated with the presence of the HLA class II allele DRB3*0101.

A number of polymerase chain reaction (PCR)-based assays have been used for HPA genotyping, including restriction fragment length polymorphism (RFLP) analysis, allele-specific oligonucleotide hybridization (ASO), oligonucleotide ligation assays (OLAs), single-strand conformational polymorphism (SSCP), and preferential homoduplex formation assay ***(5)***. However, the sequence-specific primer PCR (PCR-SSP) ***(6)*** or amplification refractory mutation system (ARMS-PCR) ***(7)*** are currently the most widely used methods.

The principle of the PCR-SSP is based on the lack of a 3'→5' exonuclease activity in *Taq* polymerase to correct mismatched basepairs. A primer terminal 3' mismatch therefore significantly inhibits template amplification. In PCR-SSP, paired reactions are carried out for each antigen with a primer binding to a sequence common to both alleles and a second primer with a terminal 3' nucleotide corresponding to one or other of the two alleles. Amplification occurs only where both the common primer and the allele-specific primers anneal. An *a/a* homozygous individual will therefore amplify only with the *a*

primer mix, a *b/b* individual with the *b* mix, but a heterozygous individual will amplify with both the *a* and the *b* primer mixes. Each primer mix also includes primers for a human growth hormone sequence to act as an internal control for the PCR.

2. Materials

1. Commercial kit for DNA extraction such as Promega Wizard™ or Whatman BioScience Genomic DNA Purification System (Promega; Madison, WI).
2. Autoclaved ultrahigh purity (UHP) water.
3. HotStarTaq DNA Polymerase [deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7.] (Qiagen; ***(8)*** Crawley, UK).
4. 10X PCR buffer: Tris-HCl, KCl, $(NH_4)_2SO_4$, 15 m*M* $MgCl_2$; pH 8.7, 20°C (supplied with the Hot Start Polymerase).
5. 50-µL aliquots of 25 m*M* dNTP made from equal aliquots of 100 m*M* dATP, dCTP, dTTP, and dGTP.
6. Premade primer mixes for HPA 1-5: National Institute for Biological Standards and Controls (NIBSC; Potters Bar, UK) (*see* **Note 2**).
7. Applied Biosystems 9600 or 9700 thermocycler ***(9)***.
8. 96-Well PCR plates suitable for use in an Applied Biosystems 9600 or 9700 thermocycler.
9. Molecular biology grade agarose.
10. 10X Tris-borate buffer (TBE) diluted 1:10 with distilled water to give 1X TBE.
11. 0.1 mg/mL of Ethidium bromide in H_2O (*see* **Note 3**).
12. 1X TBE running buffer.
13. Sucrose–cresol red loading buffer: 60% (w/v) sucrose with 0.04% cresol red in H_2O.

3. Methods

3.1. Isolation of Genomic DNA

The isolation of genomic DNA must be carried out under strictly controlled conditions as the integrity, quality, and concentration of the DNA template isolated will have a profound effect on the accuracy and efficiency of the PCR. Particular care must be taken to avoid sample cross-contamination (*see* **Note 4**).

3.1.1. DNA from Whole Blood

Genomic DNA is isolated from 1.0 mL of well mixed EDTA anticoagulated blood samples or from 1.0 mL of buffy coat from 4–6 mL of blood (*see* **Note 5**). The DNA can be isolated from blood samples using the Promega Wizard™ genomic DNA purification kit or the Whatman BioScience Genomic DNA Purification System following the manufacturer's protocols (*see* **Note 6**). The DNA is rehydrated in TE buffer (supplied with Promega Wizard™) or ultrahigh purity water to a concentration of 50–100 ng/mL.

3.1.2. DNA from Amniotic Cells

Genomic DNA can be prepared from amniotic cells directly using a phenol–chloroform extraction procedure or the Promega or Whatman kits (*see* **Note 6**). Alternatively, in cases in which the number of amniocytes is low (*see* **Note 7**), the amniocytes are grown in culture for 10–14 d to expand the number of cells and thereby increase the amount of recoverable DNA (*see* **Note 8**). The amniocytes grow as adherent cells and care must be taken to ensure that DNA is recovered from the cells in the culture vessel (*see* **Note 9**). The amniocyte DNA can be isolated using any of the preceeding techniques.

3.2. PCR-SSP Protocol

3.2.1. Preparation of Megamix for 10-µL Volume PCRs

Component	Volume/PCR	Volume/100 PCRs
Ultrahigh purity water	1.8 µL	180 µL
10X PCR buffer	1.0 µL	100 µL
25 m*M* dNTPs	0.2 µL	20 µL
5 U/µL of *Taq* polymerase	0.07 µL	7 µL

Prepare a "megamix" volume suitable for one more reaction than is required to allow for pipeting errors, for example, if eight reactions need to be performed, prepare sufficient "megamix" for nine reactions.

3.2.2. Setting Up the PCR-SSP

The PCRs are set up in a 96-well plate format using thin walled eight-tube strips (**Fig. 1**). For routine genotyping, in a mainly Caucasian population, only HPA-1,-2,-3, and -5 genotypes are set up (*see* **Note 10**). This format allows 10 patients or donors plus a "no template" control (ultrahigh purity H_2O) and a positive control (heterozygous donor) to be set up per 96-well "plate."

1. Pipet 5 µL of primer mix into the bottom of 0.2-mL PCR tubes (eight-tube strip) in a 96-well plate format (*see* **Note 11**).
2. Add 3 µL of megamix to the side of the tube. Do not touch the primers mix with the tip.
3. Add 2 µL of DNA, using a new aerosol barrier tip for each DNA sample, to the side of the tube without touching any of the other reagents.
4. Cap the tubes securely.
5. Centrifuge at about 100*g* for 30 s to mix the reagents at the bottom of the tubes.

3.2.3. Thermal Cycling

The PCRs are run in an Applied Biosystems 9600 or 9700 thermocycler ***(9)*** with a heated lid (*see* **Note 12**) using the following program (*see* **Note 13**):

Step no.	Temperature (°C)	Time	No. of cycles
1	95°C	15 min	1
2	96°C	60 s	1
3.1	96°C	25 s	30
3.2	68°C	45 s	
3.3	72°C	30 s	
4	72°C	1 min	1
5	4°C	Hold	Hold

3.3. Analysis of PCR Product by Agarose Gel Electrophoresis

3.3.1. Preparation of 1.5% w/v Agarose Gels

1. For each 50-mL gel dissolve 0.75 g of electrophoresis grade agarose in 50 mL of 1X TBE buffer by heating to the boiling point in a microwave oven.
2. Allow to cool for 10 min. Add 5µL of 1 mg/mL ethidium bromide in H_2O and mix well by swirling without creating bubbles (*see* **Note 14**).
3. Ensure the gel former is horizontal using a spirit level.
4. Pour a 10 cm × 8 cm × 0.6 cm horizontal gel (width × length × thickness) with 2 × 16-well combs (3 mm × 1 mm wells). This is sufficient for running four paired alleles from four patients/donors per gel. Allow the gel to set for 30 min.

3.3.2. Running of Gels

1. Add 2.5 µL of loading buffer to 10 µL of each of the PCR reaction products and mix.
2. Flood the gel with 1X TBE running buffer.
3. Load 3 µL of each PCR product into a well in the 1.5% w/v agarose gel.
4. Run the gel at a constant current of 90 mA for 15 min at room temperature (*see* **Note 15**).

3.4. Analysis and Interpretation of Gels

1. Examine the gels under ultraviolet (UV) transillumination.
2. Capture and store result as a digital image (*see* **Note 16** and photograph [**Fig. 1**]).
3. The presence of a specific band indicates the presence of the gene for that allele.
4. If the control HGH band is not present, the absence of an HPA band cannot be interpreted, as the absence of the gene for that allele and the PCR should be repeated (*see* **Note 17**).

4. Notes

1. A table of HPA and other platelet-specific alloantigens can be found on the NIBSC Web site ***(10)***.
2. The primer mixes for HPA-1 to HPA-5 can be obtained from the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK). Each mix

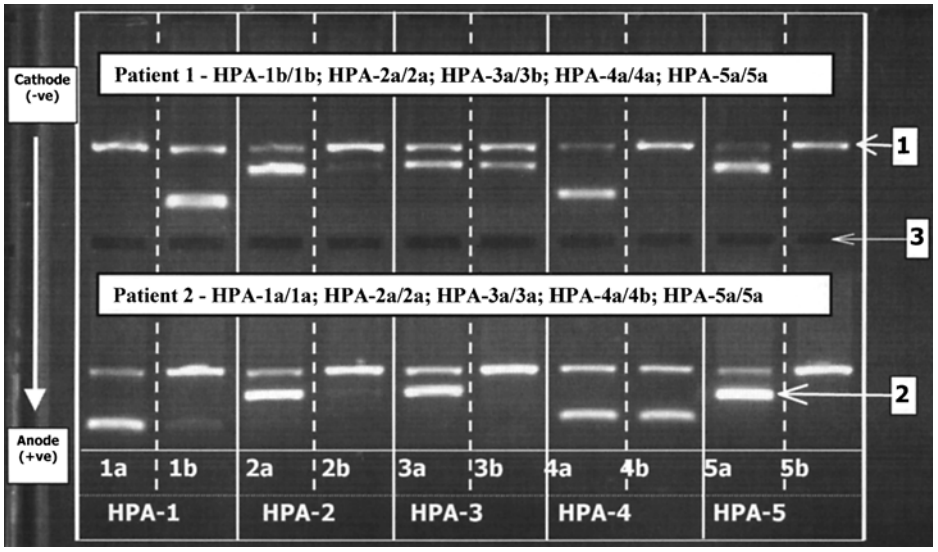


Fig. 1. HPA-1, -2, -3,-4 and -5 genotypes of two patients. 1.5% w/v agarose gel; 3 µL of PCR product/loading buffer per well. Run for 15 min at 90 mAmp constant current. 1, Control HGH band 429 bp. 2, Specific HPA bands 90-267 bp. 3, Sample loading well. The arrow indicates direction of migration of PCR products under electrophoretic gradient.

includes the common primer, the HPA allele specific primer, and the two HGH primers. Primer sequences are given in **Table 1**.

3. Ethidium bromide is a carcinogen and mutagen and should be disposed of according to local regulations. Ethidium bromide can be removed from small volumes of buffer solutions by activated carbon filtration (e.g., Extractor® Scleicher & Schuell).
4. Gloves should be worn to minimize cross-contamination during DNA isolation, preparation of reagents, and while setting up the PCR reactions. Change gloves whenever necessary. Filter pipet tips should be used to prevent aerosol contamination during pipeting.
5. EDTA, citrate, or heparin anticoagulated blood samples may be used as a source of genomic DNA.
6. Other DNA extraction procedures may also be suitable.
7. The yield of amniocytes is often low if the sampling is performed before 15 wk of gestation.
8. Amniocytes should be cultured in an accredited cytogenetics laboratory. The amniocytes should be placed in culture as soon as possible.
9. DNA extraction from adherent cultured monocytes is maximized by adding a lysing agent directly to the culture flask after removal of the culture medium.

Table 1
Primer Sequences for PCR-SSP Genotyping for HPA -1, -2, -3, -4, -5 and Gov *(10,11)*

HPA type	Primers	Sequence	Final PCR conc. (µmol/L)	Product size (bp)
HPA-1	HPA-1a	5'-TCACAGCGAGGTGAGGCC**A**-3'	0.35	
	HPA-1b	5'-TCACAGCGAGGTGAGGCC**G**-3'	0.35	90
	Common	5'-GGAGGTAGAGAGTCGCCATAG-3'	0.35	
HPA-2	HPA-2a	5'-GCCCCCAGGGCTCCTGA**C**-3'	0.35	
	HPA-2b	5'-GCCCCCAGGGCTCCTGA**T**-3'	0.35	258
	Common	5'-TCAGCATTGTCCTGCAGCCA-3'	0.35	
HPA-3	HPA-3a	5'-TGGACTGGGGGCTGCCCA**T**-3'	0.50	
	HPA-3b	5'-TGGACTGGGGGCTGCCCA**G**-3'	0.50	267
	Common	5'-TCCATGTTCACTTGAAGTGCT-3'	0.50	
HPA-4	HPA-4a	5'-GCTGGCCACCCAGATGC**G**-3'	0.50	
	HPA-4b	5'-GCTGGCCACCCAGATGC**A**-3'	0.50	120
	Common	5'-CAGGGGTTTTCGAGGGCCT-3'	0.50	
HPA-5	HPA-5a	5'-AGTCTACCTGTTTACTATCAAA**G**-3'	0.50	
	HPA-5b	5'-AGTCTACCTGTTTACTATCAAA**A**-3'	0.50	246
	Common	5'-CTCTCATGGAAAATGGCAGTA-3'	0.50	
Gov	Gova	5'-TTCAAATTCTTGGTAAATCCTG**T**-3'	0.50	
	Govb	5'-TTCAAATTCTTGGTAAATCCTG**G**-3'	0.50	225
	Common	5'-ATGACCTTATGAGACCTATTC-3'	0.50	
HGH	HGH	5'-GCCTTCCCAACCATTCCCTTA-3'	0.20	429
	control	5'-TCACGGATTTCTGTTGTGTTTC-3'	0.20	

10. The HPA-4 genotypes are not routinely set up because our mainly Caucasian populations are HPA-4a/4a. A 12 × 8 allele PCR conveniently fits a 96-tube format and a 16-well gel format.
11. For convenience, the primer mixes (HPA-1a, 1b, 2a, 2b, 3a, 3b, 5a, 5b) can be stored in 5-µL aliquots in the eight tube-strips ready for use.
12. For PCR machines without a heated lid, it will be necessary to add 25 µL of mineral oil to each tube.
13. Alternative thermal programs have also been successfully used and these have a specific application if the yield of specific amplicons is low—*see* the NIBSC website ***(9)***.
14. The final ethidium bromide concentration of the gel is 0.1 µg/mL.
15. Room temperature: 18–22°C.
16. Alpha Innotech AlphaImager™ running 'AlphaEase' v5.5 software.
17. The PCR is a competitive reaction between the HPA allele specific reaction and the HGH reaction. On occasion, the HPA allele specific reaction can consume

sufficient reagents so that the HGH reaction product is not visible. In those cases, when the HPA allele specific product of the appropriate molecular weight is visualized but the HGH control has not been amplified, it is not necessary to repeat the reaction.

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6

Multiplex PCR for the Detection of the Factor V Leiden and Prothrombin 20210A Mutations

Anne M. Sproul and Elizabeth A. Chalmers

1. Introduction

An extensive published literature now exists on the role of heritable thrombophilia in adult thromboembolic disease ***(1)***. Activated protein C resistance (APCR) secondary to the factor V (FV) Leiden mutation is a relatively common genetic defect, occurring in 2–15% of Caucasian populations and 20–50% of adults with a first episode of venous thromboembolism ***(2)***. Similarly, the PT20210A mutation, which results in increased levels of prothrombin, has a prevalence of approx 2% in the normal population and 6% in unselected adults with venous thrombosis ***(3)***.

Thrombotic problems are uncommon during childhood but are increasingly recognized particularly in tertiary care pediatric populations and represent a spectrum of disorders different from those seen in adults ***(4)***. Although heritable thrombophilia is likely to be a significant risk factor in some children with symptomatic thrombosis, the overall prevalence varies in different clinical settings and there is currently only limited evidence on the risks associated with individual prothrombotic defects ***(5)***. While recommendations on thrombophilia screening in children are likely to evolve with time, at present screening is usually undertaken following clinically significant thrombosis, including unexplained ischemic skin lesions, and should include molecular analysis for the FV Leiden and PT20210A mutations.

The FV Leiden mutation is the result of a G→A transition at nucleotide 1691 ***(6)***. This causes an arginine to glycine substitution at amino acid position 506 in the activated protein C (APC) cleavage site. The mutation results in the

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

loss of an *Mnl*1 recognition site, thus altering the band pattern obtained when the digested polymerase chain reaction (PCR) product is analyzed by electrophoresis and compared to a specimen lacking the FV Leiden mutation. The PT 20210A mutation results in a G→A transition in the 3' untranslated region of the prothrombin gene ***(3)***. The mutation is detected by Hind III digestion of PCR products produced using an oligonucleotide primer designed to introduce a *Hin*dIII site in the presence of the G→A transition.

This protocol is for use either with purified genomic DNA or directly from whole blood or buffy coat. A "hot start" thermostable DNA polymerase is used in a single reaction that amplifies both the FV and prothrombin genes. Three percent agarose or 10% polyacrylamide gels can be used to analyze the digested PCR products. Agarose is used routinely for convenience and safety.

2. Materials

All reagents should be prepared using high-quality distilled or deionized water. All chemicals should be AnalaR or molecular biology grade.

2.1. DNA Preparation

1. 10X Red cell lysis buffer: 0.83% NH_4Cl, 0.1% $KHCO_3$, 0.0001 *M* EDTA. Store at room temperature.
2. White cell lysis buffer: 25 m*M* EDTA, 2% sodium dodecyl sulfate, pH 8.0. Store at room temperature.
3. Protein precipitation solution: 10 *M* NH_4 acetate. Store at room temperature.
4. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA. Store at room temperature.
5. Isopropanol. Store at room temperature in solvent cabinet.
6. 70% Ethanol. Store at room temperature in solvent cabinet.
7. Absolute ethanol. Store at room temperature in solvent cabinet.

2.2. PCR

1. DNA polymerase: "HotStar Taq" 2.5 U/µL (Qiagen). Store at –20°C.
2. 10X reaction buffer supplied with enzyme. Store at –20°C.
3. 2 m*M* dNTP stock: Use 100 m*M* solution of dNTPs (Amersham Pharmacia Biotech) and dilute using sterile distilled H_2O. Store in 0.5-mL aliquots at –20°C.
4. PCR primers at a working concentration of 100 pmol/µL. Store in 100-µL aliquots at –20°C. The primer sequences used for FV are 5'-CAT GAG AGA CAT CGC CTC TG and 5'-GAC CTA ACA TGT TCT AGC CAG AAG-3' ***(7)***. For Prothrombin mutation detection the oligonucleotide primers are 5'-ATA GCA CTG GGA GCA TTG AAG C-3' and 5'-TCT AGA AAC AGT TGC CTG GC-3' ***(3)***. Store in 100-µL aliquots at –20°C.
5. Positive control DNA. Store in 100-µL aliquots at 4°C.
6. Programmable thermal cycler.
7. Electrophoresis equipment: Horizontal Submarine Gel kit and power pack.

8. Loading buffer: 15% Ficoll (Amersham Pharmacia Biotech) (w/v), 0.25% xylene cyanol (w/v), 0.25% orange G. Store at room temperature.
9. Molecular weight markers: 100-bp ladder or pBR 322 *Hae*lll. Use approx 0.1 µg per lane. Store main stock at –20°C and working aliquot at room temperature.
10. Agarose: SeaKem LE (Biowhittaker UK Ltd). Prepare 3% gels in 1X TBE buffer.

2.3. Restriction Digestion of PCR Products

1. *Mnl*1 (New England Biolabs Inc). Store at –20°C.
2. *Hin*dlll (Life Technologies). Store at –20°C.
3. Reaction buffers: Supplied with enzymes. Store working aliquots at 4°C.
4. Bovine serum albumin (BSA) 10% (w/v). Supplied with *Mnl*1. Store at –20°C.

3. Methods

3.1. DNA Extraction (see Notes 1–3)

3.1.1. Red Cell Lysis

1. Add 3 mL of blood to 9 mL of 1X red blood cell lysis solution in a 15-mL polypropylene centrifuge tube. Mix well and leave at room temperature for approx 10 min until red cells are lysed (*see* **Note 4**).
2. Centrifuge for 5 min at 1215*g* at 20°C in an appropriate benchtop centrifuge.
3. Carefully pour off the supernatant and resuspend the white cell pellet in 1X 12 mL RBC lysis solution.
4. Centrifuge as described earlier.
5. Pour off supernatant retaining a small amount. Vortex-mix or flick the tubes to resuspend the cell pellet.

3.1.2. White Cell Lysis

1. Add 3 mL of 1X white cell lysis solution and invert the tube several times to mix well until cells are lysed. Ensure that all of the cells are lysed and that the lysate is clear.

3.1.3. Protein Precipitation

1. Add 1 mL of protein precipitation solution and mix well, but do not vortex-mix as this will shear the DNA. A flocculent white precipitate will form; continue mixing gently until no viscous areas remain.
2. Centrifuge 3000*g* for 20 min at 20°C. A large pellet containing the protein and sodium dodecyl sulfate (SDS) will be formed.

3.1.4. DNA Precipitation

1. Pour off the supernatant into a 15-mL tube containing 3 mL of isopropanol. Mix well by inverting the tube several times until the DNA threads are visible. Spool DNA on the sealed fine end of a Pasteur pipet (*see* **Note 5**).
2. Discard isopropranol from the tube and add 5 mL of 70% ethanol. Leave DNA on the inverted pipet for 2 min to wash DNA.

3. Discard 70% ethanol and add 100% ethanol and repeat wash as described in the preceding.
4. Air-dry the DNA on the inverted pipet. Do not allow to become too dry as this makes the DNA difficult to dissolve.

3.1.5. DNA Resuspension (see ***Note 6***)

1. Add 0.2 mL of TE buffer to a 1.5-mL microcentrifuge tube and place DNA still on the pipet into the tube, cover tube with parafilm, and leave at room temperature until DNA comes off the pipet.
2. Remove the pipet and leave the tube at room temperature until DNA is evenly dissolved. Occasional flicking of the tube will speed up this process. Store the DNA solution at 4°C.

3.2. PCR Amplification of the FV and Prothrombin Genes (see Note 7)

3.2.1. Preparation of PCR Mastermixes

1. Assembling the major components of PCR reactions in bulk reduces interreaction variability and saves time in setting up reactions. The mastermix consists of: 10X concentrated reaction buffer (supplied with enzyme), 2m*M* dNTPS, Distilled H_2O, and PCR amplimers (sense and antisense; 25 pmol/reaction). For 5 mL of mix: 500 µL 10X Reaction buffer, 3.75 mL H_2O, 500 µL 2 m*M* dNTP, and 25 µL of forward and reverse primers (100 pmol/µL).

3.2.2. Selection of DNA To Be Analyzed

1. It is advisable to include known heterozygotes for FV Leiden and PT20210A.. A negative control with no DNA added is essential to control for DNA contamination of reagents.

3.2.3. Thermal Cycling Parameters

1. The following program should be entered onto the thermal cycler:
   a. 95°C for 15 min to activate the *Taq* polymerase.
   b. 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s for 35 cycles.

3.2.4. Setting Up the PCR

1. Calculate the volume of mastermix required (49 µL per reaction). Include an extra 10% to allow for losses in pipeting the final mix which contains detergent and protein.
2. Thaw the required number of aliquots of mastermix.
3. Label the required number of 0.5-mL microcentrifuge tubes (*see* **Note 8**). Allow for positive and negative controls.
4. Assemble the test specimens and controls. Have an empty test tube rack on hand. Replace each DNA specimen in this rack after addition to the reaction tube to avoid errors.

5. Add 0.25 µL (1.25 U) per reaction of HotStarTaq to the mastermix. Vortex-mix briefly and dispense 49 µL to each tube.
6. Add 1 µL of DNA (or whole blood) from the first specimen to the first reaction tube. Close the specimen tube and replace in the spare rack. Move the reaction tube back one row to avoid adding another specimen in error. Add DNA to all the appropriate tubes and 1 µL of TE buffer to the negative control. Add two drops of light mineral oil and close the caps. Centrifuge for 1 min at full-speed revolutions per minute in microfuge. Transfer to thermal cycler immediately if possible, but genomic DNA template reactions can be left at room temperature for up to 2 h until a thermal cycler is free (*see* **Note 9**).
7. Confirm the order of the specimen tubes and check that it corresponds to your records before replacing the DNA specimens in the storage racks at 4°C.
8. PCR products are stable at room temperature for some time but for long-term storage –20°C is recommended.

3.3. Mnl*1 and* Hin*dlll Digestion of the PCR Products*

Ten microliters of PCR product is digested in a total volume of 20 µL using 2 U of enzyme per reaction. The enzyme is usually supplied at 10,000 U/mL, but this can vary.

1. Prepare *Mnl*1/*Hin*dlll mastermix:
   10X Buffer 2 (New England Biolabs), that is, 2 µL per reaction, H_2O to final volume of 10 µL, 10% Bovine serum albumin, 1 µL per reaction, *Mnl*1 (2 U per reaction), 0.2 µL per reaction *Hin*dlll (2 U per reaction), 0.2 µL per reaction.
2. Restriction Digestion:
   a. Dispense 10-µL aliquots to 0.5-mL reaction tubes. Take the prepared tubes to the post-PCR analysis area and add 10 µL of PCR product. Mix well with brief vortex-mixing.
   b. Incubate at 37°C for at least 2 h. This incubation may be carried out in the thermal cycler or in a water bath.

3.4. Agarose Gel Electrophoresis

1. Set up the gel tray with suitable combs (remember that there will be a digested and undigested specimen for each sample). Place the casting gates in place.
2. Prepare a 3% agarose gel. Add 3 g of agarose to 100 mL of TBE buffer in a 500-mL conical flask. Microwave on full power for 2 min and carefully check that the agarose has melted (*see* **Note 10**). Add 2.5 µL of 10 mg/mL ethidium bromide, swirl, and pour into the casting tray. Allow to set for approx 30 min.
3. Dispense 3 µL of gel loading mix to the required number of wells in a V-bottomed microtiter dish.
4. Remove digested PCR products from the water bath or PCR machine. Centrifuge briefly in a microfuge to remove condensation from caps and walls of the tubes. Place tubes in test tube rack beside the corresponding tubes containing the undigested PCR product.

5. Load 10 µL of diluted markers to outside lane of each set.
6. Remove 10 µL from the first specimen and mix with the loading buffer in the first well of the microtiter dish. Load directly into the appropriate well on the gel. Place the sample tube in an empty rack. Repeat until all the specimens are loaded.
7. Place the cover on the electrophoresis tank and connect electrodes to the power pack. DNA is negatively charged and will run from anode to cathode. Adjust the voltage to 125 V and run for approx 45 min.
8. Examine the gel on a UV transilluminator (*see* **Note 11**). If bands are sufficiently resolved, photograph. If necessary run the gel further before photographing.

3.5. Interpretation

It is helpful to include a known heterozygote for each assay and a known FV wild-type with the test specimens

3.5.1. Factor V

Amplification with these primers results in a 147 basepair (bp) fragment that contains 3 *Mnl*1 sites in the G1691 homozygote (G/G). This generates three fragments of 25 bp, 35 bp, and 85 bp. An A at position 1691 will result in two bands from the mutated allele of 25 and 122 bp. In a heterozygous (G/A) individual, however, it is important to remember that both alleles will be present giving rise to four bands of 25, 35, 85, and 122 bp. An A/A homozygote will have only two bands of 25 and 122 bp (*see* **Fig. 1**). Ensure that the wild-type control is fully digested, as partial digestion will result in misinterpretation.

Other mutations may cause loss of *Mnl*1 recognition sites but without affecting the amino acid sequence ***(8)***. Mutations at other positions in the APC cleavage site may also result in APC resistance but will not change the *Mnl*1 cleavage pattern ***(9)***. Both of these events are, however, thought to be rare.

3.5.2. Prothrombin

Amplification with these primers results in a band of 345 bp. Digestion of the PCR product with *Hin*dlll results in digestion of the 3' end of the PCR product if the G→A mutation is present. This produces two major fragments of 345 bp and 322 bp in a heterozygote for the 20210A allele; the 23-bp fragment is not visible on ethidium bromide stained agarose gels. The 322-bp fragment corresponds to the 20210A allele and the 345-bp fragment to the PT2021G allele. In an individual homozygous for the 20210A allele only the lower 322-bp band will be visible following *Hin*dlll digestion. Confirm that the control heterozygote specimen is fully digested to be sure that the genotypes are correctly assigned.

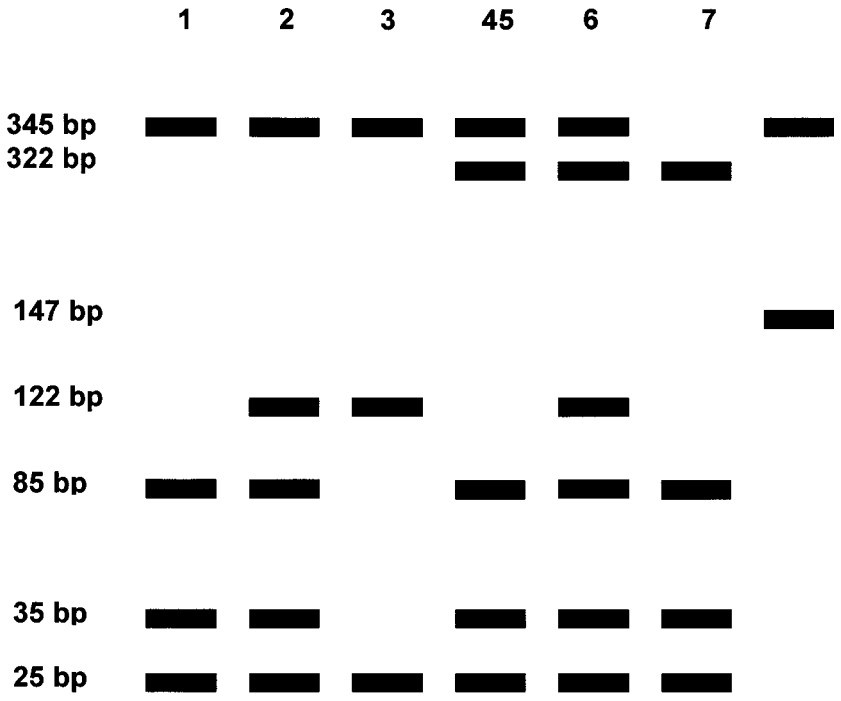


Fig. 1. Schematic representation of the most common genotypes obtained following multiplex PCR. *Lane 1*, normal genotype; *lane 2*, FV Leiden heterozygote, PT20210A wild-type; *lane 3*, FV Leiden homozygote, PT20210A wild-type; *lane 4*, FV Leiden wild-type, PT20210A heterozygote; *lane 5*, FV Leiden heterozygote, PT20210A heterozygote; *lane 6*, PT202101A homozygote, FV Leiden wild-type; *lane 7*, undigested PCR products.

3.5.3. Multiplex PCR

When the PT and FV PCR reactions are multiplexed, some small restriction fragments are produced by *Mnl*1 digestion of the prothrombin product. These sites do not affect the detection of the 20210A allele and are probably polymorphic. The *Mnl*1 fragments from the prothrombin PCR product do not interfere with the interpretation of the FV PCR if 3% gels are used. *Hin*dlll does not cut the FV PCR product (*see* **Fig. 1**).

This multiplex PCR may performed with modified primers to enable the sole use of *Hin*dlll mutation detection in both prothrombin and FV genes ***(10)***. However, the use of *Mnl*1 in the multiplex digestion indicates that the PCR products are digestible as there are *Mnl*1 sites in the prothrombin product.

4. Notes

1. Genomic DNA is extracted from buffy coat prepared from whole blood collected into sodium citrate (following the removal of plasma for plasma based assays) or whole blood specimens collected into EDTA. The PCR assay may also be performed on whole blood stored at –70°C.
2. Specimens can be stored at 4°C for up to 1 wk prior to DNA extraction.
3. It is vital to avoid prevent cross-contamination when preparing DNA specimens for PCR. Gloves must be worn and changed frequently, separate pipets and tips must be used for each specimen, and reagents aliquoted to prevent accidental contamination of stocks.
4. The volumes specified in this procedure assume a 3 to 5 mL blood specimen with the white cell count in the normal range. The working volumes should be altered proportionally for higher or lower cell numbers.
5. If DNA threads are not visible or too diffuse to spool, the DNA should be recovered by centrifugation (3000*g* for 20 min at 20°C). The resulting DNA pellet should be washed with 5 mL of 70% ethanol and centrifuged (3000*g* for 5 min at 20°C). Discard the 70% ethanol wash and repeat with 100% ethanol and centrifuge (3000*g* for 5 min at 20°C). Carefully pour off the supernatant and allow the tube to drain by standing upturned on a tissue. Allow the DNA pellet to dry before resuspending in TE buffer.
6. Reduce the volume of TE accordingly for small pellets from patients with low white blood cell counts.
7. To avoid contamination it is vital that prealiquoted reagents are used for PCR analysis. Aerosol-resistant tips should be used for all stages of preparation and PCR setup and analysis. If possible DNA isolation, PCR setup and post-PCR analysis should be carried out in different laboratories. If this is not possible, separate areas should be designated for each process.
8. Different colored tubes are available. Use a different color for PCR and subsequent digestion of PCR products.
9. "Hot start" *Taq* polymerases are modified such that the enzyme is inactive until heated at high temperature for several minutes, usually 95°C for 15 min. This feature improves PCR specificity by minimizing potential PCR artefacts due to mispriming at suboptimal annealing temperatures and allows PCR reactions to be assembled at room temperature. Hot start PCR is also convenient in laboratories in which thermal cyclers are shared as reactions can be set up and the reactions will not be compromised by delay in starting the PCR program. However, reactions that use blood or buffy coat cells as a template should be placed on the thermal cycler immediately.
10. Microwaving agarose solutions can result in superheating. Exercise extreme caution when handling molten agarose.
11. Low-wavelength UV is damaging to skin and eyes. Protect the eyes and face with an appropriate visor, ensure that gloves and labcoat are worn, and that no skin is exposed to the UV source.

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7

Molecular Diagnosis of Congenital Immunodeficiency

David Eastwood, Kimberly C. Gilmour, and Hubert B. Gaspar

1. Introduction

The congenital immunodeficiencies are a group of inherited conditions in which there are defects of immune function. The last decade has seen the identification of most of the genes defective in these disorders ***(1)***. Diagnostic genetic assays have been developed for many of these conditions so that a molecular diagnosis can be assigned to individuals and genetic testing offered for carrier status and antenatal diagnosis. Most genetic testing now involves screening the gene for the defective exon using one of a number of techniques, the most popular of which is single-stranded conformational polymorphism (SSCP) analysis ***(2)***. Once the affected region is identified, direct sequence analysis can be performed to identify the precise mutation. This procedure has a number of disadvantages. If a gene has many exons, screening the gene using SSCP can be time consuming, and in cases where the clinical picture is ambiguous or atypical, can result in diagnostic delay. The sensitivity of the SSCP technique is also only 85% and thus a number of affected cases may be left undiagnosed.

Over the last few years, protein-based techniques have been developed for diagnosis of these conditions. These have proved to be extremely valuable approaches in that they offer a high level of specificity and sensitivity with a more rapid turnaround than genetic assays. Our studies on a number of conditions demonstrate that >90% of mutations will result in absent or abnormal protein expression ***(3–7)***. Thus by looking for the expression of the relevant protein in peripheral blood mononuclear cells (PBMCs) of patients with suspected immunodeficiency, an unambiguous molecular diagnosis can be made

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

rapidly. For example, the defective gene in X-linked agammaglobulinaemia (XLA) is *Btk* and thus PBMCs from boys with a clinical suspicion of XLA can be analyzed for expression of *Btk* ***(3)***. The analysis of expression can be performed by immunoblotting or by flow cytometric analysis (FACS) for proteins expressed on the cell surface. Intracellular FACS analysis has been used for the analysis of cytoplasmic proteins but this relies on the availability of an extremely specific antibody and is not routinely used in our laboratory ***(8)***. In certain conditions such as XLA, we have immunoprecipitated the antibody from PBMCs prior to immunoblotting to enhance protein retrieval and specificity of the signal. In other conditions, stimulation of cells is required to allow optimal expression of the cells prior to FACS analysis (e.g., hyper-IgM syndrome) ***(9)***. **Table 1** details a list of primary immunodeficiencies, the defective protein, and the techniques used for protein expression analysis currently used in our laboratory.

2. Materials

2.1. Equipment

1. Electrophoresis equipment: 0.8-mm spacers, 20-lane comb, glass plates, binder clamps, gel tank, power supply.
2. Semidry transfer device.
3. Whatman 3MM filter paper.
4. Spectrophotometer.
5. Nitrocellulose: MSI—Micron Separations Inc.
6. Saran wrap.
7. Rolling mixer/rocking platform.
8. Autoradiography film: Kodak Biomax™ Light—1 (13 × 18 cm) or equivalent (light sensitive).
9. X-ray film cassette.
10. Film processor/dark room.
11. Scalpel.
12. Flow cytometer.
13. FACS tubes.
14. Immunofuge.
15. Class II tissue culture cabinet.
16. 37°C incubator.

2.2. Reagents

1. Lymphoprep™ (Axis Shield).
2. RPMI buffer: 1640 medium with 25 m*M* *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer, L-glutamine.
3. NP40 lysis buffer: 4.1 mL of water; 500 µL of 10% Nonidet P-40 (NP-40); 100 µL of 1 *M* Tris-HCl, pH 8.0; 130 µL of 5 *M* NaCl; 100 µL of 500 m*M* NaF; 50 µL of

Table 1
Genes Defective in Specific Immune Disorders and the Protein-Based Diagnostic Tool Used

Disease	Defective gene	Method for protein-based diagnosis
X-linked agammaglobulinaemia (XLA)	Bruton's tyrosine kinase (Btk)	Btk immunoprecipitation and immunoblot analysis
Wiskott–Aldrich syndrome (WAS)	WAS protein (WASP)	WASP immunoblot analysis
X-linked severe combined immunodeficiency (XSCID)	Common gamma chain (γc)	γc expression by FACS analysis
JAK-3 SCID	JAK-3	JAK-3 immunoprecipitation and immunoblot analysis
X-linked hyper-IgM syndrome	CD154[a] (CD40 ligand)	CD154 expression by FACS analysis following PMA/PHA stimulation
X-linked lymphoproliferative disease (XLP)	SLAM associated protein (SAP)	SAP immunoblot analysis

[a]X-linked hyper-IgM syndrome was initially shown to arise from defects in the gene encoding CD40 ligand (a TNF receptor-like molecule expressed on activated T cells). Recent nomenclature changes have designated this protein as CD154.

100 m*M* phenylmethylsulfonyl fluoride (PMSF); 10 µL of Na_3VO_4, 5 µL of 1 *M* dithiothreitol (DTT), 1 µL of 5000X leupeptin 5 mg/mL, 1 µL of 5000X pepstatin 2 mg/mL, and sterile filter (store at 4°C, stable for 1 mo).

4. Antibodies: *See* **Table 2** (Aliquot and store at 4°C, remainder freeze at –70°C).
5. Secondary antibodies: *See* **Table 2** (Aliquot and store at 4°C, remainder freeze at –70°C).
6. Protein A/G Plus agarose beads (0.5 mL of agarose/2.0 mL)—Santa Cruz Biotechnology (store at 4°C).
7. Protein quantification rabbit Ig: Concentration of 1.44 µg of protein/µL.
8. Bio-Rad protein assay reagent.
9. 2X Sodium dodecyl sulfate (SDS) buffer: 0.76 g of Tris, 0.5 mg of bromphenol blue, 10 mL of glycerol, 20 mL of water, 5 mL of 20% SDS. Adjust the pH to 6.8 with HCl, fill to 50 mL with water, and sterile filter.
10. SDS Sample buffer: 3 mL of 2X SDS buffer + 60 µL of β-mercaptoethanol. **Note:** Toxic/harmful.
11. Gel marker: Full Range Rainbow™ - RPN 800—Amersham (store at –20 to –70°C).
12. PBS-T: 500-mL bottle of phosphate-buffered saline (PBS) without Ca, Mg, and $NaCO_3$ + 500 µL of Tween.
13. 2.5% milk in PBS-T: 1.25 g of powdered nonfat (skimmed) milk, 50 mL PBS-T (store at 4°C for 1 wk).

Table 2
Antibody Combinations and Appropriate SDS-PAGE Gel Percentages for Screening Specific Lymphocyte Cell Proteins

Disease	Primary antibody	Secondary antibody	Percentage gel
JAK3 SCID	Anti-JAK3 (C-21) (Santa Cruz)	Anti-rabbit HRP (Sigma)	8%
Wiskott–Aldrich Syndrome	Anti-WASP (D-1) (Santa Cruz)	Anti-mouse HRP (Dako)	10%
XLA	Anti-Btk (gift from S. Tsukada)	Anti-mouse HRP (Dako)	8%
XLP	Anti-SAP (474) (gift from K. Nichols)	Anti-rabbit HRP (Sigma)	12.5%
Control antibody	Anti-β-actin (Sigma)	Anti-mouse HRP (Dako)	

HRP, horseradish peroxidase.

14. 30% Acrylamide: 37.5:1. **Note:** Carcinogen/toxic.
15. Main gel buffer: 91.06 g of Tris, 400 mL of water; adjust the pH to 8.8; add 2.5 mL of 20% SDS; fill to 500 mL with water.
16. TEMED: *N,N,N',N'*-Tetramethylethylenediamine.
17. 10% Ammonium persulfate (APS): 0.5 g of APS, 5 mL of water (store at 4°C, stable for 3 mo).
18. Stacking buffer: 30.35 g of Tris, 400 mL of water; adjust the pH to 6.8, and 2.5 mL 20% SDS solution. Fill to 500 mL with water.
19. 10X transfer buffer: 29.1 g of Tris, 14.65 g of glycine, 450 mL of water; pH 9.2. Fill to 500 mL with water and sterile filter.
20. Transfer buffer: 10 mL of 10X transfer buffer, 70 mL of water, 20 mL of methanol (make fresh as required).
21. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer: 3 g of Tris, 14.4 g of glycine, add 997.5 mL of water, 5 mL of 20% SDS (make fresh as required).
22. Western blotting detection reagents—ECL™: Amersham Pharmacia Biotech (light sensitive, store at 4°C, 6-mo shelf life).
23. FACS lyse.
24. FACS wash.
25. FACS fix.
26. Anti-common gamma chain antibody conjugated to PE (Tug, Becton Dickinson).
27. Isotype control IgG PE antibody (Becton Dickinson).
28. Anti-CD25 PE antibody (Becton Dickinson).
29. Anti-CD69 PE antibody (Becton Dickinson).
30. Anti-CD45 FITC antibody (Dako).

31. Anti-CD154 PE antibody (Alexis).
32. 320 µg/mL of Phytohemagglutinin (PHA).
33. 5µg/mL of Phorbol 13-myristate 12-acetate (PMA).

3. Methods

The protocols have been broken down into the following categories.

1. Flow cytometric diagnostic analysis (for hyper-IgM syndrome and XSCID).
2. Immunoblot analysis (for XLA, XLP, and WAS; *see* **Table 1**) including details on immunoprecipitation for enrichment of protein.

3.1. Flow Cytometric Analysis

FACS analysis involves staining cells with fluorochrome-labeled antibodies, detecting the stained cells using a flow cytometer, and analyzing the collected events using appropriate computer software. The preparation of blood and staining of cells is described (in **Subheadings 3.1.1.** and **3.1.2.**) as well as a summary of collection and analysis of events (*see* **Subheading 3.1.2.**).

3.1.1. Blood Sample Preparation

A control sample from a healthy individual should always be prepared alongside the patient sample. All blood should be collected in EDTA tubes and is stable for 24 h. No preparation is required for gamma chain staining and the blood can be used directly. For cell surface detection of CD40L, the cells must be stimulated as follows:

1. Using sterile technique in a class 2 tissue culture cabinet, take 1 mL of blood and 1 mL of RPMI and mix. Place 1 mL of blood/RPMI in a tube labeled with patient's initials/unstimulated. Place 1 mL of blood/RPMI in a second tube labeled with patient's initials/stimulated.
2. To the stimulated tube, add 18.5 µL of PHA and 4 µL of PMA.
3. Loosely cover both tubes and place at 37°C overnight.

3.1.2. Staining, Analysis, and Acquisition

Samples are stained with fluorochrome antibodies; individual cells are then detected by FACS and analyzed using computer software as described in the following.

1. Take 100-µL aliquots of blood (or blood/RPMI) and place in labeled FACS tubes (indicating patient initials, antibody combinations, and stimulated/unstimulated). Add 5 µL anti-CD45 FITC to each tube and the indicated secondary antibodies (*see* **Note 1**). Mix well and incubate at room temperature for 10 min.
2. Add 500 µL of FACS lyse to each tube, mix, and incubate for 10 min at room temperature.
3. Centrifuge cells in an Immunofuge for 45 s. Tip the supernatant down the sink.

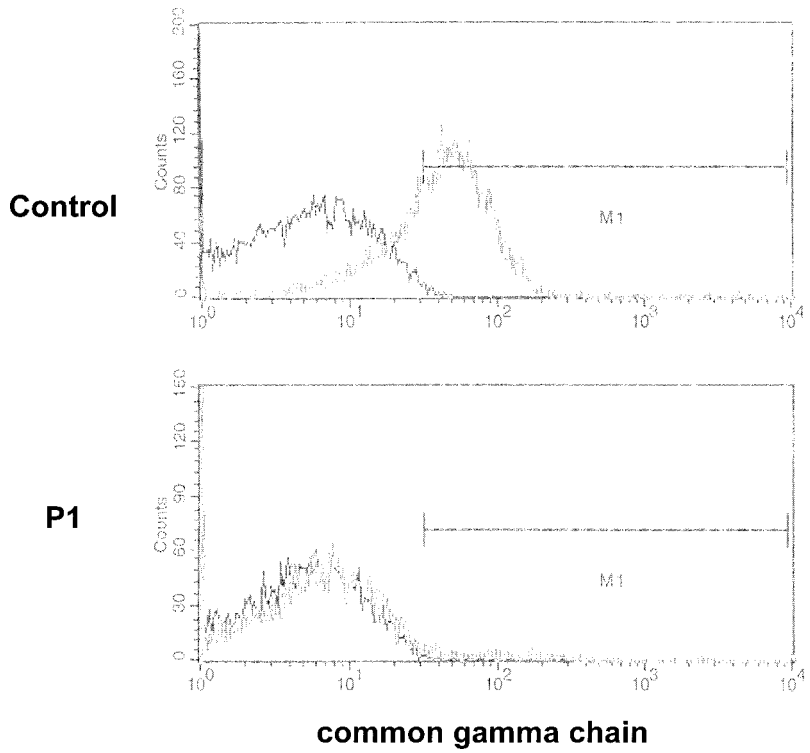


Fig. 1. Diagnosis of XSCID by analysis of common gamma chain expression. Flow cytometric analysis shows normal gamma chain expression in comparison to the isotype control in the control sample. No shift from isotype control is seen in P1; this indicates complete lack of gamma chain expression and thereby confirms the diagnosis of XSCID.

4. Add 500 µL of FACS wash to each tube, and pellet cells as described in **step 3**.
5. To cells add 250 µL of FACS fix and mix by tapping the tube.
6. Acquire 5000 $CD45^+$ lymphocyte events according to the flow cytometer manufacturer's directions.
7. Analyze samples by gating on $CD45^+$ lymphocytes. Using histograms, overlay the antibody stainings onto the isotype controls. Determine the percentage of positively stained cells, that is, cells shifted beyond the isotype control. For CD40L analysis, two histograms are required, one of unstimulated cells and one of stimulated cells.
8. Healthy individuals should have at least 50% gamma chain positive cells. Fewer than 20% gamma chain staining cells is abnormal and indicative of X-linked severe combined immuno-deficiency disease (XSCID) (*see* **Fig. 1**). For CD40L, compare stimulated to unstimulated histograms. Patients expressing CD40L

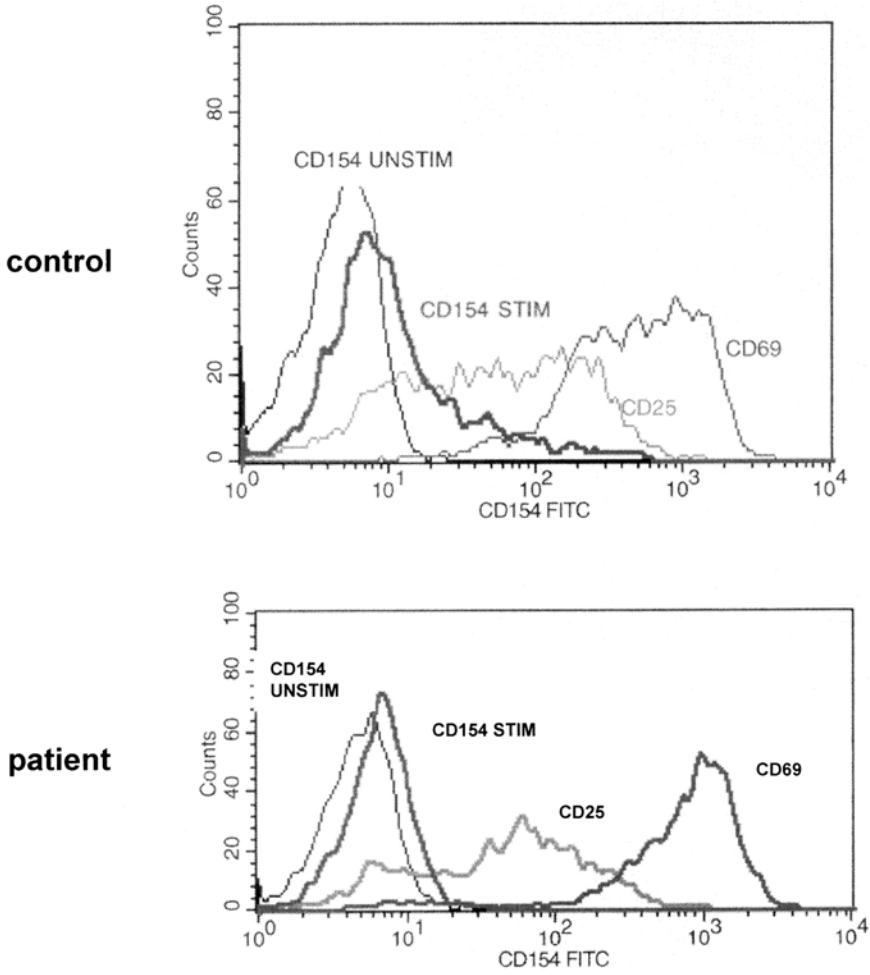


Fig. 2. Diagnosis of CD40L (CD154) deficiency by analysis of CD154 expression on activated T cells. Activated T cells were stained for CD154 and the activation markers CD25 and CD69. In the control there is normal upregulation of CD154 cell surface expression. In the patient sample, despite normal CD25 and CD69 upregulation on activation, there is only minimal CD154 expression indicating an abnormality in CD154.

should have a shift in the stimulated sample beyond that of the isotype controls and the CD40L observed in the unstimulated cells. If CD25 and CD69 are not upregulated by PMA and PHA stimulation, then the results are uninterpretable. Cells lacking CD40L surface expression suggests the patient has hyper-IgM syndrome (*see* **Fig. 2**).

3.2. Immunoblot Analysis

Immunoblot analysis first involves the preparation of a PBMC lysate followed by direct immunoblotting or an initial immunoprecipitation step and immunoblotting. The isolation and preparation of specific cell lysates are outlined in **Subheadings 3.2.1.–3.2.2.** The procedure for protein quantification of the cellular lysate, prior to gel loading, is described in **Subheading 3.2.3.** This technique can be used to establish equal sample protein concentrations for direct comparison.

3.2.1. Ficoll Hypaque™ Density Centrifugation for Obtaining PBMCs

1. Transfer a mixture of 5–10 mL of peripheral blood collected in EDTA and 5 mL of room temperature tissue culture media (e.g., RPMI) gently onto the surface of 10 mL of Lymphoprep. This is best achieved by first coating the side of the vessel with Lymphoprep followed by tilting both vessels while pouring the blood/culture suspension onto the Lymphoprep (*see* **Note 2**).
2. Centrifuge the sample (1000*g*, 15 min), with the centrifuge brake disengaged. Take care while moving sample vessels to avoid mixing.
3. Using a plastet, remove the cell layer at the Lymphoprep–plasma interface to a universal tube (approx 5 mL of cells) and fill with up to 25 mL of room temperature RPMI to wash cells. The cellular layer consists mainly of lymphocytes and monocytes.
4. Centrifuge the PBMCs and RPMI mixture (350*g*, 7 min). The centrifuge brake can be reengaged during this step. A creamy pellet should be observed.
5. Discard supernatant, leaving the cell pellet. Remove as much supernatant as possible using a Gilson pipet, being careful not to remove loose cells. Proceed as in **Subheading 3.2.2.** or store pellet at –70°C.

3.2.2. Lysis of the Cell Pellet

1. For both PBMC and tissue culture derived cells, lyse pellet in 200 µL (for approx 10 million cells) of cold NP40 lysis buffer (4°C). Pipet the lysis buffer around the base of the sample tube to obtain any cells attached away from the pellet, and transfer lysate to a 1.5-mL Eppendorf tube and place on ice or in a chilled container for 10 min to 1 h. Maintain at 4°C to reduce the action of host cell proteases.
2. Centrifuge (100*g*, 5 min) at 4°C to remove cellular debris. A white pellet should be visible.
3. Transfer the supernatant to a fresh tube and obtain protein concentration (*see* **Note 3**).
4. For future use in a standard SDS-PAGE protocol, remove 50 µL of the lysate and add to the equivalent volume of 2X SDS (50 µL); store at –20°C until use. *Note:* Ensure the removal of an adequate volume of lysate for subsequent sample repeats and storage.

3.2.3. Lysate OD—Protein Quantification

1. Label clean plastic disposable cuvets 0, 1, 2, 4, 10 plus additional cuvets with lysate sample information.
2. Add 0.8 mL of water to all cuvets. Also, to each of the numbered cuvets add the equivalent volume (in microliters) of IgG, that is, 0 µL into cuvet 0, 1 µL into cuvet 1, and so forth, each microliter of IgG being equal to 1.44 µg of protein (*see* **Note 4**).
3. To each sample cuvet add 1 µL of lysate (*see* **Note 5**).
4. Add 200 µL of Bio-Rad protein buffer to all cuvets. It is important that equal volumes of Bio-Rad buffer are added; this can be obtained by allowing excess buffer to drain from the pipet tip for a few seconds before addition. Cover with parafilm and invert each cuvet five times. Leave for 5–60 min at room temperature.
5. Ensure the spectrophotometer is set to absorbance and adjust wavelength to 595 λ or calibrate according to the manufacturer's protocol.
6. Calibrate the spectrophotometer by inserting cuvet 0 and pressing "set ref."
7. Remove the cuvet and replace with the next (cuvet 1), read, and record the value. Continue with all remaining cuvets. Ensure that each cuvet is positioned in the correct orientation and that the light receiving surfaces are clean.
8. Calculate the average slope for cuvets 1, 2, 4, and 10 or produce a graph of micrograms of protein against OD readings (*see* **Note 4**).
9. Take each sample absorbance reading and divide by the slope value to obtain the yield in micrograms of protein per microliter of lysate (*see* **Note 4**).

3.3. Immunoprecipitation (for Btk Analysis in Patients With Suspected XLA)

3.3.1. Lysate "Precleaning"

The reduction of nonspecific proteins "cleaning" within the lysate sample is an additional stage of the procedure but is optional. The addition of rabbit serum and protein A/G beads allows nonspecific proteins to be centrifuged from the lysate prior to immunoprecipitation. (Rabbit serum is used in precleaning prior to immunoprecipitation of *Btk* but in general the precleaning serum is dependent on the species of the immunoprecipitating antibody.)

1. Add 2 µL of rabbit serum to each lysate sample (150–200 µL of lysate, produced from 5×10^6 to 1×10^7 cells) and rotate for 1 h at 4°C.
2. While at 4°C, add 20 µL of protein A/G beads and rotate for a further 45 min. Ensure the protein beads have been resuspended in solution by inversion before addition.
3. Centrifuge each sample (100*g*; 10 s), transfer the supernatant to a fresh tube, and repeat **steps 2** and **3**. Obtain as much supernatant as possible without disturbing the bead pellet.

3.3.2. Reagent Addition

1. Add 30 µL of immunoprecipitation antibody (Btk), to each sample. Rotate at 4°C for 2 h to overnight (*see* **Note 6**). Maintain at 4°C and add 25 µL of protein A/G beads, rotate for 45 min. Once again ensure the protein beads have been resuspended in solution by inversion before addition.
2. Pellet the beads by centrifugation (100*g*; 20 s). Remove the supernatant to a fresh tube and store at –20°C for possible further immunoprecipitation.
3. Wash the bead pellet twice with 50 µL of NP40 lysis buffer by flicking the tube to redisperse the pellet followed by a pulse centrifuge and lysis buffer removal. To avoid disturbing the bead pellet during both washing steps, remove and discard NP40 lysis buffer using a pipet.
4. Add 30 µL of 2X SDS to the final bead pellet. The volume of 2X SDS required depends on the subsequent number of repeat immunoblots (a single gel well lane may contain 20–40 µL of immunoprecipitation/SDS sample).
5. Boil for 5 min. If not proceeding directly with immunoblotting, store at –20°C (*see* **Note 7**).

3.4. SDS-PAGE and Electroblotting

By denaturing proteins using heat, β-mercaptoethanol, and SDS, disulfide bonds are broken and their structure becomes exposed to the binding of SDS itself. In turn, the more SDS bound, the more negatively charged the protein molecule will become. On this basis, proteins of different sizes can be separated using electrophoretic methods such as SDS-PAGE.

3.4.1. SDS-PAGE Preparation

1. Using masking tape, mark on one side of each glass plate. By using the same internal surface of each glass plate during SDS-PAGE, conditioning of the plates will occur following further gel runs.
2. Clean the inside (untapped) surface of each glass plate with 70% ethanol.
3. Position spacers on each side of the large plate. Attach all spacers together interlocking with the bottom section. Place the second glass plate on top, and ensure that both plates hold the spacers at their edge. Push the spacer sponges to the top edge of the smaller plate and clamp together using binder clamps. Stand apparatus upright by folding the arms of the lower clamps against the glass.
4. To produce a 12.5% gel, mix in a universal tube 6.4 mL of water, 5 mL of main gel buffer, 20 µL of TEMED, and 200 µL of 10% APS. Finally add 8.4 mL of 30% acrylamide, immediately pipet the mixture into the glass plate apparatus to approximately three quarters full, and cover with 3–5 mL of water. Leave a small volume of gel reagent mixture in the universal for **step 5** (recipes for different percentage gels are given in **Table 3**).
5. Establish that the gel has polymerized by shaking the remainder of the mix in the universal and also looking for the refractile line at the gel–water interface. Once

Table 3
Reagent Components for the Preparation of Various SDS-PAGE Gel Percentages

Material	8% Gel	10% Gel	12.5% Gel	4% Stacking gel
30% Acrylamide	5.3 mL	6 mL	8.4 mL	1.3 mL
Main gel buffer	5 mL	5 mL	5 mL	2.5 mL stack buffer
Water	9.7 mL	8.8 mL	6.4 mL	6.1 mL
TEMED	20 µL	20 µL	20 µL	10 µL
10% APS	200 µL	200 µL	200 µL	100 µL

the gel has polymerized, tip out the water from the gel apparatus and blot with tissue.

6. Make a 4% gel (**Table 3**). Immediately pipet the solution on top of the main gel until full. Insert comb, and leave to polymerize.
7. Once the gel has polymerized, remove clamps, bottom spacer, and comb carefully. Label each well on the back of the large glass plate with a nonpermanent marker pen.
8. Transfer the gel apparatus to the running tank by placing the bottom right corner into the lower reservoir containing running buffer. Gently lower the other corner until the lower plate edge is submerged. This step is needed to remove all air bubbles from the bottom of the gel, allowing an equal current flow through the gel.
9. Tighten clamps and fill the top reservoir with running buffer until each empty well has been filled plus an additional 1–2 cm above the stacking gel. Ensure there is no leakage from top to bottom reservoir.

3.4.2. Sample Loading and Running

1. Boil samples for 3–5 min and centrifuge (pulse).
2. Load 10 µL of 2X SDS buffer in three empty lanes on either side of the samples using gel loading tips.
3. Add 10 µL of rainbow marker to the first well followed by 30 µL of sample into subsequent wells (well volume will vary depending upon spacer and comb thickness—a thicker gel spacer set will allow a greater maximum loading volume).
4. Attach leads to the power supply. Set voltage to 150 V and run until the marker begins to separate. Voltage may then be increased to 250 V at 50–100 mA. To run gel overnight, set voltage to 25 V at 8 mAmps.

3.4.3. Electroblotting

The now separated and denatured proteins can at this stage be transferred onto nitrocellulose membrane electrophoretically. Transverse electrophoresis "blotting" of the antigen onto the nitrocellulose matrix allows the antigen to be maintained for subsequent immunoblotting.

1. During the final stages of electrophoresis, estimate the size of the gel region containing the marker and all samples. Cut six equally sized pieces of Whatman 3MM paper and one piece of MSN nitrocellulose to a size slightly greater than that of the gel region.
2. Wet the Whatman 3MM and nitrocellulose in transfer buffer.
3. Dismantle the gel apparatus and separate the two glass plates. Using a scalpel, gently cut away the stacking gel, excess gel on either side of the samples, and the region of gel below each sample lane.
4. Assemble the transfer stack on the semidry transfer apparatus as follows: three pieces of wet Whatman and nitrocellulose. Using fingertips wetted in transfer buffer, gently place the gel on top of the stack. Add three more layers of Whatman paper. Carefully remove air bubbles after each Whatman addition by rolling with a tube or pipet (do not roll across the nitrocellulose). Remove excess buffer around the stack to allow consistent current flow.
5. Transfer for 20 min at 12 V and 200–250 mAmps. Transfer time and mAmp settings vary with protein size and the number of lanes to be transferred.
6. Following transfer, turn the stack over and remove three layers of Whatman to reveal the nitrocellulose membrane. Ensure the marker has visibly transferred onto the membrane and indicate each point on the side of the membrane away from the gel, using an insoluble ink pen.
7. Block membrane in 3 mL of 2.5% milk-PBS-T. Rotate at room temperature for 15 min to 1 h (*see* **Note 8**).

3.5. Immunoblotting

As described below, the procedure requires the addition of primary antibody to detect specific to antigen. Following addition of peroxidase-labeled secondary antibody, detection of the antigen–antibody interaction can be obtained by visualization using light-emitting nonradioactive ECL Western blotting detection reagents and autoradiography film.

1. After blocking, add primary antibody and rotate at room temperature for 2–4 h (*see* **Notes 9** and **10**).
2. Wash five times with PBS-T, and place in 3 mL of milk-PBS-T.
3. Add 1:1000 secondary antibody and place rotating at room temperature for 30–45 min (*see* **Note 10**).
4. Wash five times with PBS-T and place the blot unmarked side up (protein side) in a flat-bottomed dish.
5. Mix equal volumes of ECL reagent 1 and 2 (1–2 mL is sufficient) and gently pipet over the blot for 1 min. It is important to work quickly following addition of ECL reagents as the chemical reaction has a limited half-life.
6. Drain off excess ECL reagent and place protein side down onto Saran Wrap. Fold over and cover with Wrap. Turn the blot over and gently smooth out air pockets and creases by wiping with a tissue.
7. Secure the blot, protein side up in the film cassette. Continue in a dark room by placing an autoradiography film on top of the membrane and closing the cassette

(before exposing the film, fold the bottom right corner; *see* **step 8**). Initially expose the film for 10–15 s and develop. On the basis of this result, it may be necessary to adjust the exposure time for a second film. When handling film, use powder-free gloves to avoid hand contact and powder interference with ECL reagents (*see* **Notes 12–16**).

8. After film development, replace the film back on top of the blot (folded corner placed bottom right to ensure correct film orientation) and mark the positions of each marker for later interpretation of results.
9. To verify protein loading and integrity, wash the membrane once with PBS-T, incubate with 3 µL of anti-actin antibody in 2.5% milk, and continue from **step 3** above, with the exception of using anti-mouse horseradish peroxidase (HRP) secondary antibody. This immunoblot will indicate the quantity of protein loaded and the extent of protein degradation (if any) within the samples.
10. The absence of protein (*see* **Fig. 3a**) or presence of an abnormally sized band (**Fig. 3b**) suggests a significant abnormality of protein expression and can indicate a mutation in the gene coding for that protein (*see* **Note 17**).

4. Notes

1. For common gamma chain staining add 5 µL of IgG PE or 5 µL of anti-common gamma chain PE to each CD45 FITC tube. For detection of CD40L add 5 µL of anti-CD25 PE, 5 µL of anti-CD 69, 5 µL of anti-CD154 (CD40L) or 5 µL of IgG PE to each CD45 FITC tube.
2. To avoid mixing, blood and RPMI can be pipetted onto the surface of the Lymphoprep at a slow rate if this method is preferred.
3. On occasion, a gelatinous precipitate may form in the sample prior to supernatant transfer, this can be removed using a pipet tip leaving the cellular lysate.
4. When calculating the average slope during optical density calibration, any unusual readings that do not closely correspond to the concentration gradient may be ignored, and the final slope calculated using fewer reference points.

Average slope	$y = mx + b$	($b = 0$ since the line goes through 0,0)
	$y = mx$	(y = absorbance)
	$y/x = m$ (average slope)	(x = µg of protein)
	$x = y/m$	(µg of protein = absorbance/slope)

Example calculation of lysate protein concentration using all four reference concentrations:

µL of IgG added	Protein concentration	Spectrophotometer readings
1	1.44	0.030
2	2.88	0.058
4	5.76	0.130
10	14.40	0.310

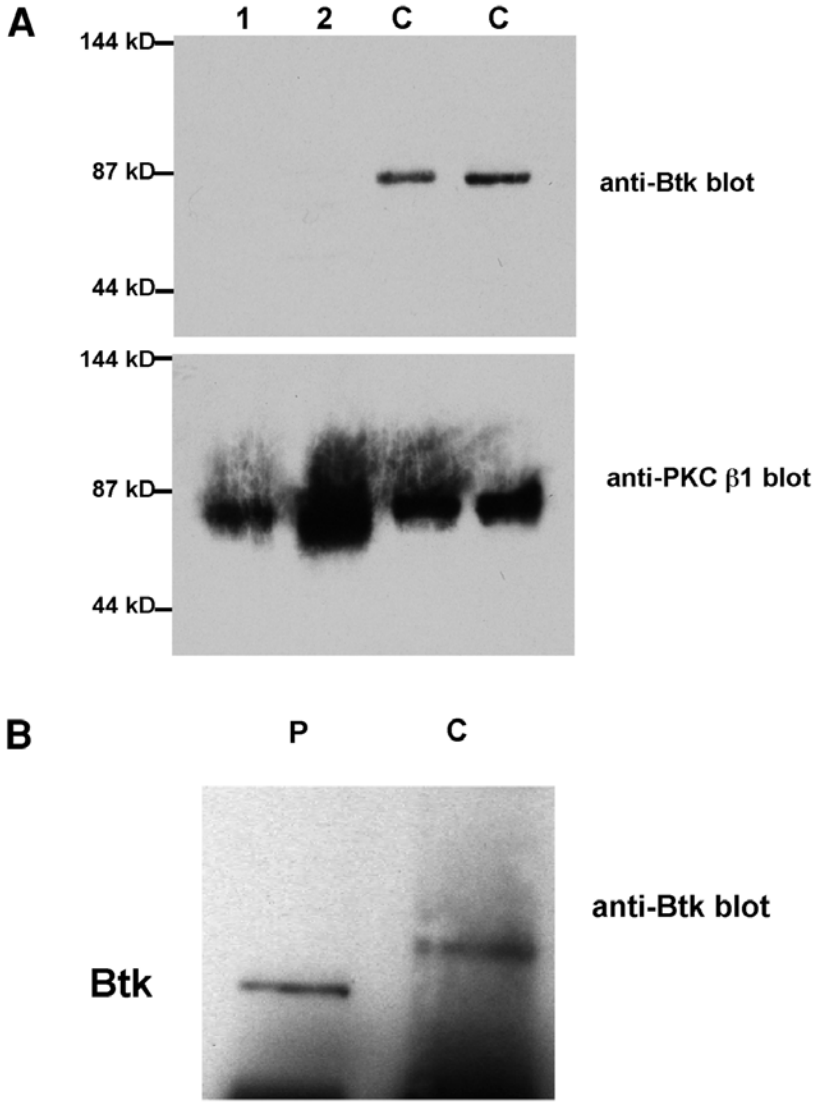


Fig. 3. Diagnosis of XLA by absence of Btk expression. (**A**) Peripheral blood mononuclear cell lysates from two control samples (C) and from patients 1 and 2 with suspected XLA were analyzed by Western blotting for expression of Btk. Anti-Btk Western blots show normal Btk expression in the control samples but complete absence of expression in patient samples, thereby confirming the diagnosis of XLA. Expression of a control protein (in this case PKC-β1 - but normally β-actin) was normal. (**B**) In this case the patient (P) shows the presence of an abnormally migrating Btk band. The smaller molecular weight indicates a truncated species that was confirmed at the genetic level.

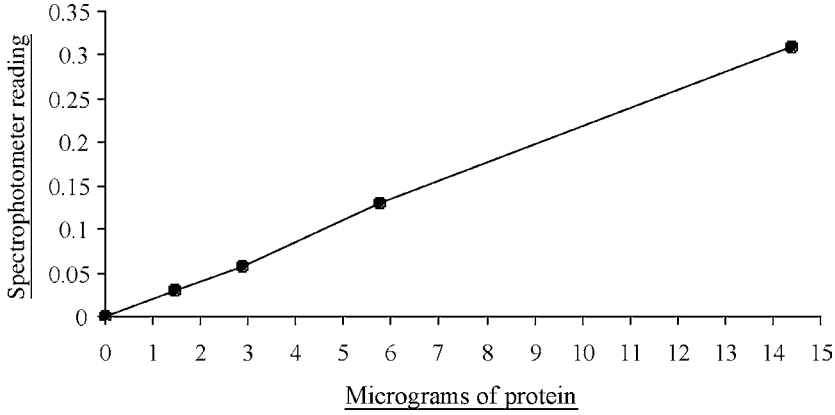


Fig. 4. Estimation of protein concentration in cell lysates following graphical representation of reference protein spectrophotometer readings. Using the linear relationship between protein concentration and spectrophotometer values, a cell lysate sample with an OD reading of 0.15 is estimated to have a concentration of approx 7 µg of protein/µL of lysate.

0.030 ÷ 1.44 = 0.0208

0.058 ÷ 2.88 = 0.0201

0.130 ÷ 5.76 = 0.0226

0.310 ÷ 14.4 = 0.0215

0.0850

0.0850 (4 = 0.0213 Divide each lysate reading by 0.0213 = µg protein/µL of lysate.

For example, sample lysate reading = 0.150

0.150 (0.0213 = Approximately 7 µg of protein/µL of lysate.

An alternative method is to present graphically the measured protein Ig spectrophometer values and extrapolate each sample reading to obtain protein concentration (*see* **Fig. 4**).

5. If the lysate contains traces of hemoglobin derived from blood sample preparation (*see* **Subheading 3.1.1.**), the resulting OD will be inaccurate; therefore these samples cannot be quantified by this method.

6. Improving band signal strength when using the immunoprecipitation technique can be obtained by increasing initial protein concentration or lysate volume. In addition, a greater volume of immunoprecipitation antibody can improve signal. For many antibodies, 1 µL of antibody (at a concentration of 1µg/µL) per 100 µL of lysate is sufficient.
7. *Note:* Optimization of this procedure is advised by varying antibody concentration, incubation times, and protein A/G bead volume, in some cases, the elimination of the procedures in **Subheading 3.3.1.** Lysate "precleaning" may improve the intensity of the final protein signal obtained, although clarity of result may be reduced. This process can be more useful when using some antibodies rather than others.
8. Alternative blocking reagent can produce a cleaner/clearer blot depending on antibody used, for example, bovine serum albumen (BSA). The blocking of nonspecific binding may be improved by leaving the blot covered in blocking reagent overnight at 4°C.
9. The use of polyclonal antisera for immunoblotting may increase the chance of binding to antigen, as some protein epitopes can be destroyed during protein denaturation and therefore reduce binding of specific antibody.
10. Successful immunoblotting depends greatly on optimization of antibody concentrations. Prepare a number of primary antibody dilutions and incubate duplicates of the same cell lysate for 1 h. Wash as described in **Subheading 3.5.**, **step 2**; perform a number of secondary antibody dilutions; and incubate membranes for 45 min and detect as described in **Subheading 3.5.**, **steps 4–8**. Optimizing the secondary antibody can reduce the background "noise" of the membrane. In addition, do not incubate with secondary antibody for longer than 45 min, as this will increase background interference.
11. Initial immunoprecipitation experiments may produce blots containing large visualized bands around 50 and 25 kDa (depending on the secondary antibody used). This is caused by heavy and light chain visualization on the blot. To reduce this immunoglobulin band effect, lower the concentration of primary antibody during the immunoblotting procedure (*see* **Subheading 3.5.**), or use different species of antibody to immunoprecipitation and blot.
12. Simply repeating the ECL stage of the procedure may rectify a weakly visualized signal. Wash off old ECL reagents and repeat **Subheading 3.5.**, **step 5**.
13. On occasion, antibody binding may not occur as efficiently as expected, resulting in only faint or nonvisible bands after exposure to film. This may appear as a lack of protein within the sample. By washing the blot to remove ECL reagents followed by reincubation with secondary antibody for approx 1 h, a signal may be obtained.
14. Following on from **Notes 12** and **13**, a lack of signal could also be caused by lack of primary antibody binding. Reincubation of the membrane with primary antibody first requires the blot to be stripped of previous antibody. Wash the blot once in PBS-T and place in stripping buffer (41 mL of water, 3.12 mL of Tris, pH 6.7–6.8; 5 mL of 20% SDS solution, 0.347 mL of β-mercaptoethanol).

Leave the blot at 50°C for a maximum of 30 min. If the signal was very weak, reduce the incubation time as the membrane may become stripped of protein. Remove the blot, wash 5 times with PBS-T, and block in milk for 30 min at room temperature. Follow on with immunoblotting (*see* **Subheading 3.5.**). Alternatively incubate with primary antibody over night at 4°C or for 1 h at 37°C.

15. ECL reagents have a limited shelf life; therefore protein signals may be obtained from previously negative blots by using new ECL reagents. ECL degradation may be identified by the need for an increased film exposure time to obtain a visible result.
16. Insufficient band signal film exposure may also be due to film processor chemical degradation that can occur when fixer and developer eventually become exposed to too much light or generally go past their shelf life. Regular film processor maintenance and the changing of developer and fixer chemicals can avoid this problem.
17. Membranes can be stored at 4°C covered in Saran Wrap (to maintain their moisture) for 6–12 mo. These blots can be reused for immunoblotting with other antibody combinations.

Acknowledgments

The authors thank the Clinical Immunology Laboratory at Great Ormond Street Hospital NHS Trust for their help in drawing up the standard operating procedures for analysis of CD40 ligand deficiency. We would also like to thank Dr. S. Tsukada and Dr. K. Nichols for their kind gifts of anti-Btk and anti-SAP antibodies respectively. David Eastwood is supported by a grant from the Primary Immunodeficiency Association, to whom we are extremely grateful.

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8

Molecular Techniques to Improve Outcome in Childhood ALL

Frederik W. van Delft and Vaskar Saha

1. Introduction

During the last two decades, survival in cases of childhood acute lymphoblastic leukemia (ALL) has improved from 50% to approx 80%. This has been achieved primarily by intensifying therapy, particularly for high-risk groups ***(1)***. During this period, biological features of the disease have been investigated for prognostic significance, and along with clinical features, define patient groups for risk-adapted therapy ***(2)***. Although the prognostic significance of these variables is dependent on the type and intensity of the treatment regimen, in parallel there have been unprecedented advances made in our understanding of the biology of the disease ***(3–5)***.

The concept that double-stranded DNA could be denatured, and on annealing complementary nucleotides would hybridize to each other was inherent to the Watson–Crick model of DNA. It was the ability to use this principle by immobilizing DNA to a solid phase and hybridizing in a liquid phase, as demonstrated by Ed Southern in 1975, that ushered in the era of the modern understanding of the genetic changes of the leukemic cell. The next step forward at the end of the 1980s was the development of the polymerase chain reaction (PCR). Elaborate modifications of these techniques such as the use of fluorescence rather than radioactive primers and probes and high-throughput technology with automation led to the ambitious Human Genome Project. Cancer researchers have been quick to adapt the tools and technologies unleashed by this task and in a few years have moved from a unigene to a genomic understanding of cancer. So far the biological tools have had little influence on the treatment of ALL and its outcome. This is about to change.

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

2. Chromosomal Analyses

In 1960, Nowell and Hungerford observed an unusually small G-group chromosome that they named the Philadelphia chromosome. It was only in 1973, after G-banding techniques had been introduced, that Janet Rowley showed that the Philadelphia chromosome was the result of a balanced translocation between the long arms of chromosomes 9 and 22. As the technique became more refined, more numerical and structural abnormalities were identified. The identification of these nonrandom chromosomal abnormalities in association with specific leukemia subtypes underpins our understanding of the pathogenesis of the disease. A database that collects and catalogs these changes is available at http://cgap.nci.nih.gov/Chromosomes/Mitelman.

It was in the 1970s as well that the use of combination chemotherapy began to produce cures in childhood ALL. The first observation that the chromosomal changes within the leukemic cell could influence the outcome of chemotherapy was made in the late 1970s when Seckel-Walker reported that children with hyperdiploid lymphoblasts appeared to have a better outcome *(6)*. As shown in **Table 1**, intense investigation of chromosomal changes in leukemic cells over the next decade identified a number of nonrandom chromosomal translocations associated with childhood ALL *(7)*. Junctional breakpoint analysis of these translocations revealed that they resulted in either juxtaposition or an in-frame fusion of two genes from disparate chromosomal territories *(7)*. The genes involved are either transcription factors or kinases involved in cell regulation.

3. Fluorescent *In Situ* Hybridization (FISH)

The Human Genome Project has generated a large library of probes that are being used to map chromosomal breakpoints, offering cytogeneticists more powerful analytical tools. Chromosomes fixed onto a glass slide are the equivalent of fixing DNA onto a solid state. Sequence-specific fluorescently labeled DNA probes can then be hybridized and visualized using a fluorescent microscope. Although G-banding is still the mainstay of cytogenetic analysis, it has its limitations. Metaphases obtained in lymphoblasts are short and stubby and G-banding may result in indistinct morphology. In our laboratory, we are also unable to obtain metaphase spreads in about 6% of cases and therefore no structural analyses are possible. In such cases, interphase FISH with specific probes can often be diagnostic. FISH has the advantage that the aberrant signals are visualized within the abnormal cell, enabling the observer to ignore the background of normal cells. Centromeric probes are ideally suited to the detection of whole chromosome gains seen in hyperdiploidy. Similarly G-banding is unable to pick out subtle telomeric changes. An example of this is the most common

Table 1
Genetic Subtypes of Childhood ALL

Subgroup	Frequency (%)	Estimated 5-yr event-free survival (%)
B cell (rearranged *MYC*)	2	75–85
B-cell precursor		
Hyperdiploidy >50	25	80–90
TEL-AML1	22	85–90
E2A-PBX1	5	75–85
BCR-ABL	4	20–40
MLL-AF4	2	20–35
Hypodiploidy <45	1	25–40
MLL rearrangement	5	30–50
T cell		
HOX11	3	80–90
TAL1	6.5	30–40
LYL1	1	30–40

translocation seen in childhood ALL, the t(12;21)(p13;q22). This is rarely detected by G-banding, and in most centers ALL samples are routinely probed using FISH for this translocation. FISH studies of the t(12;21) have also played a role in our understanding of the pathogenesis of the disease. The translocation results in a fusion of *TEL* on 12p with *AML* on 21q ***(8)***. In approx 40% of patients, FISH reveals the loss of the second *TEL* allele ***(9)***. Careful analysis using loss of hereozygosity and expression studies has shown that in patients with a *TEL–AML1* fusion, there appears to be no normal *TEL* expression ***(10)***.

Where specific probes are not available whole chromosome paints may be used. These were initially derived from flow-sorted chromosomes or mouse somatic radiation hybrids. These days, fluorescently labeled DNA probe pools for each chromosome are commercially available. These can be labeled with different dyes or assigned pseudocolors and detected either by laser (multiplex fluorescent *in situ* hybridization [M-FISH]) or by an interferometer (spectral karyotyping [SKY]). The images are digitized and a pseudocolor is assigned to each chromosome. The computer then displays this as an image showing each chromosome as a different color. Not only does this allow confirmation or correction of a tentative karyotype but it has proved to be useful in identifying translocations not detected by traditional methods ***(11,12)***. The technique of comparative genomic hybridization (CGH), on the other hand, has not contributed largely to our understanding of ALL.

Chromosomal analyses classify hyperdiploidy and the t(12;21) into the favorable and near haploidy and the t(9;22) into the poor prognostic group in childhood ALL. However, even in the so-called favorable group, children relapse after receiving what should have been adequate therapy. The strongest predictive factor to date has been the speed of response to therapy during the first 4–5 wk of therapy (induction) ***(13)***. Morphological assessment using light microsocopy to identify blasts suggests that those who have low levels of disease by wk 2 and no detectable disease at the end of induction have a favorable outcome. Morphology is at best able to detect disease at a 1–5% level, and as discussed later, one needs to be able to detect disease at the 10^{-4} level to be able to identify therapeutically significant risk groups ***(14)***. FISH is not suitable for this task. First, using a single probe, FISH detects disease at the 1–0.1% level. The majority of children who will ultimately relapse will have disease below this level at the end of induction and will therefore be negative by FISH. Southern blotting, in which digested tumor DNA is fixed on a membrane and probed with a gene-specific probe, is more sensitive than FISH but is still only at the 10^{-2}–10^{-3} level. Second, for the large-scale screening required for clinical trials, the technique(s) used must permit the monitoring of at least 90% of cases. Again given the heterogeneity of chromosomal changes, neither Southern blotting nor FISH is applicable.

4. Polymerase Chain Reaction (PCR)

PCR has the capacity to tackle the sensitivity issue. This technique enzymatically amplifies in a cyclical fashion a piece of DNA. From the second cycle, each cycle doubles the product, and theoretically at the end of a 40-cycle PCR reaction it is possible to amplify the target by 10^{12}. The product can then be visualized using a variety of techniques. The whole process lends itself to automation and high throughput. There are two possible targets for amplification in lymphoblasts. The most obvious are the fusion genes created by chromosomal translocations. The chimaeric product can be amplified by first reverse transcribing RNA and then amplifying (RT-PCR). Often the chimaeric product is present in abundance and the sensitivity of the PCR technique can approach one in a million cells ***(15)***. The BIOMED-1 concerted action has standardized the RT-PCR analyses of the commonly occurring fusion gene transcripts in leukemia ***(16)***. The use of standardized primer pairs and PCR conditions makes it easy to replicate the results between laboratories. In translocations in which there is no fusion but a juxtaposition of two genes, primers can be designed to amplify the genomic fusion point, and this can be used to track the clone. This is not always possible, as the breakpoint cluster regions may be quite large and carry repeats. Some genes such as *MLL* have numerous partners ***(17)*** and it is not always possible to have suitable primer pairs of probes

to identify all partner genes. Moreover, approx 30% of ALLs do not have identifiable chromosomal translocation that can be used as a target. For a test to be able to monitor patients on a trial, it needs to be able to do so in at least 90% of patients. This means that as a single strategy, the use of RT-PCR lacks sufficient power to be used in clinical trials in ALL.

5. Minimal Residual Disease

Modal changes and translocations are not the only changes taking place in the genome of the lymphoblast. In the majority of cases, these cells were destined to be either T or B cells and have undergone a maturation arrest. During the early differentiation of B and T cells, the germline variable (V), diversity (D), and joining (J) gene segments of the immunoglobulin (Ig) and T-cell receptor (TCR) gene complexes undergo rearrangement under the control of the V(D)J recombinase enzyme system. The random insertion/deletion of nucleotides at the junction sites of the V, D, and J gene segments is unique for each cell. In the majority of childhood ALLs differentiation arrest occurs after these rearrangements have taken place. As disease is clonal, it is possible to use these as targets for PCR-based techniques to monitor disease. Curiously, the majority of leukemias of B-cell origin also have a clonal TCR rearrangement. This has allowed the development of PCR-based strategies to detect and monitor disease levels in response to therapy ***(18)***. The problems of clonal evolution caused by secondary rearrangement processes mediated via ongoing activity of the V(D)J recombinase enzyme system have been largely circumvented by using a panel of primers able to detect clonality in Ig heavy chain (IgH), Ig kappa light chain (Igκ), the kappa deleting element (IgK-κde), and T-cell gamma (TCRγ) and delta (TCRδ) gene rearrangements ***(19)***. PCR products obtained are analyzed to confirm that they originate from the malignant clone and not from the normal cell population. The products are then sequenced to allow the design of a junction-specific oligonucleotide. This can be used either as a probe or as PCR primer to detect the clone in subsequent samples. Such targets can be identified in >95% of patients and allow disease to be tracked in approx 90%. The sensitivity of these techniques is approx 10^{-4}–10^{-5}. Several studies have now shown the application of these techniques to monitor response to therapy in childhood ALL. Multivariate analyses have shown that the level of MRD after induction is the single most powerful prognostic factor, independent of age, blast count, and chromosomal rearrangements ***(20)***. It is able to distinguish between low-risk patients who are MRD negative and have a 5-yr relapse rate of 2% and an intermediate group who have an MRD level of $<10^{-3}$ and a 5-yr relapse rate of 22% from a high-risk group with an MRD level of $\geq 10^{2}$ and a 5-yr relapse rate of 80% ***(21)***. Moreover, there is also evidence to suggest that a low or nondetectable disease level,

as measured by MRD prior to bone marrow transplantation, is a prerequisite for successful outcome in ALL patients ***(22,23)***.

If the speed of response to treatment is the critical factor, then we need to go one step further and calculate the slope of response. Standard PCR, or end point PCR, has the ability to amplify target DNA up to a plateau, but it is not possible to determine exactly where this is reached using standard techniques. Consequently it is not possible to determine precisely the initial amount of target DNA. Real-time PCR (RQ-PCR) allows this to be achieved as analysis is performed at the start of the exponential phase of amplification, when reaction components are not limiting. This permits accurate quantification over a wider dynamic range than end point quantitation assays permit ***(24)***. The chief drawback of the technique is the cost of the fluorescent probe and the detection systems. If probes have to be designed for each patient, this would preclude their use in a large-scale clinical trial. However, consensus probes that can be used for the majority of patients are being devised and standardized across laboratories in Europe. It is probable that many of the large clinical trials in childhood will incorporate the use of MRD to stratify therapy and some of these will use RQ-PCR. Those with persisting disease at the molecular level will receive more aggressive therapy and there is the potential to decrease therapy for those who are quick to become MRD negative.

6. Gene Expression Analysis

So far we have been discussing molecular techniques that have helped in identifying different types of ALLs (cytogenetics) and in the molecular monitoring of disease levels. The latter approach will allow stratification of therapy and a more stringent risk classification. Analysis of gene expression, using microarrays, in ALL is bringing a new understanding of the disease process.

As mentioned earlier, chromosomal translocations target genes that appear to be transcription factors or kinases. One such kinase is ABL. The t(9;22) translocation results in the fusion of the gene *BCR* to *ABL*, and results in the activation of the ABL tyrosine kinase. Imatinib mesylate is a small tyrosine kinase inhibitor that is active against the tyrosine kinase subclass III family, which includes ABL, c-KIT, PDGF, and ARG ***(25)*** Imatinib occupies the kinase pocket, prevents access to ATP, and therefore prevents phosphorylation of substrate. Clinically when used as a single agent, it results in sustained remission in Ph+ chronic myeloid leukaemia (CML) in chronic phase and short remissions in CML in blast phase as well as in Ph+ ALL patients ***(26,27)***. In the latter category, drug resistance is largely due to mutations in the *BCR–ABL* fusion gene or gene amplification occurring in a highly proliferative cell population. This is the first time that therapy has been designed to target specifically the expression of an abnormal gene and it is remarkable that "switching off"

the expression of the gene product stops proliferation and induces apoptosis and in some instances loss of the malignant clone ***(25)***.

To design more such molecules, we need to have a better understanding of the genes dysregulated in ALL. This task has been made easier by the advent of microarrays. Basically, human genes, either as cDNAs or oligonucleotides, are spotted onto a solid phase (nylon membranes or glass slides). Currently almost 25,000 genes, half the predicted human genome, are available as arrayed slides. Hybridization of leukemic RNA to these arrays provides an expression profile of each type of leukemia. Predictably the arrays distinguish clearly between different types of ALL and AML ***(28)***, as these are differentiated by cell surface markers that in turn reflect the unique genetic signature of the cell. However, the expression analyses are also able to identify with a high degree of accuracy the chromosomal rearrangements seen in B lineage ALL ***(29)***. Seventy percent of genes found to be expressed in hyperdiploid leukemias are encoded by chromosomes 21 and X. This is not explained by numerical abnormalities of these chromosomes, but may indicate a regulatory mechanism that operates at the level of the whole chromosome ***(29)***. B-ALL cases that lacked the recurrent B-ALL chromosomal abnormalities shared a large gene expression signature that distinguished them from other leukemic subtypes. These case constitute a novel leukemic sybtype for which the oncogenic event is currently unknown. Finally, 20% of B lineage ALL cases could not be sorted into any currently known leukemic subtype by gene expression. As some chromosomal translocations in B-ALL are infrequent, it may be necessary to profile many more ALLs, possibly thousands, to define rare leukemia subtypes to which these cases belong ***(29)***.

In contrast to the high frequency of recurrent translocations in B-ALL, only 30% of T-ALL cases have chromosomal translocations. Gene expression profiling of T-ALL provides an explanation for this disparity ***(30)***. The oncogenes *HOX11*, *TAL1*, and *LYL1* seen to be overexpressed in T-cell ALLs with translocations are also overexpressed in T-cell leukemias that do not appear to have an identifiable translocation, suggesting a common pathway for leukemogenesis in T-cell ALL ***(30)***.

Another technique that can be used for similar studies is serial analyses of gene expression (SAGE) ***(31)***. This has the advantage of using RNA from the tissue type being studied as the source and therefore has the potential to identify expression differences of genes not currently spotted on available arrays. In brief, the method is based on the isolation of unique sequence tags from individual transcripts and concatenation of tags serially into long DNA molecules. Rapid sequencing of the concatemer clones reveals individual clones, allowing quantification and identification of the gene expression signature of the tissue under study.

From a clinical standpoint these gene expression analyses represent a quick and viable alternative to immunophenotyping, cytogenetics, and FISH in diagnosing leukemic subtypes, particularly as they are able to identify cryptic changes. The fact that they also predict treatment outcome makes this an attractive candidate for single-platform analyses. As more data become available, it is likely that a customized "leukemia" chip will establish diagnosis and replace MRD in the risk stratification for therapy.

The greater hope is that these studies will go on to identify new "smart" molecules for ALL therapy. The marked differences in expression profiles in ALL suggest different pathways. As only a limited number of growth control mechanisms need to be subverted to result in cellular transformation, these multiple pathways may converge onto common regulatory mechanisms, which when dysregulated lead to ALL and which in turn are potential targets for molecular therapy.

7. The Host Polymorphism

Until now we have been discussing the molecular advances in understanding the biology of the disease and the advances in monitoring therapeutic response. However, host-related factors such as pharmacodynamics and pharmacogenomics can strongly affect treatment effectiveness and influence clinical outcomes in individual patients. A diversity of genes encode for drug-metabolizing enzymes, drug transporters, or drug receptors. Polymorphisms in these genes can affect the kinetics and dynamics of numerous drugs and result in interindividual variations in drug response, toxicity, and clinical outcome. The most well known of this is the polymorphism of the thiopurine methyltransferase enzyme. This enzyme methylates thiopurines, preventing the incorporation of metabolites into the nucleus. Patients who have a homozygous or heterozygous deficiency of the enzyme tend to have a better therapeutic response because of the higher levels of thipourine metabolites achieved ***(32)***. However, they also have increased hematopoietic and hepatic toxicities, and homozygous-deficient patients may even have a fatal outcome if treated with standard doses of thiopurines ***(33)***. These children also have an increased incidence of developing therapy-related acute myeloid leukemia ***(34)*** and irradiation-induced brain tumors ***(35)*** as a consequence of antimetabolite therapy. Glutathione *S*-transferase (GST) detoxifies a wide range of anticancer drugs by catalyzing their conjugation to glutathione, and the absence of both alleles in the *GSTM1*, *GSTT1*, and the *GSTP1 Val105/Val105* genotypes are associated with a lower rate of relapse ***(36)***. Similarly polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) enzyme may result in increased toxicity and sensitivity to methotrexate ***(37)***. In addition to these enzymes, polymorphisms in the cytochrome P450 (CY P450) enzyme systems, which

detoxify many drugs, may lead to unwanted drug interactions. For example, anticonvulsants such as phenytoin activate CY P450 and result in lower than expected levels of chemotherapeutic agents. Similarly azoles can suppress the activity of CY P450, leading to increased toxicity with vinca alkaloids, anthracyclines, and epipodophyllotoxins ***(38)***. Clearly, forthcoming trials in childhood leukemia will begin to screen children prospectively for polymorphisms and devise strategies to decrease toxicity and improve outcome.

8. The Future

We are moving from the genomic to the proteomics era. To understand the disease process and to be able to manipulate the outcome at a biological level we will need to understand the posttranslational changes that occur in the transcriptome and the effect of oncogenic processes on the function of these proteins. Time-of-flight mass spectrophotometry has the potential for being adapted to assess many of the parameters discussed in this chapter and indeed may replace most gene-based molecular techniques in the years to come ***(39)***. Proteomic chips for detection of cancer cells are already available and offer considerable promise as they are able to detect cancer cells in body fluids and do not require tumor tissue ***(40)***. For example, prostate cancer can be detected from urine samples or bowel cancer from a fecal specimen. It is not difficult to imagine that it could be adapted to examine for minimal residual disease in children with ALL from a sample of peripheral blood rather than bone marrow.

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9

Molecular Cytogenetics in Childhood Leukemia

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1. Introduction

In the last decade molecular cytogenetics, or fluorescence *in situ* hybridization (FISH), has become an important complementary procedure to routine chromosomal analysis. The most significant consequence from cytogenetic studies in childhood leukemia has been the association of specific chromosomal abnormalities with different patient subgroups, particularly in relation to prognosis. In childhood acute myeloid leukemia (AML), the rearrangements t(8;21)(q22;q22), t(15;17)(q22;q12), and inv(16)(p13q22) are associated with a good outcome. Conversely, deletions of the long arm of chromosome 5, monosomy 5 or 7, in association with a complex karyotype, are related to a poor prognosis ***(1)***. Karyotypes with a favorable outcome in childhood acute lymphoblastic leukemia (ALL) include high hyperdiploidy (51–65 chromosomes) and the translocation, t(12;21)(p13;q22). The Philadelphia translocation, t(9;22)(q34;q11), rearrangements involving the *MLL* gene and near haploidy (23–29 chromosomes) are associated with a short overall survival ***(2)***. Metaphase and interphase FISH are increasingly being used to screen routinely for such chromosomal abnormalities in childhood leukemia. Metaphase FISH also plays a role in the identification of new nonrandom chromosomal changes of prognostic significance.

This chapter provides details of a series of basic protocols for labeling of probes, slide preparation and pretreatment of target DNA, hybridization of probes to target DNA, and detection of signals that can be adapted for a range of FISH applications (**Fig. 1**). The preparations and pretreatments described relate specifically to these blood and bone marrow cell suspensions prepared

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

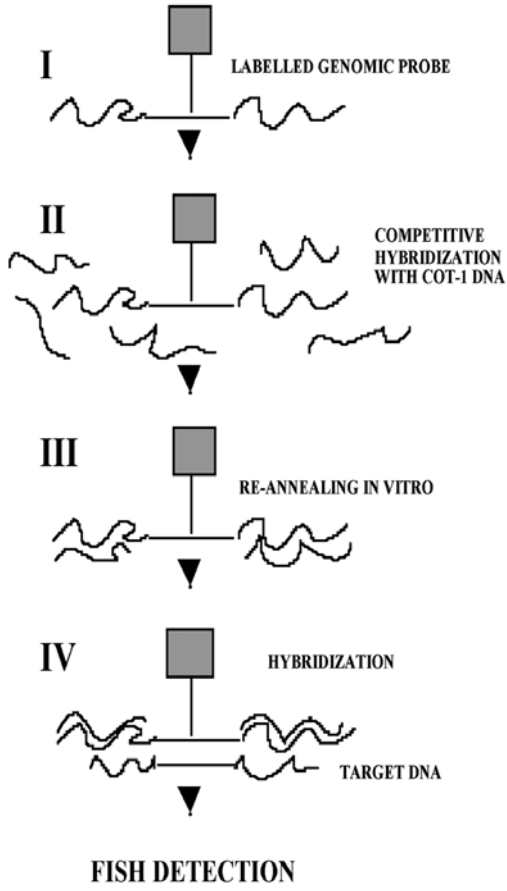


Fig. 1. Schematic illustration of the basic procedures of FISH.

for cytogenetic studies or as smears for morphological review. Procedures presented here are applicable to the three main types of probes: unique sequence, repetitive sequence, and chromosome painting probes ("paints"). An important consideration of successful FISH analysis is the appropriate choice of probe in relation to the abnormality under investigation and the target DNA being used (*see* **Note 1**).

2. Materials

2.1. Nick Translation Labeling of Probes

1. Purified probe: Either cosmid, P1 artificial chromosome (PAC), bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC) DNA (*see* **Note 1**).

2. 100 µg/mL of RNase A (Sigma R 4642, Poole, UK) (*see* **Note 2**).
3. Alternative labeling molecules (*see* **Note 3**):
   a. For direct labeling: 25 nmol of Cy3, Cy5 dUTP (Amersham Biosciences, Little Chalfont, UK).
   b. For indirect labeling: Biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics, Lewes, UK).
4. 1 *M* Tris, pH 8.0: 121.1 g of Tris base in 800 mL of distilled water. Concentrated HCl added to the desired pH (42 mL for pH 8.0) (*see* **Note 4**).
5. 10X Nick translation buffer: 0.5 *M* Tris-HCl (diluted from stock solution described above in **step 4**), 50 m*M* $MgCl_2$, 0.5 mg/mL of nuclease-free bovine serum albumin (BSA) (Sigma, Poole, UK).
6. 10X dAGC mix: 0.5 m*M* each dATP, dCTP, dGTP (Roche Diagnostics, Lewes, UK).
7. 0.1 *M* β-mercaptoethanol: 0.1 mL of β-mercaptoethanol in 14.4 mL of distilled water.
8. DNase I stock solution: 20,000 U/µL of DNase I (RNase-free Grade I pure, Roche Diagnostics, Lewes, UK).
9. DNase I dilution buffer: 50% Glycerol, 0.15 *M* NaCl, 20 m*M* sodium acetate, pH 5.0 (diluted from 3 *M* sodium acetate, *see* **Subheading 2.3.**).
10. 10 U/mL of DNA polymerase I (New England Biolabs, Bishops Stortford, UK).
11. 0.5 *M* EDTA, pH 8.0: 18.6 g disodium EDTA·$2H_2O$ added to 80 mL of distilled water and pH adjusted to 8.0 (*see* **Note 5**).
12. 10X TBE stock solution: 108 g of (89 m*M*) Tris base, 55 g of (89 m*M*) boric acid, 40 mL of 0.5 *M* EDTA, pH 8.0, in 1 L of distilled water.
13. Agarose gels: Agarose type I, low EEO (Sigma A-6013, Poole, UK) (*see* **Note 6**).
14. 10% Sodium dodecyl sulfate (SDS) stock solution: 100 g of electrophoresis grade SDS dissolved in 1 L of distilled water at 68°C (*see* **Note 7**).
15. Column buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 0.1% SDS (diluted from stock described in **step 14**).
16. Sephadex G-50 resin for spin columns: 30 g in 400 mL of column buffer (*see* **step 15**), hydrated at 95–100°C for 1.5 h (or 1 d at 37°C).

2.2. Preparation and Pretreatment of Target DNA (Chromosomes and Nuclei) on Slides

1. Fixative: Absolute methanol–glacial acetic acid 3:1, freshly prepared and cooled to 20°C prior to use.
2. 20X Saline sodium citrate (SSC) stock solution: 3 *M* NaCl, 0.3 *M* sodium citrate (175.3 g of NaCl and 88.2 g of sodium citrate pH adjusted to pH 7.0 with NaOH. Stored at room temperature.
3. 100 mg/mL of pepsin stock solution. Use this to make up:
   a. 30 µg/mL of working solution: 150 µL of pepsin stock in 50 mL of 0.01 *N* HCl, made up freshly before use.
   b. 50 µg/mL of working solution: 250 µL of pepsin stock in 50 mL of 0.01 *N* HCl, made up freshly before use.
4. 1X Phosphate-buffered saline (PBS): Either: 8 g of NaCl, 0.2 g of KCl, 1.4 g of Na_2HPO_4, and 0.24 KH_2PO_4 dissolved in 1 L of distilled water, pH adjusted to

7.4 with HCl and autoclaved before use, *or*: 10X PBS tablets (Sigma P-4417, Poole, UK) dissolved in 1 L of distilled water and stored at 4°C.
5. PBS–50 m*M* $MgCl_2$: 50 mL of 1 *M* $MgCl_2$ with 950 mL of 1X PBS added.
6. PBS–50 m*M* $MgCl_2$–1% formaldehyde: 2.7 mL of formaldehyde in 100 mL of PBS–$MgCl_2$, made up freshly each time (*see* **Note 8**).
7. Ethanol series: 70%, 95%, absolute ethanol. Made up weekly (absolute ethanol changed daily).

2.3. Hybridization

1. 70% (v/v) formamide, pH 7.0 (denaturing solution): 35 mL of purified formamide (Fluka, Gillingham, UK), 5 mL of 20X SSC, pH 5.2; 10 mL of distilled water. The pH must be checked and the solution must be made fresh (*see* **Note 8**).
2. 50% (v/v) formamide, pH 7.0 (for formamide washes): 75 mL of purified formamide (Fluka, Gillingham, UK) and 15 mL of 20X SSC, pH 5.2, mixed in 60 mL of distilled water. This makes enough wash solution for three Coplin jars and must be made fresh (*see* **Note 8**). Although using 20X SSC, pH 5.2, ensures that the resulting formamide solutions will be at approx pH 7.0, pH should be checked using pH paper.
3. 1 mg/mL *Cot-1* DNA (Gibco BRL, Paisley, UK).
4. 3 *M* sodium acetate: 40.8 g of sodium acetate·$3H_2O$ in 80 mL of distilled water, stirred until clear and pH adjusted to 8.0 with NaOH pellets. This dissolves only as it nears the correct pH. Make up to 100 mL, autoclave, and store at 4°C.
5. 50% Dextran sulfate stock solution: 10 g of dextran sulfate (sodium salt mol wt approx 500,000) (Sigma D-8906, Poole, UK) added slowly to a 50-mL sterile tube containing 10 mL 2X SSC (diluted from 20X SSC stock solution; *see* **Subheading 2.1.**), heated at 65°C, and vortex-mixed frequently until the dextran sulfate has dissolved (20–30 min). Make up to 20 mL with 2X SSC, aliquot, and freeze at –20°C.
6. Hybridization buffer: 10 mL of formamide AR (Fluka, Gillingham, UK), 4 mL of 50% dextran sulfate (*see* **step 5**), 2 mL of 20X SSC, pH 7.0; 2 mL of 10% Tween 20 (Sigma Poole, UK); and 2 mL of distilled water. Mix, aliquot, and store at –20°C (*see* **Note 8**).

2.4. Posthybridization Washes and Detection

1. SSCT: 2X SSC (diluted from 20X SSC stock solution, *see* **Subheading 2.2.**) with 0.05% Tween 20 (Sigma, Poole, UK).
2. Blocking solution: 3% BSA in SSCT (1.8 g of BSA (Sigma, Poole, UK) in 60 mL of SSCT.
3. Single-color detection (for biotin-labeled probes):
   a. 1 mg/mL of avidin-DCS (Vector Laboratories, Peterborough, UK).
   b. 0.5 mg/mL of biotinylated anti-avidin D (Vector Laboratories, Peterborough, UK).

4. Dual-color detection (for biotin and digoxigenin-labeled probes):
   a. Avidin-DCS-Texas red (Vector Laboratories, Peterborough, UK).
   b. 0.5 mg/mL of biotinylated anti-avidin D (Vector Laboratories, Peterborough, UK).
   c. Monoclonal anti-digoxigenin (Sigma D8156, Poole, UK).
   d. Rabbit anti-mouse immunoglobulin-fluorescein isothiocyanate (Ig-FITC) (Sigma F9137, Poole, UK).
   e. Monoclonal anti-rabbit-FITC (Sigma F4890, Poole, UK).
5. Diluent for antibodies: blocking solution (SSCT + 3% BSA), filtered through a 0.45-µm syringe filter. Store stock antibody solutions at –20°C (*see* **Note 9**).
6. Vectashield mountant: Vectashield (Vector Laboratories, Peterborough, UK) containing 1.0 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) (1 µL of 1 mg/mL stock solution) (Sigma, Poole, UK).

2.5. Viewing and Analysis of Results

1. Epifluorescence microscope with appropriate filter sets: For example, DAPI, FITC, Texas red, dual (FITC, Texas red), triple (DAPI, Texas red, FITC).
2. FISH imaging system (*see* **Note 10**).

3. Methods

3.1. Nick Translation Labeling of Probes

3.1.1. Labeling

1. Select DNA to be used as a probe (*see* **Note 1**).
2. Treat the DNA with RNase (optional; *see* **Note 2**): For each 1 µg of probe DNA add 200 ng of RNase A into an Eppendorf tube. Incubate the DNA for 30 min at 37°C in a water bath. Stop the digestion by placing the tube on ice.
3. Add the following (in order) to a new 1.5-mL Eppendorf tube on ice:
   a. 1 µg of probe DNA (RNA-free) (from **step 2**).
   b. For direct labeling: 1 µL (1 nmol) of Cy3 dUTP or 1 µL of Cy5 dUTP. For indirect labeling: 1 µL (1 m*M*) biotin-16-dUTP or digoxigenin-11-dUTP (*see* **Note 3**).
   c. 5 µL of 10X nick translation buffer.
   d. 5 µL of 10X dAGC mix.
   e. 5 µL of 0.1 *M* β mercaptoethanol.
   f. Sterile distilled water to make up to a final volume of 50 µL.
   g. 4 µL of DNase I. (Dilute stock DNase I solution 1:2000 with DNase I dilution buffer just before use: discard remainder after use.)
   h. 1 µL of 10 U/µL DNA polymerase I.
4. Mix well.
5. Incubate the tubes at 16°C in a water bath for 1.5–2 h.
6. Interrupt the reaction by placing the tubes on ice.

7. On agarose gel electrophoresis equipment, run a 5-µL aliquot on a 2% TBE agarose gel (2.1) at 100 V for 1 h, with *Phi*Xi74 *Hae*III as a size marker (*see* **Note 11**).
8. When the DNA is the correct size (approx 300 bp), completely arrest the reaction by adding 1.5 µL of 0.5 *M* EDTA and incubating at 68°C for 10 min.
9. Store at –20°C until required.
10. For chromosome "paints" (*see* **Note 12**).

3.1.2. Column Purification of Labeled Probe

1. Prepare Sephadex G-50 centrifuge columns by plugging 1-mL syringes with filter wool to the 0.1-mL mark. Add Sephadex G-50 solution gently while holding the syringe at an angle to avoid introducing air bubbles. Fill to the top of the syringe. Place the column in a 15-mL centrifuge tube and centrifuge at 100*g* for 5 min. Discard the eluate. Top up the column with more Sephadex G-50 solution and repeat the centrifugation (*see* **Note 13**).
2. Wash the column with 200 µL of column buffer and centrifuge at 100*g* for 5 min.
3. Repeat these washes five or six times.
4. Transfer the column to a new 15-mL centrifuge tube with an Eppendorf tube at the bottom to catch the eluate from the column. Load a maximum of 150 µL of the labeled probe onto the center of the column. Centrifuge at 100*g* for 5 min. This eluate contains the labeled probe.
5. Measure the probe concentration in a fluorometer. Store the purified, labeled probe at –20°C until required.

3.2. Preparation and Pretreatment of Target DNA

3.2.1. Slide Preparation of Cells Used for Cytogenetic Analysis

1. Slides for FISH will normally be made from cell suspensions prepared for cytogenetic analysis. Wash the cells in fresh fixative by adding fixative to tube and mixing the cell suspension thoroughly with a disposable pipet.
2. Centrifuge the tube at 200*g* for 5 min.
3. Remove the supernatant and replenish with fresh fixative to obtain the optimum cell suspension.
4. Slides for FISH are made by placing a small drop of cell suspension onto a restricted area of the slide and air-drying. The cells should not be too concentrated; nuclei should be flat and not overlapping (*see* **Note 14**).

3.2.2. Pretreatment of Target DNA on Slides

1. Fix freshly prepared bone marrow or peripheral blood smears in fresh fixative for 10 min (*see* **Notes 15** and **16**).
2. Put the slides through a graded alcohol series of 10 min each.
3. Air-dry the slides.
4. Place 100 µL of RNase A on slides under a 24 × 50 mm coverslip and incubate in a humid chamber (*see* **Note 17**) at 37°C for 1 h.

5. Wash twice (3 min each, with agitation) in 2X SSC (diluted from 20X SSC stock solution with distilled water; *see* **Subheading 2.2.**) at room temperature.
6. Incubate the slides in 30–50 µg/mL of pepsin at 37°C for 2–5 min depending on the amount of cytoplasm present (*see* **Note 18**).
7. Wash twice (5 min each) in 1X PBS with agitation.
8. Place slides in PBS–50 m*M* $MgCl_2$ for 5 min.
9. Fix in PBS–50 m*M* $MgCl_2$/1% formaldehyde for 10 min (*see* **Note 8**).
10. Wash in 1X PBS for 5 min with agitation.
11. Dehydrate slides through a graded alcohol series and allow to air-dry (*see* **Notes 19** and **20**).
12. Pretreatment may not be necessary for all slides, particularly if commercial probes are being used (*see* **Note 20**).

3.3. Hybridization

1. Heat 70% formamide to 70°C in a Coplin jar in a water bath under a fume hood. Place the slides in the heated formamide to denature the chromosomes for 2 min exactly (*see* **Notes 8**, **21**, and **22**).
2. Transfer the slides to ice-cold 70% ethanol. Leave for 4 min. Transfer to cold 90% ethanol and then 100% ethanol for 4 min each.
3. Air-dry the slides.
4. Dry down the appropriate concentration of probe and competitor either in a vacuum desiccator or by ethanol precipitation (*see* **Table 1**), for example, 100 ng of labeled cosmid or 400 ng–1 µg YAC, PAC with 2.5 µg (2.5 µL) *Cot-1* DNA (for cosmids), or 5 µg (for YACs, PACs), 0.1 volumes of 3 *M* sodium acetate, and 2 volumes of ice-cold ethanol.
5. Allow to precipitate for 1–2 h at –70°C.
6. Resuspend the pellet in 11 µL of hybridization buffer.
7. Denature the probe mixture at 95°C on a hotblock for 10 min. Plunge the tubes on ice for a few minutes, then centrifuge briefly in a microcentrifuge.
8. Reanneal the probe mixture at 37°C in a water bath for 15 min to 1 h.
9. Centrifuge the probe mixture quickly to pull the liquid to bottom of tube. Place this mixture on the slide containing denatured chromosomes and cover with a 22 × 32 mm coverslip (do not let the drop dry). Seal the coverslip with rubber solution and place the slides in a humid chamber (*see* **Note 17**) at 37°C to hybridize (*see* **Note 23**).

3.4. Posthybridization Washes and Detection

1. Carry out the following posthybridization washes (5 min each) at 42°C in a water bath in the fume hood:
   a. Three washes in 50% formamide (*see* **Note 24**).
   b. Three washes in 2X SSC (diluted from 20X SSC stock solution; *see* **Subheading 2.2.**).
   c. One wash in SSCT.

   For directly labeled probes, proceed directly to **step 12**.

Table 1
Amount of Labeled Probe and Unlabeled Competitor DNA for Hybridization (for 10 µL of Hybridization Mixture)

Type of probe	Labeled probe DNA	Unlabeled *Cot 1* DNA
Plasmid (single copy sequences)	100–200 ng	
Cosmid	50–100 ng	2.5 µg
PAC, P1	200–400 ng	3–5 µg
BAC	200–400 ng	3–5 µg
Total yeast DNA containing YAC	400 ng–1 µg	5–7.5 µg
Centromere (repeat sequences)	10 ng	
Paint, DOP-PCR amplified	100ng	5–7.5 µg

2. Place the slides in blocking solution for 10–30 min at room temperature.
3. Wash the slides briefly in SSCT at room temperature.
4. Drain the slides briefly without drying out. Apply the first detection layer:
   a. For biotin-labeled probes: Dilute 2.5 µL of stock avidin DCS-FITC in 1 mL of blocking solution (final concentration 5 µg/mL).
   b. For dual biotin- and digoxigenin-labeled probes: Dilute 1.5 µL of mouse monoclonal anti-digoxigenin and 1 µL of avidin–Texas red in 1 mL of blocking solution.
5. Place 100 µL of the respective solution on the slide. Cover with a 24 mm × 50 mm coverslip (*see* **Note 25**). Incubate at 37°C for 20 min.
6. Wash in SSCT at room temperature (RT) for 5 min. Drain the slides without drying out. Apply the second detection layer:
   a. For biotin-labeled probes: Dilute 10 µL of stock biotinylated anti-avidin D in 1 mL of blocking solution (final concentration 5 mg/mL).
   b. For dual biotin- and digoxigenin-labeled probes: Dilute 10 µL of stock biotinylated anti-avidin D and 1 mL of rabbit anti-mouse FITC in 1 mL of blocking solution.
7. Place 100 µL of the respective solution on the slide. Cover with a 24 mm × 50 mm coverslip. Incubate at 37°C for 20 min.
8. Wash in SSCT at RT for 5 min. Drain the slides without drying out. Apply the third detection layer:
   a. For biotin-labeled probes: Dilute 2.5 µL of stock avidin DCS–FITC in 1 mL of blocking solution (final concentration 5 µg/mL).
   b. For dual biotin- and digoxigenin-labeled probes:. Dilute 1 µL of avidin–Texas red and 10 µL of monoclonal anti-rabbit–FITC in 1 mL of blocking solution.
9. Place 100 µL of the respective solution on the slide. Cover with a 24 mm × 50 mm coverslip. Incubate at 37°C for 20 min.
10. Wash in SSCT at RT for 5 min.

11. Wash twice in 1X PBS for 5 min at room temperature.
12. Dehydrate the slides through the alcohol series.
13. Mount the slides in Vectashield with DAPI.
14. Cover with a 24 mm × 50 mm coverslip and seal with glue or nail varnish (*see* **Notes 26** and **27**).

3.5. Analysis of Results

1. Using the DAPI filter select metaphase or appropriate area of interphase cells using a low-power objective lens (×10 or ×20). Ignore damaged, overlapping, or closely clumped cells.
2. Move to the high-power objective lens (×100) and examine signals using the appropriate single- or dual-color bandpass filters.
3. Determine the hybridization efficiency (*see* **Note 28**) and cutoff levels for false-positive and false-negative results (*see* **Note 29**) for all probes being used.
4. In interphase single-color FISH score the number of signals in 200 nuclei, wherever possible. Beware of cross-hybridization (*see* **Note 30**).
5. In interphase dual-color FISH score the number of individual and fused red/green signals in 200 cells. Be aware of false-positive results (*see* **Note 29**).

4. Notes

1. The preparation of probe DNA for FISH can be carried out using standard alkaline lysis and purification by phenol–chloroform extraction or cesium chloride density centrifugation, using standard molecular biology protocols ***(3)***. This note also provides advice on the choice of probes for a range of applications (**Fig. 2**). Unique sequence probes target specific single-copy sequences. They may be cloned in a range of vectors from plasmids (1–10 kb) to PACs, BACs, and YACs (80 kb–1 Mb). These probes are used to detect defined chromosomal rearrangements in both interphase and metaphase. Interphase FISH has the advantage that it does not rely on the presence of metaphases. The most successful application of unique sequence probes has been in dual-color hybridizations, using probes for partner genes involved in translocations labeled in different colors. The presence of a resultant fusion gene is identified by the colocalization of two differentially colored signals within interphase nuclei or on the derived chromosome in metaphase cells. For example, the *TEL–AML1* fusion signal is evident on the derived chromosome 21 of the t(12;21)(p13;q22) (**Fig. 3**). Alternatively, for genes with multiple chromosomal partners, such as the *MLL* gene, two differently colored unique sequence probes, located to sequences on either side of the breakpoint, may be used, which separate as the result of a translocation. Chromosome-specific subtelomeric probes, with long and short arms labeled in different colors, are also included in the unique sequence probe category. They have been used to detect submicroscopic balanced chromosomal rearrangements ***(4)***. Repetitive sequence probes target the repeated sequences of, for example, individual chromosomal centromeres. Chromosome-specific centromere probes are appropriate for the rapid enumeration of numerical chromosomal abnormali-

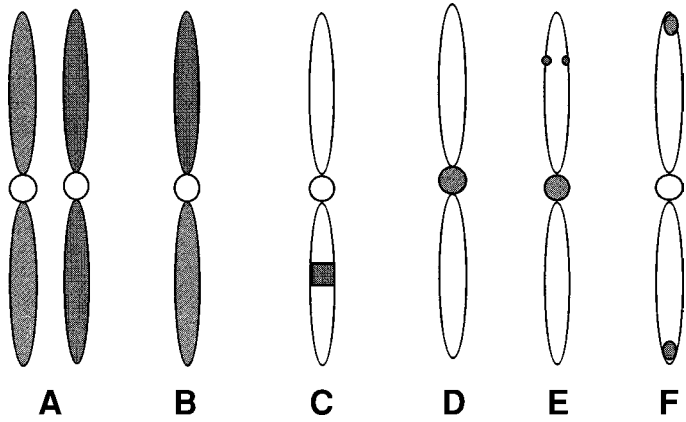


Fig. 2. Types of probes available for FISH. (**A**) Whole chromosome paints; (**B**) chromosome arm paints; (**C**) chromosome region specific paint; (**D**) chromosome specific centromeric repetitive sequence probe; (**E**) centromeric plus unique sequence probe; (**F**) subtelomeric probes.

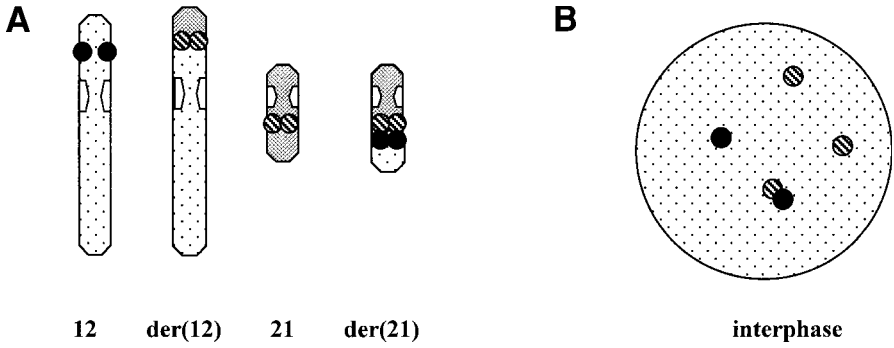


Fig. 3. *TEL–AML1* fusion with specific sequence probes. (**A**) Diagramatic representation of the translocation, t(12;21)(p13;q22), by dual-color FISH on metaphase chromosomes. The normal chromosome 12 (12) shows paired signals indicating the presence of the *ETV6* gene on the short arm (*solid circles*). This probe covers exons 1–4 only. The normal chromosome 21 (21) shows signals for the *AML1* gene (*hatched circles*). This probe spans the whole gene. As a result of the translocation, exons 1–4 of *ETV6* are moved onto the derived chromosome 21 [der(21)], therefore, no *ETV6* signal is observed on the derived chromosome 12. The *AML1* signal splits, part remains on the derived chromosome 21 [der(21)] which fuses with the *ETV6* signal, indicating the presence of the *ETV6–AML1* fusion gene, and part moves onto the der(12). (**B**) The individual *ETV6*, *AML1*, and fusion signals can be readily visualized in interphase.

ties in interphase and metaphase cells as they exhibit strong compact signals that are easily evaluated. "Paints" are derived from one chromosome type, arm, or region, generated by flow sorting or microdissection to produce a probe that highlights an entire chromosome or specified region. These probes are used to clarify cytogenetically visible structural or numerical abnormalities in metaphase. Commercially produced probes of excellent quality are now available for diagnosis of the majority of recurrent translocations and inversions in leukemia, proving a reliable alternative approach. These are provided with their own protocols designed to be simple and at the same time provide consistent results. However, it is important to be aware of the composition of commercial probes, particularly for interphase analysis. Commercial locus-specific probes are often a combination of cosmids or phage from the region of interest. These are not necessarily contiguous clones, and may give split signals in interphase nuclei. It is imperative that each probe is evaluated on a series of normal controls for the type of cell being investigated.

2. If probe DNA is prepared by alkaline lysis without any further purification, the DNA must be treated with RNase before nick translation is carried out. This step is not required for phenol–chloroform or cesium chloride purified DNA.
3. Indirect labeling methods (i.e., biotin- or digoxigenin-labeled probes detected by avidin–fluorochrome conjugates and anti-digoxigenin antibodies, respectively) give the most robust results. However, the sensitivity of FISH techniques is such that the detection of directly fluorochrome-labeled probes is now feasible. Directly labeled probes are essential for any quantitative analysis as they produce a lower level of background signals. However, direct labeling is most useful for probes that detect a large target (e.g., PACs, BACs), or a repetitive sequence (e.g., centromere probes) or whole chromosome paints. Some dyes, for example, Cy3.5 and Cy5.5, cannot be used as substrates for DNA polymerases, and therefore indirect labeling methods must be used. We find that labeling either indirectly with a hapten such as digoxigenin or biotin, or directly with fluorochrome requires essentially the same nick translation conditions. The basic protocols presented in this chapter assume the use of one or a maximum of two to be used in single- and dual-color hybridizations. These protocols, however, will allow the detection of up to four differentially colored chromosomes in a single hybridization using digoxigenin-FITC, Cy3 dUTP, biotin-Cy3.5, and Cy5-dUTP together.
4. Make up to 1 L, autoclave, and store at room temperature. The pH meter must have an electrode suitable for Tris.
5. The pH is adjusted to 8.0 with NaoH pellets. The solution must be stirred until the solution clears, as it dissolves only as it nears the correct pH. Make up to 100 mL, autoclave, and store at room temperature.
6. The desired amount of agarose should be added to a volume of 1X TBE (diluted from 10X TBE stock) sufficient to construct the gel. Agarose is melted in a microwave oven and cooled to 55°C in a water bath before pouring.
7. SDS powder is harmful by inhalation; therefore, avoid weighing out in the open laboratory. Buy 100-g lots and add the entire amount to water in a fume hood.

8. Formaldehyde is harmful and formamide causes harm to the unborn child; therefore, a fume hood should be used both for making up these solutions and for the incubation steps.
9. While in constant use, antibody stocks should be stored at 4°C. However, for prolonged storage, antibody stocks can be stored at –20°C. Avoid repeated freeze–thawing of antibodies, as this may promote deconjugation of fluorochrome. To overcome this, antibody solutions should be vortex-mixed and stored at 4°C for at least 10 min, then centrifuged in a microcentrifuge for 15 min and only the supernatant used.
10. The ease of storage and the ability to apply image processing have led to the increased popularity of digital imaging systems. However, the choice of imaging system needs to be carefully considered, depending on the application. In the early days of FISH imaging, highly cooled (–20°C) charge-coupled-device (CCD) cameras were widely employed. These are now considered to be unnecessary, with ambient temperature (+14°C), video-rated CCD cameras are suitable for most applications. Most FISH imaging packages include automated karyotyping and the ability to create a G-banded image from the DAPI stained chromosomes on which the probe signal can be overlaid. The multicolor FISH techniques, multiplex-FISH (M-FISH) ***(5)*** and spectral karyotyping (SKY) ***(6)***, both require sophisticated equipment and a dedicated computer. For M-FISH, a set of specific, narrow bandpass filter sets is required. Most major microscope manufacturers now incorporate a 7 or 8 position filter turret to accommodate such filter sets.
11. The correct fragment size is required for successful labeling. Running a gel to determine the size of fragments is the best method. The optimum size for labeled fragments is 100–500 bp (average 300 bp). If the DNA is larger than this it will need to be reincubated with DNase for a further 30 min. If the DNA is smaller than 100 bp, it needs to be relabeled, using a lower concentration of DNase 1.
12. An alternative method for labeling whole chromosome paints is amplification using a degenerate oligonucleotide primer (DOP-PCR) method ***(7)***. Sensitive methods of imaging in conjunction with carefully selected combinations of fluorochromes have led to multiple color painting and the ultimate achievement of visualizing the entire chromosome complement simultaneously in 24 different colors using only five fluorochromes. Both M-FISH and SKY, which discriminate fluorochrome combinations, have achieved this goal and successfully identified cryptic chromosomal abnormalities within complex and ill-defined karyotypes. The generation of combinatorially labeled probes for M-FISH or SKY is a complex procedure, beyond the scope of most diagnostic laboratories. High-quality commercially produced 24-color whole chromosome painting sets are now available (Vysis, Richmond, UK), which are simple to use and provide a reliable alternative. Dedicated computer software is also required that translates the unique labeling combinations for each chromosome into pseudocolors and generates multiple colored karyograms. This type of analysis remains restricted to specialized or research laboratories.

13. Purification of the labeled probe by running through a column in this way removes any unincorporated labeled fragments and thus increases the signal-to-noise ratio.
14. If the cells are too concentrated it may be necessary to add a few additional drops of fixative to the suspension. Alternatively, if they are too dilute the cells will need to be washed again and resuspended at a higher density. The size of the drop added to the slide is dependent on the number of cells required for analysis. For interphase FISH several drops may be placed on different areas of the side. These may be a number of drops from the same patient to whom a range of probes may be applied, or drops from different patients to be hybridized with the same probe. Applying the probes under small circular coverslips prevents mixing of probes during hybridization.
15. Sometimes smears may have a high proportion of contaminating red blood cells. These may be lysed by leaving the slides in fixative overnight at 4°C prior to pretreatment.
16. For blood or bone marrow smears previously stained in May Grünwald–Giemsa or Wright's stain the stain may be removed prior to use by incubating slides for 10 min in 10 m*M* Tris-HCl, pH 7.5 (diluted from stock solution, *see* **Subheading 2.1.**), 1 m*M* EDTA (diluted from stock solution, *see* **Subheading 2.1.**) for 5 min at 37°C before fixation. For smears older than 2 wk, this incubation should be extended to overnight.
17. The humid chamber can be a plastic microscope slide storage box with a lid (Raymond A. Lamb, Eastbourne, UK), floated in a water bath. In this case it is not necessary to add any moisture into the chamber. However, if hybridizing in a dry oven some moist (not wet) tissue will need to be placed in the bottom of the box with the slides suspended above this on a rack. Alternatively, immunology slide staining trays (Raymond A. Lamb, Eastbourne, UK) may be used, placed in a water bath.
18. To ensure that all the cytoplasm has been removed cells should be checked under phase contrast microscopyat this stage. If cytoplasm persists, **steps 4–10** of **Subheading 3.2.2.** may be repeated.
19. If not required immediately, slides can be stored desiccated at 4°C for up to 1 mo before use.
20. If after the slides are prepared there is no evidence of residual plasma membrane and the isolated nuclei or metaphases are flat (not phase bright) then the complete pretreatment protocol may not be necessary. In this case a shortened pretreatment may be carried out involving **steps 5** and **11** only of **Subheading 3.2.2.**
21. If slides are at room temperature prior to placing them into the heated formamide, the temperature of the formamide will decrease by 0.5°C for each slide used. The temperature should be adjusted accordingly. For example, for three slides, the formamide should be heated to 71.5–72°C. Alternatively, the slides may be heated in an oven or on a hotblock to 65–70°C for about 5–10 min before placing them in the denaturing solution.
22. Some laboratories prefer to denature target DNA in a small amount of 70% formamide held in contact with the cells under a sealed coverslip to reduce the risk of

harmful effects and eliminate the requirement for a fume hood. For this alternative procedure the slide with the encased formamide is placed in prewarmed 0.5X SSC (diluted from 20X SSC stock solution; *see* **Subheading 2.2.**) at 37°C for 15 min then transferred to 72°C in a water bath for at least another 15 min. The seal and coverslip are removed from the slide in 2X SSC (diluted from 20X SSC stock solution, *see* **Subheading 2.2.**) at room temperature.

23. Unique sequence probes normally require 1–2 d to hybridize completely; for repetitive sequence probes a few hours hybridization is sufficient. For large unique sequence probes (e.g., YACs) overnight hybridization is usually required. For commercial probes follow the instructions of the manufacturer.
24. The posthybridization washes may be carried out without formamide. Instead of 50% formamide the slide is washed in 0.5X SSC (diluted from 20X SSC stock solution; *see* **Subheading 2.2.**) at 72°C for 5 min, then SSCT (*see* **Subheading 2.4.**) at room temperature for 1 min. In this case the stringency of the wash has to be increased by raising the temperature from 42°C to 72°C. This type of wash may not be appropriate for small probes with reduced signals and it may increase the risk of cross-hybridization with repetitive sequence probes.
25. Alternatively a piece of Parafilm™ cut to size may be used, which is easier to remove and reduces the risk of damage to the cells.
26. If a high level of background signal can be detected then the coverslip can be removed and the slides rewashed. If the signals are weak a second round of detection, repeating **steps 3–15** of **Subheading 3.4.**, can be carried out.
27. It is important to protect light-sensitive probes, hybridized slides, antibodies, and DAPI from light.
28. Wherever possible, probes chosen for interphase analysis should have high hybridization efficiencies (>90%), particularly when being used to assess the presence of deletions. The hybridization efficiency is calculated by the application of the probe of interest, in combination with a second probe to act as an internal control, to a series of control samples of the same type as the experimental material. The hybridization efficiency is the percentage of cells in the control population that show the expected normal signal pattern.
29. It is important that cutoff levels for all interphase FISH analyses are defined for each probe used and by each individual scoring the results. This requires hybridization of the probes under test to a series of normal controls, to determine the mean. A useful cutoff level is the mean ± 3 standard deviations. When viewing probes in single color incomplete hybridization or the overlap of two signals when viewing a three-dimensional nucleus in two dimensions will lead to the false assessment of monosomy. This type of false-positive result occurs more frequently than false trisomy (which is caused by counting background fluorescent spots as true signals). Therefore the cutoff levels for loss of a signal are often higher. Cohybridization with a second control probe lowers the cutoff level considerably. The complexity of control probe should be of similar to that of the target, and localized to a region not likely to be involved in a chromosome rearrangement in the particular malignancy under investigation. When labeled with

two different florochromes, a fusion gene is identified by a red–green fusion signal representing the translocation, and a single red and green signal corresponding to the normal chromosome homologs. However, the chance colocalization of fluorescent signals resembles a red–green fusion signal and this can occur in up to 5% of normal cells. Thus cutoff levels to rule out a false-positive result must be established as described previously for single-copy probes, and applied when scoring diagnostic samples in which the same dual-color probes have been used.

30. It is important to be aware that certain chromosome-specific centromere probes that hybridize to the repetitive sequences of the alpha-satellite regions continue to cross-hybridize in spite of highly stringent washes. This applies in particular to chromosomes 1, 5 and 19, chromosomes 13 and 21, and chromosomes 14 and 22. This can be overcome by using a probe specific for the beta-satellite region of chromosome 1 and unique sequence probes for the other chromosomes when screening for numerical chromosomal changes.

Acknowledgments

The authors wish to thank the members of their respective laboratories for their contributions to protocol development. We wish to acknowledge the Medical Research Council, Leukaemia Research Fund, and Yorkshire Cancer Research for financial support.

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Fluorescent IgH Fingerprinting to Assess Minimal Residual Disease in Childhood B-Lineage ALL

Paul A. S. Evans and Roger G. Owen

1. Introduction

Minimal residual disease (MRD) refers to the presence of clonal cells in the bone marrow at a level below morphological detection. It is possible to detect low levels of bone marrow disease in acute lymphoblastic leukemia (ALL) by flow cytometry, ploidy studies, fluorescent *in situ* hybridization (FISH), Southern blotting, and reverse transcriptase-polymerase chain reaction (RT-PCR) for specific chromosomal translocations. These techniques, however, suffer from a lack of applicability and/or sensitivity ***(1)***. Clonal IgH rearrangements are the most applicable clonal markers in B-lineage ALL as they are demonstrable by simple PCR-based techniques in approx 90% of cases ***(2–4)*** .

Assessment of MRD in ALL has traditionally used two distinct approaches. The first involves sequencing of the IgH rearrangement and the generation of sequence- or allele-specific oligonucleotides (ASOs) that may be used as probes or PCR primers. These techniques are highly sensitive, as they are capable of detecting one leukemic cell in a background of 100,000 normal cells (0.001%) ***(5–10)***. Despite their sensitivity, ASO techniques have a number of disadvantages; they are time consuming, labor intensive, and expensive. They are not readily suited to routine laboratory practice and cannot easily provide results in real time for clinical decision-making. In addition, they may not be able to detect clonal change, which can occur in up to 30% of cases ***(11)***.

A second, non-sequence-based technique exploits the distinct size of the PCR product at presentation and compares this to PCR products amplified from follow-up samples. The most widely used technique in this type of analysis is IgH fingerprinting, which initially involved the resolution of radiolabeled PCR

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

products on denaturing polyacrylamide gels. This procedure is applicable to approx 70% of patients using a single consensus Framework 3 (Fr3) primer set and approx 90% of patients if VH family specific Framework 1 (Fr1) primers are also employed ***(2–4)***.

Fluorescence-based DNA fragment analysis and sequencing systems have now largely replaced radioisotope incorporation techniques. We originally described the use of this technology to size accurately IgH rearrangements in 1993 ***(12)***. The strategy involves end-labeling of the JH primer (via an amino–hexyl link) with a fluorochrome and analyzing the products with an automated fragment analyzer equipped with "genescanning" software ***(12–20)***. This technique is more sensitive than nested PCR strategies ***(18)***, achieving a sensitivity of at least 0.1% in the majority of cases ***(16)***. The system provides for accurate and reproducible sizing of PCR products, as the software is able to size rearrangements to within a single basepair. In addition, the system is able to size each peak within a fingerprint, which simplifies the identification of clonal rearrangements of known size when they are present within a polyclonal background. It is ideally suited to routine laboratory practice; as analysis of large numbers of samples is possible and results are generally available within one working day.

Fluorescent IgH PCR techniques are undoubtedly less sensitive than sequence-specific approaches. We have assessed this by directly comparing fluorescent IgH PCR with allele-specific oligonucleotide probing (ASOP) in 19 children with B-lineage ALL. In the analysis of logarithmic dilutions of presentation BM samples, ASOP was clearly more sensitive, achieving a sensitivity of 0.01% or greater in 94% of cases. However, comparable sensitivities were obtained in 38% of cases, the fluorescent Fr3 PCR achieving a sensitivity of 0.1% or greater in 88% of cases ***(16)***. In the same series of patients 57 follow-up samples were also examined using both techniques and concordant results were obtained in 93% of the samples. We have also sequentially assessed MRD in 42 children treated according to the MRC UKALL XI trial protocol and found positivity at wk 20 of therapy was highly predictive of relapse ***(17)***. These results correlate very well with those obtained by ASOP in a similarly treated group of children with standard risk B-lineage ALL ***(6)***.

In this chapter we describe the fluorescent IgH fingerprinting method and its application in the detection of MRD in childhood B-lineage ALL.

2. Materials

1. HEX-labeled consensus JH primer: ACCTGAGGAGACGGTGACCAGGGT
2. IgH Framework primers:
   a. Fr3 consensus primer: CCGAGGACACGGC(C/T)(C/G)TGTATTACTG

b. VH Fr1 family specific primers:
   VH1: CCTCAGTGAAGGTCTCCTGCAAGG
   VH2: GAGTCTGGTCCTGCGCTGGTGAAA
   VH3: GGTCCCTGAGACTCTCCTGTGCA
   VH4: TTCGGA(G/C)ACCCTGTCCCTCACCT
   VH5: GAGGTGAAAAAGCCCGGGGAGTCT
   VH6: CCTGTGCCATCTCCGGGGACAGTG
c. Globin control gene primers:
   Globin 1 primer: ACACAACTGTGTTTCACTAGC
   Globin 2 primer: CAACTTCATCCACGTTCACC

3. 10X PCR buffer: 100 m*M* Tris, pH 9.1; 500 m*M* KCl.
4. 25 m*M* $MgCl_2$ solution.
5. 25 m*M* dNTP solution.
6. *Taq* polymerase (Supertaq, HT Biotechnology Ltd., Cambridge UK).
7. Agarose.
8. Lambda *Hin*dIII marker (Gibco BRL).
9. Automated DNA sequencer with GENESCAN collection and analysis software (Applied Biosystems, Foster City, USA).
10. Fluorescent size standard (GENESCAN-500 ROX™, Applied Biosystems).
11. Glass plates with "load to read distances" of 12 cm and 24 cm (Applied Biosystems) and equipped with 0.4-mm spacers and 36-well combs.
12. 6% Polyacrylamide sequencing gel (Gibco BRL).
13. 0.1% Ammonium persulfate (APS) (Gibco BRL).
14. 1% Aqueous dextran blue (Pharmacia).
15. Formamide (Gibco BRL).
16. 10X TBE buffer (1.0 *M* Tris, 0.9 *M* boric acid, 0.01 *M* EDTA).
17. 0.4-mm Flat pipet tips (Bioquote Ltd., York, UK).

3. Methods

The methods described in the following subheadings outline (1) DNA extraction, (2) assessment of DNA quality using β-globin PCR, (3) PCR amplification across the *CDRIII* region of the IgH gene using a consensus Fr3 primer and family-specific Fr1 primers, (4) analysis of the products using both 373 and 377 ABI DNA sequencers with GENESCAN collection and analysis software, and (5) interpretation of electrophoretograms.

3.1. DNA Extraction

All established DNA extraction methods appear to be suitable for use with fluorescent fingerprinting techniques. DNA can be obtained if necessary from archival bone marrow smears but these specimens require extended digestion (at least 5 d) with proteinase K.

3.2. Assessment of DNA Quality Using β-Globin PCR

1. In a 1.6-mL Eppendorf tube make 1 mL "mastermix" containing: 2000 picomol of primers 1 and 2, 100 µL of 10X PCR buffer, 60 µL of $MgCl_2$ solution, 5 µL of dNTP solution, and 40 U of *Taq* polymerase. Molecular biology grade water to 1000 µL.
2. Add 1 µg of test DNA and 25 µL of mastermix to a labeled 0.6-mL PCR tube.
3. Overlay with mineral oil.
4. Recap the tube and amplify according to the following program: 95°C, 1 min; 65°C, 1 min; 72°C, 1 min (repeated for a total of 31 cycles); and 72°C, 10 min.
5. Run the products of each reaction on 2% agarose gel; a 355-bp fragment is produced when amplifiable DNA is present.
6. Include a water-only control with each batch of reactions.

3.3. Fluorescent PCR Amplification Across the CDRIII Region of the IgH Gene

Initially, all presentation samples are screened using the Fr3 consensus PCR strategy. This will detect a clonal rearrangement in up to 70% of presentation ALL cases. If a clonal rearrangement cannot be demonstrated, the VH family specific Fr1 PCR technique is used. By utilizing both PCR strategies it is possible to demonstrate clonal rearrangements in up to 90% of cases of childhood B-lineage ALL. This detection rate may be improved further by designing multiple primers to each of the three conserved framework region sequences. This has recently been addressed by a European Biomed concerted action project ***(21)***.

3.3.1. Fluorescent Framework 3 PCR

1. In a 1.6-mL Eppendorf tube make 1-mL "mastermix" containing: 400 picomol of Fr3 and JH consensus primers, 100 µL of 10X buffer, 60 µL of $MgCl_2$ solution, and 5 µL dNTP of solution. Molecular biology grade water to final volume of 1000 µL. This is sufficient for a complete gel of 36 samples.
2. Add approx 1 µg of test DNA to a labeled 0.6-mL PCR tube.
3. Add 20 µL of mastermix and overlay with mineral oil.
4. Add 1 U of *Taq* polymerase to 5 µL of mastermix.
5. Heat the reaction mixtures and hold at 95°C for 3 min (*see* **Note 1**).
6. Add 5 µL of diluted *Taq* polymerase to each tube under the oil layer.
7. Recap the tubes and amplify according to the following program (*see* **Note 2**): 95°C, 1 min; 60°C, 1 min 30 s (repeated for a total of 35 cycles); and 72°C, 10 min.
8. Controls: Three controls should be included with each batch of PCR reactions: normal peripheral blood DNA, a positive control at a dilution of 0.1%, and a water-only "PCR blank." The amplicons produced using this PCR strategy measure 60–165 bp in size.

3.3.2. Fluorescent Framework 1 Family-Specific PCR

1. In a 1.6-mL Eppendorf tube make 1-mL mastermix for each VH family containing: 400 picomol of VH family-specific primer and JH consensus primer, 100 µL of 10X buffer, 60 µL of $MgCl_2$ solution, and 5 µL of dNTP solution. Molecular biology grade water to 1000 µL.
2. Add approx 1 µg of test DNA to each of six labeled 0.6-µL PCR tubes.
3. Add 20 µL of VH family specific mastermix to the appropriately labeled tube and overlay with mineral oil.
4. Add 1 U of *Taq* polymerase to 5 µL of appropriate mastermix.
5. Heat the reaction mixtures and hold at 95°C for 3 min (*see* **Note 1**).
6. Add 5 µL of diluted *Taq* polymerase to appropriate tubes.
7. Recap the tubes and amplify according to the following program (*see* **Note 3**): 95°C, 1 min; 68°C, 1 min (reduced in consecutive cycles by 1°C to 62°C); 72°C, 1 min (repeated for a total of 35 cycles); 72°C, 10 min.
8. Controls: Include the following controls with each batch of reactions: normal peripheral blood DNA, a known VH3 rearrangement at a dilution of 0.1%, and a water-only PCR "blank." The amplicons produced using this PCR strategy measure 280–380 bp in size.

3.4. Fragment Analysis

3.4.1. Gel Preparation

1. Clean and assemble glass plates with 0.4-mm spacers. Lay the plates horizontally on a small box with the "rabbit ear plate" uppermost. Apply bulldog clips along each side.
2. Prepare the gel mixture as follows:
   a. 12-cm gel: 35 mL of polyacrylamide, 211 µL of APS.
   b. 24-cm gel: 50 mL of polyacrylamide, 300 µL of APS.
   Fr3 PCR products are analyzed on 12-cm gels and Fr1 products on 24-cm gels.
3. Pour the gel and insert a 36-well comb.
4. Allow the gel to polymerize for at least 1 h.

3.4.2. Sample Preparation, Loading, and Analysis

1. Prepare 4 µL of loading mixture for each sample consisting of 3 µL of formamide, 0.5 µL of fluorescent size standard, and 0.5 µL of aqueous dextran blue.
2. Add 1 µL of PCR product and 4 µL of loading mixture to a labeled 0.6-mL PCR tube.
3. Denature each mixture at 95°C for 3 min. Snap cool and hold at 4°C prior to loading.
4. Insert the gel into the analyzer with the "rabbit ear plate" innermost. Assemble the buffer tank and fill with 1X TBE.
5. Flush unpolymerized gel from the wells with a 200-µL pipet.
6. Prerun each gel for 10 min.

7. Load samples from left to right using 0.4-mm flat pipet tips avoiding carry over to the next well.
8. Run 12-cm gels for 2 h and 24-cm gels for 8 h.
9. Analyze the data with GENESCAN collection and analysis software according to the manufacturer's instructions.
10. Store the gel image, sample data, and electrophoretograms on an optical disk (or equivalent) for future reference.

3.5. Interpretation of Electrophoretograms

The GENESCAN collection and analysis software converts the gel image for each sample into what is termed an "electrophoretogram" that consists of a number of peaks, the heights of which correspond to the intensity of fluorescence on the gel. A polyclonal pattern, or fingerprint, appears as a normally distributed series of peaks, each separated by 3 bp. Clonal rearrangements appear as distinct peaks either alone or within a polyclonal background (*see* **Fig. 1** and **Note 4**).

3.5.1. Definition of a Clone Size at Presentation

Good quality presentation material is essential for the demonstration and sizing of clonal rearrangements. The lack of a clearly defined clonal rearrangement at presentation precludes subsequent MRD analysis. In practice clonal rearrangements are easily identified as single peaks with no evidence of a polyclonal background (**Fig. 1A**). Multiple rearrangements are common (40%) and all may be sized and used in subsequent analysis. Overamplification of clonal rearrangements is commonly observed. In such instances the PCR products should be diluted and the fragment analysis repeated as this provides a more accurate value for the product size.

3.5.2. Defining a Positive Result in Follow-Up Material

A follow-up sample is considered positive when a peak of a size identical to that demonstrated in the presentation material is detected in a follow up bone marrow specimen (*see* **Fig. 2** and **Note 5**). This should have a level of fluorescence of least 250 absorbance units (fivefold higher than background) and a peak height twice that of adjacent peaks when it is present within a polyclonal background ***(17)***.

3.5.3. Definition of a New Clone in Follow-Up Material

New clones can emerge over time in ALL, usually by the process of VH–VH replacement. Such rearrangements will, by their very nature, be missed by sequence-specific MRD approaches. New clones are similarly

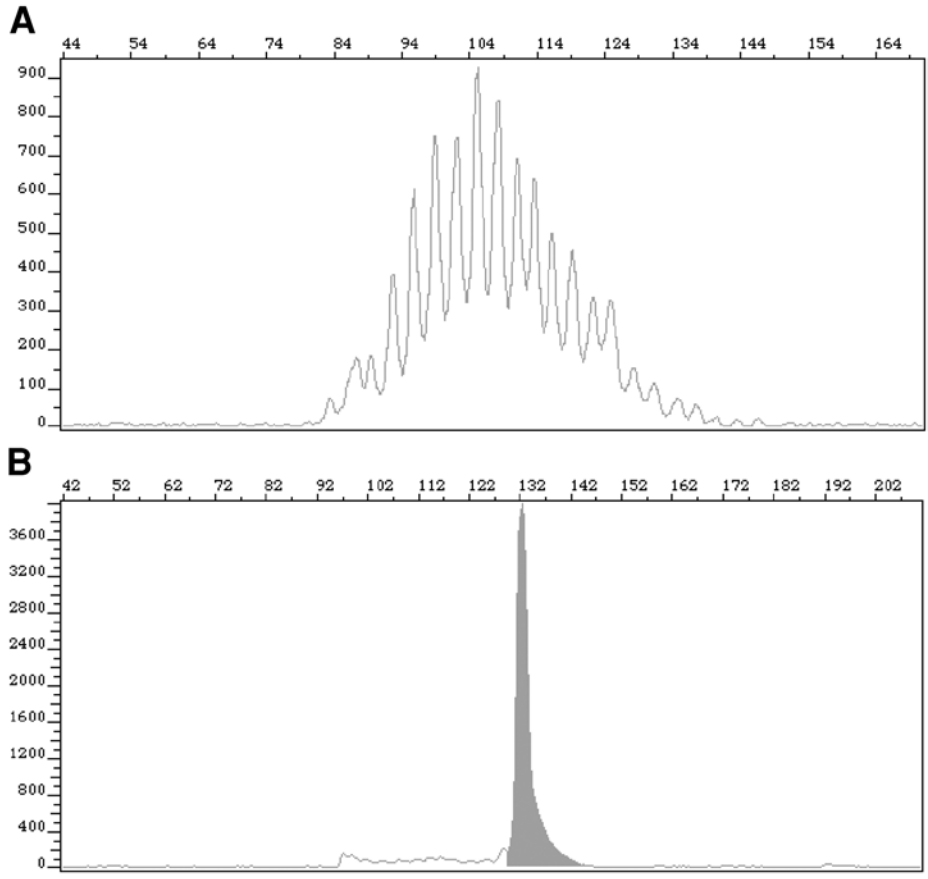


Fig 1. Typical electrophoretogram patterns generated by fluorescent Fr3 PCR. A polyclonal pattern **(A)** consists of a number of peaks separated by 3 bp and arranged in a normal distribution. Clonal rearrangements appear as distinct peaks. In this example **(B)** a single 132-bp rearrangement is clearly seen. (Reproduced with permission from the American Society of Clinical Oncology, from Evans et al. ***[17]***.)

defined as single peaks with fluorescence of at least 250 absorbance units and a peak height that is at least twice that of adjacent peaks if it is present within a polyclonal background ***(17)***. In practice new rearrangements are easily identified as they are frequently have peak heights of at least 1500 absorbance units and are rarely associated with a polyclonal background.

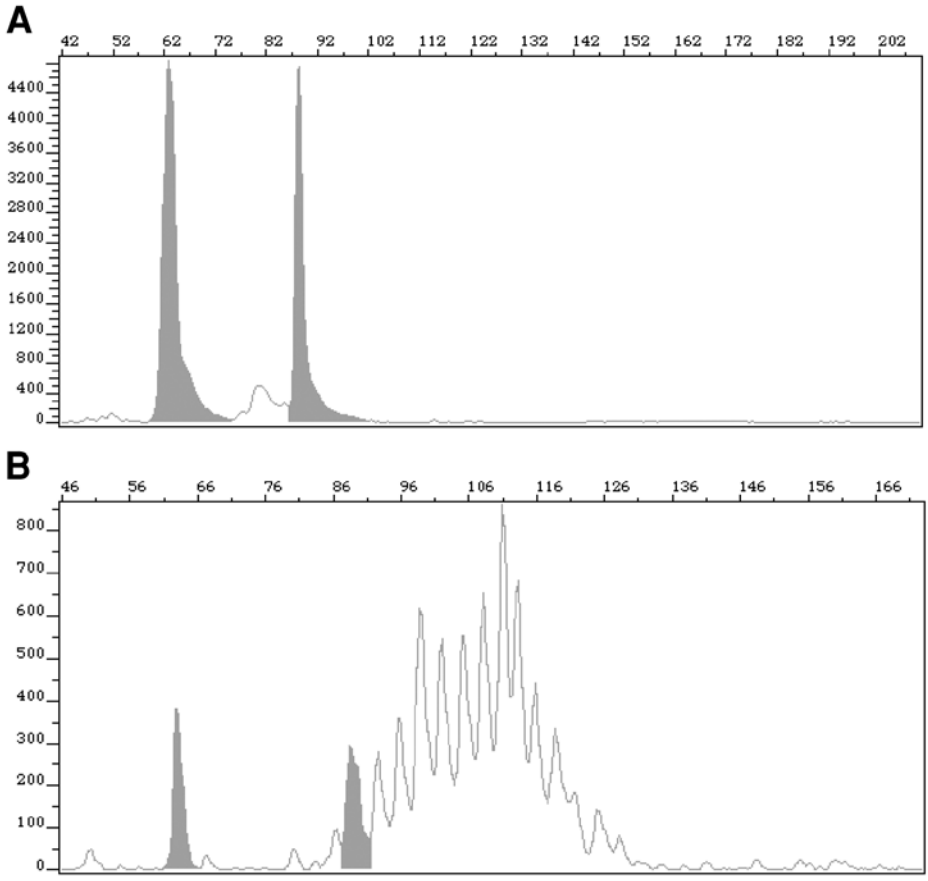


Fig 2. Demonstration of MRD in a patient with B-lineage ALL. At presentation **(A)** two clonal rearrangements are demonstrable, 62 bp and 88 bp in size. The 62-bp rearrangement is also seen in the wk 20 bone marrow sample. (Reproduced with permission from the American Society of Clinical Oncology, from Evans et al. ***[17]***.)

3.6 Establishment of Sensitivity for Each Patient

It is important to establish the sensitivity of the technique for each patient and for each rearrangement when multiple rearrangements are demonstrated at presentation. Sensitivity does vary from patient to patient but may also vary between different rearrangements within any single individual. The main determinant of sensitivity is the size of the rearrangement relative to the normal polyclonal fingerprint. Rearrangements at the extremes of the size range will be detected at lower concentrations than those that lie within the middle of the fingerprint (**Figs. 2** and **3**). Clonal rearrangements in ALL are often detected

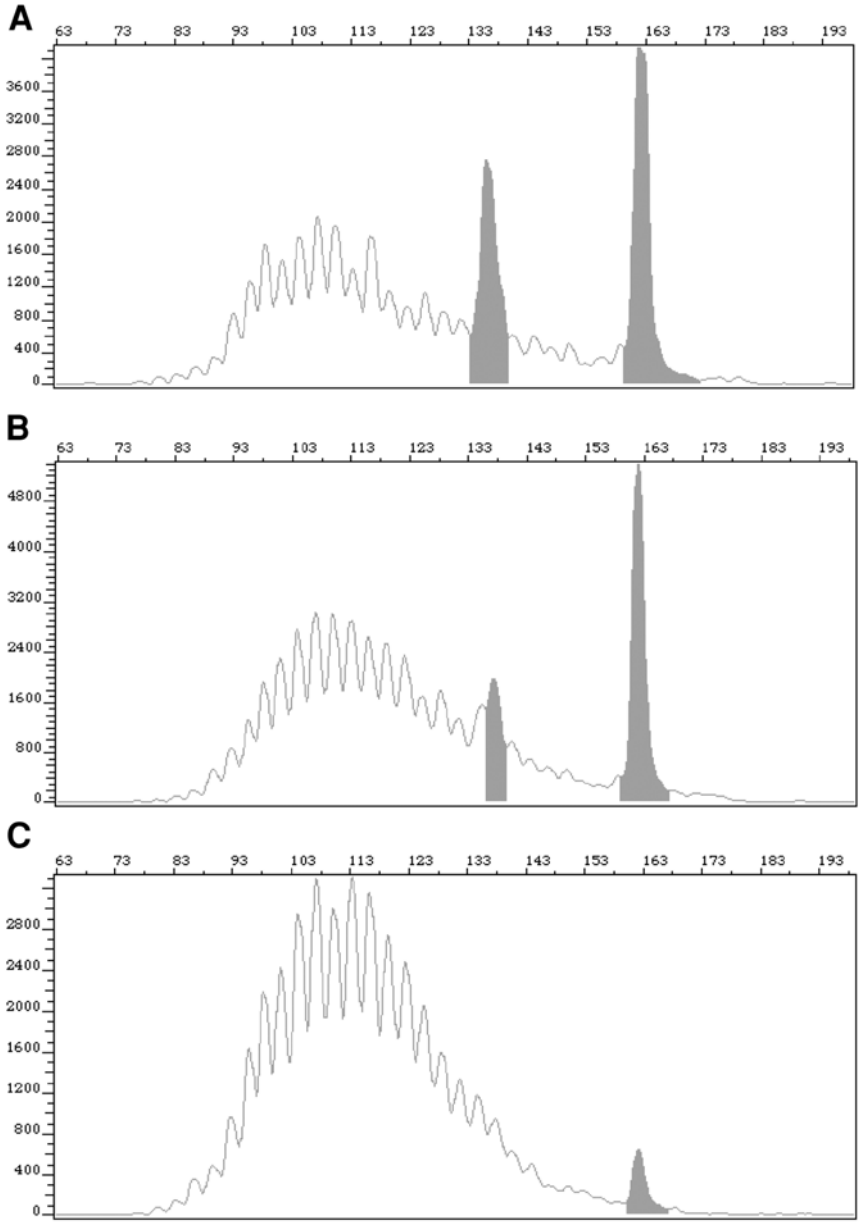


Fig 3. Sensitivity dilution series. In this example the 137-bp and 162-bp rearrangements are clearly seen in the 0.1% dilution **(A)**. The 162-bp rearrangement, which lies outside the normal fingerprint, is also demonstrable in the 0.01% and 0.001% dilutions shown in **(B)** and **(C)**, respectively. (Reproduced with permission from the American Society of Clinical Oncology, from Evans et al. ***[17]***.)

outside the normal fingerprint. This is in contrast to the pattern seen in mature B-cell disorders, in which clonal rearrangements almost invariably lie within the normal polyclonal fingerprint ***(15)***. Sensitivity is assessed for each patient by making serial dilutions of presentation sample DNA into normal bone marrow DNA. Aliquots of 1 µg of each dilution are amplified and analyzed as described earlier (*see* **Note 6**). This method achieves a sensitivity of at least 0.1% in 88% of cases ***(16)***.

4. Notes

1. The Fr3 and Fr1 PCR strategies both employ a "hot start" technique in which the sample is denatured at 95°C prior to the addition of *Taq* polymerase. This improves both the efficiency of amplification and decreases nonspecific annealing of the primers.
2. The Fr3 PCR strategy employs a two-stage amplification procedure. The product size is small and the removal of an extension step decreases nonspecific amplification.
3. The Fr1 PCR strategy employs a "touchdown" technique in which the annealing temperature is sequentially reduced by 1°C increments during the initial cycles of amplification. This is also used to decrease nonspecific amplification.
4. The presence of a clonal rearrangement or a polyclonal fingerprint confirms the presence of amplifiable DNA. However, follow-up bone marrow samples in ALL patients frequently contain very low numbers of B cells and a control PCR is therefore advisable in all cases. Follow-up bone marrow samples often yield poor quality DNA, which in some instances may preclude any meaningful analysis of MRD.
5. The reproducibility of clone sizing using this method is excellent. Reanalysis of presentation material is not therefore routinely performed when follow-up bone marrow specimens are analyzed.
6. When dilution series derived from presentation samples are analyzed it is important not to load the PCR products in adjacent lanes, as any spillover may falsely increase the sensitivity demonstrated.

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11

Real-Time Quantitative RT-PCR to Detect Fusion Gene Transcripts Associated With AML

Rajinder Flora and David Grimwade

1. Introduction

Relapse remains a major cause of treatment failure in acute myeloid leukemia (AML); however, patients destined to relapse cannot be reliably distinguished on the basis of pretreatment characteristics. Hence, there has been considerable interest in strategies for detection of minimal residual disease (MRD) as a means of identifying patients at high risk from relapse who could benefit from additional therapy. Moreover, MRD monitoring also affords the opportunity to reveal subsets of patients at low risk of relapse who could be spared excessive therapy, thereby reducing the risk of treatment related mortality and morbidity. Previous studies have confirmed that MRD monitoring using PCR- or flow cytometric-based approaches provides key independent prognostic information in a number of subtypes of leukemia and is increasingly being used for risk stratification as a means of directing treatment approach (*see* **refs. *1*,*2*** for recent reviews).

Leukemias with well characterized oncogenic fusion genes comprise approximately a third of cases of AML arising in children and younger adults (reviewed in **ref. *3***). In such cases nested reverse transcriptase-polymerase chain reaction (RT-PCR) has been the mainstay for MRD monitoring. The majority of studies to date have focused on detection of *PML–RARα*, *AML1–ETO*, and *CBFβ-MYH11* fusion transcripts, consequent on t(15;17)(q22;q21), t(8;21)(q22;q22), and inv(16)(p13q22) /t(16;16)(p13;q22), respectively (reviewed in ***4*,*5***).

The predictive clinical value of conventional RT-PCR assays is critically dependent upon the level of sensitivity achieved. Relatively insensitive assays (1 in 10^3 to 10^4), such as those typically used to detect *PML–RARα* in acute

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

promyelocytic leukemia (APL), are highly predictive of relapse (reviewed in **ref. *4***). Moreover such relapses may be averted with additional therapy. A recent study has suggested that preemptive therapy at the point of molecular relapse is associated with a superior outcome in comparison to patients treated in frank hematological relapse ***(6)***. Unfortunately the relatively insensitive conventional assays used to detect *PML–RARα* fusion transcripts fail to detect MRD in a substantial proportion of patients who ultimately relapse (reviewed in **ref. *4***). By contrast, the clinical value of more sensitive assays (1 in 10^5 to 10^6) such as those for *AML1–ETO* or *CBFβ–MYH11* fusion transcripts in AML has been hampered by the detection of fusion transcripts in long-term remission, precluding distinction of patients who are likely to be cured from those destined to relapse ***(5)***. This has prompted the development of quantitative competitive RT-PCR assays, which have suggested that risk of relapse among AML patients with *AML1–ETO* transcripts detected in clinical remission is correlated with the relative level of residual disease ***(7)***. These encouraging data, when taken along with results in other leukemias ***(8–11)*** demonstrating that the kinetics of achievement of molecular remission is also an independent prognostic factor, have underpinned the drive to employ automated quantitative PCR approaches.

So-called real-time quantitative RT-PCR (RQ-PCR) is more reproducible and less labor-intensive than competitor-based RT-PCR assays and hence is ideally suited to analyses of patients entered into large-scale clinical trials. Furthermore, this approach is not restricted solely to MRD detection of leukemia-associated fusion genes, which occur in fewer than 40% AML cases. Its wide range of applications includes determination of relative gene expression levels, thereby enabling evaluation of alternative targets that might extend MRD detection using molecular methods to a wider range of AML cases. One potential candidate is the Wilms' Tumor gene, *WT1*, which is overexpressed in approx 90% of AML (reviewed in **ref. *5***). Studies to date using qualitative RT-PCR have revealed conflicting data regarding its suitability as a marker for MRD monitoring, which is likely to reflect differences in the relative sensitivities of the assays used and variation in expression levels between patients, again underlining the need for a quantitative RT-PCR method.

RQ-PCR technologies involve measurement of the number of amplicons generated with each PCR cycle in the exponential phase of the PCR reaction detected as a proportionate rise in fluorescence intensity (for comprehensive reviews of RQ-PCR *see* **refs. *12,13***). The PCR cycle at which fluorescence is first detected above the background level (C_t or threshold cycle) is indicative of the initial number of target template copies (*see* **Note 1**). The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number (**Figs. 1** and **2**). PCR products may be detected through use of the

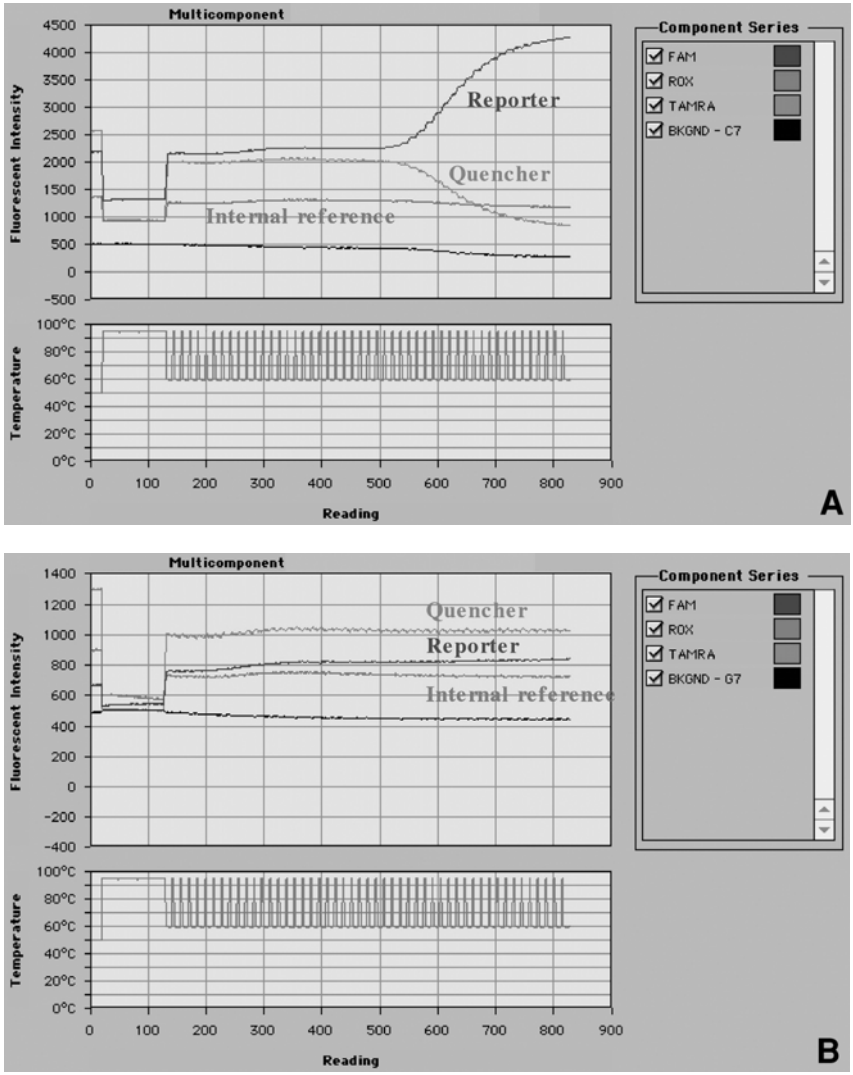


Fig. 1. Reporter, quencher, and passive reference signals during PCR in the presence or absence of target template. Multicomponent view (as seen using Sequence Detector software V1.6.4), revealing rise in reporter fluorescence (FAM, 6-carboxyfluorescein) and corresponding fall in quencher fluorescence (TAMRA, 6-carboxytetramethyl rhodamine) relative to the ROX internal standard, indicative of amplification of target sequence (**A**). In this particular reaction well, specific amplification product was first detected following 29 cycles of PCR. This contrasts with the multicomponent view of a well (**B**) in which there was no amplification of specific target gene product, as seen for No Amplification and No Template Controls (NAC and NTC; *see* **Subheading 3.4.**) and in patient samples lacking expression of the fusion gene target.

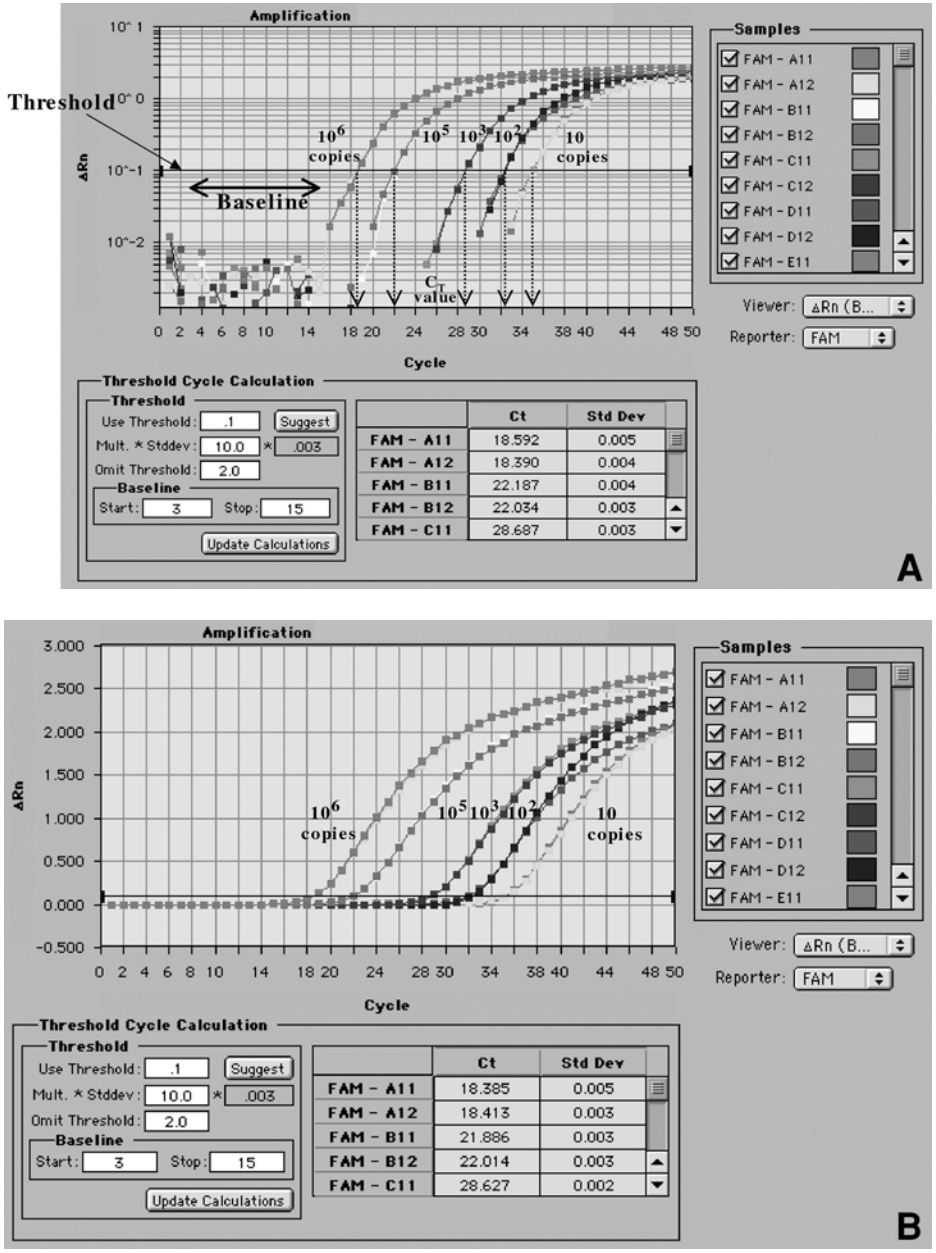


Fig. 2. Amplification plots of serial dilutions of linearized plasmid standards, for example, containing *AML1–ETO* fusion gene target sequence shown in this analysis. Normalized reporter signal (R_n) is plotted (logarithmic scale, **A**; linear scale, **B**) against PCR cycle number. The PCR cycle at which fluorescence intensity rises above the

fluorescent dye SYBR green I which binds to the minor groove of double-stranded DNA. However, this approach, although cheaper, has a number of disadvantages, in comparison to detection of PCR products with specific probes, for the purpose of monitoring for MRD. In particular, SYBR green I will also bind to nonspecific PCR products and to primer dimers, which can interfere with reliable quantification of the fusion gene target, although PCR product specificity can be confirmed by melting curve analysis. Furthermore, MRD assays using SYBR green I for amplicon detection are often relatively insensitive, potentially limiting their clinical utility. Use of SYBR green I in conjunction with a nested PCR assay has been shown to increase sensitivity for MRD detection ***(14)***, although the inclusion of a second PCR step precludes the generation of reliable quantitative data. Hence for MRD detection, PCR products are typically detected by specific hybridization or hydrolysis probes, which not only afford greater specificity but also superior sensitivity.

In RQ-PCR assays based on "hybridization probe" technology (e.g., used in conjunction with the LightCycler™ System, Roche Molecular Biochemicals), specific PCR products are detected by two separate probes labeled with donor and acceptor fluorophores, respectively. Probes are designed to hybridize to the amplified DNA fragment in a "head-to-tail" arrangement within 5 bp of each other, leading to close apposition of the fluorophores and release of fluorescent light of a particular wavelength which is measured after the annealing step.

The Taqman® approach is based on the 5'-nuclease activity of *Taq* polymerase in conjunction with a nonextendible probe specific for the target sequence, labeled with reporter and quencher dyes at its 5' and 3' ends, respectively. In the absence of the target sequence, the probe remains unbound and intact such that reporter and quencher dyes lie in close proximity and no reporter signal is detected. Conversely, amplification of specific PCR product

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(Fig. 2. *continued*) background level (defined as the "baseline" fluorescence recorded during early PCR cycles, for example, cycles 3–15 in this analysis) and crosses the "threshold" value is denoted the "cycle threshold" (C_t value, indicated by *dotted line arrows*). The threshold is set such that it lies above the background level of fluorescence detected during early phases of the PCR, but still crosses all samples within the linear region of the log amplification plot. It is essential that this latter criterion is satisfied for all samples including those with low and high levels of target sequence, so that quantification invariably relates to the exponential phase of the PCR reaction. As can be seen from this analysis, the C_t value is indicative of the initial quantity of the target sequence. RQ-PCR amplification of leukemia-associated fusion transcripts suggests that quantitation is reliable to C_t values of at least 35, beyond which PCR amplification becomes subject to sampling variability ***(16)***.

leads to hybridization of the probe which is subsequently cleaved during the extension phase of the PCR, leading to release of the reporter dye and generation of fluorescence (**Figs. 1–3**). Such hydrolysis probes can be used in conjunction with platforms such as the ABI 7700 (Applied Biosystems, methods for use of which are described in the Subheadings that follow) or the LightCycler, which have been shown to generate comparable results ***(15)***.

Gene expression levels in unknown samples as determined by RQ-PCR may be reported in "absolute" or "relative" terms. According to the former approach, quantification of gene expression in unknown samples is derived from a standard curve comprised of serial dilutions of known amounts of target DNA sequence (*see* below and **Figs. 2** and **3**). Use of plasmids for this purpose is valuable for optimizing RQ-PCR assays and providing interlaboratory standards for quality control; however, this approach does carry an increased risk of PCR contamination. Such risks are reduced if the alternative "relative quantitation" approach is adopted, whereby transcript levels are related to those detected in a reference cell line or other designated sample, for example, diagnostic bone marrow (calibrator) and expressed as a ratio or "*n*-fold difference." Although this approach evaluates the RT step as well as the PCR step, there are a number of potential disadvantages. These include (1) difficulties in inter- and intralaboratory standardization resulting from the lability of RNA-based calibrators, (2) variation in the characteristics of subclones of the same cell line, (3) differences in relative expression of fusion genes and ubiquitously expressed control genes between cell lines and patient samples, and (4) lack of available diagnostic material as a suitable calibrator for subsequent MRD evaluation in some instances. The development of quality controls mimicking clinical samples harboring MRD that are suitable as inter- and intralaboratory standards is a matter of considerable current interest. Possibilities include fusion gene positive cell line RNA, negative cell line RNA spiked with in vitro synthesized fusion gene RNA, or plasmid DNA which will serve as controls for cDNA synthesis and most importantly for the amplification of the TaqMan primer sets. Although each of these approaches has its relative advantages and disadvantages, all suffer from the limitation that they not reliably evaluate the sample handling and RNA extraction steps, which may also have an important bearing on assay sensitivity.

An important advantage of RQ-PCR technology is that it allows quantitation of fusion gene transcripts (or of other leukemia-associated target genes) in relation to ubiquitously expressed control genes, such as *ABL*, thereby affording more accurate determination of MRD levels following initial therapy and in remission. Hence it can more reliably determine the impact of kinetics of molecular response on outcome and establish whether the risk of subsequent relapse among patients in clinical remission can be predicted on the basis of

(Continued on p. 158)

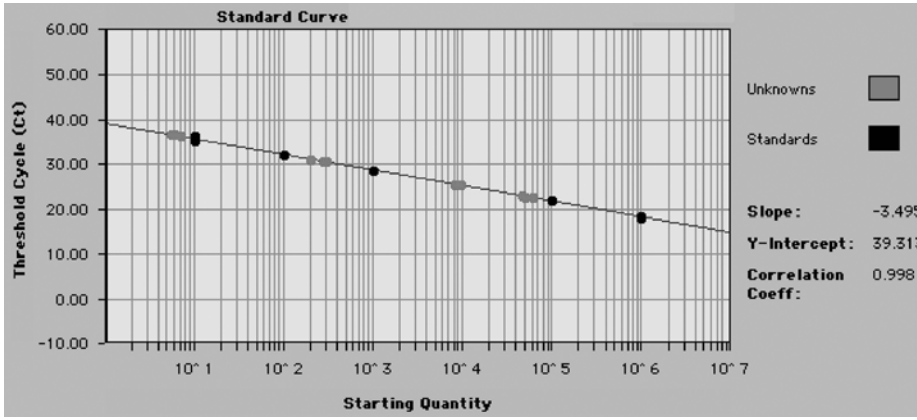


Fig. 3. Generation of plasmid standard curves for absolute quantitation. Real-time quantitative PCR analysis of linearized plasmid dilutions containing the fusion gene of interest (e.g., *AML1–ETO* shown here) may be used to generate a standard curve from which the absolute fusion gene transcript levels in unknown samples may be determined. C_t is plotted against plasmid copy number, with data points for standards shown in black and for unknown samples in gray. The *y*-intercept provides the C_t value that is equivalent to one plasmid copy. For PCR reactions of optimal efficiency a slope of –3.3 is obtained. The slope is indicative of the difference in C_t values that is equivalent to a 1 log difference in the initial quantity of target sequence. Slope values >–3.3 reflect inaccuracies in the preparation of the standards, indicating that the quantity of template in dilutions was greater than it should have been, whereas slope values of <–3.3 indicate that the assay is <100% efficient and can also be due to pipeting errors. Hence for an assay associated with a slope of –4.0, a 10-fold difference in initial template amount is equivalent to $4C_t$. For assays designed for MRD detection, the closer the assay is to 100% efficiency, the greater the sensitivity, which is also critically dependent on the fusion gene expression levels in the diagnostic sample ($CT\Delta$). The following formula provides a useful working guideline to the sensitivity of a given PCR assay:

$$\text{dynamic range (log value)} = \frac{(CT_{RQ} - CT\Delta)}{\text{slope}}$$

where CT_{RQ} is the C_t limit for reliable quantitation (approx 35 cycles for detection of leukemia-associated fusion genes ***[16]***) and the slope is equivalent to the difference in C_t value associated with a 1 log difference in starting template. Hence a fusion gene assay with $CT\Delta$ 21 and slope –3.3 may give reliable quantitative data to a dilution of 1 in 10^4, while an assay with $CT\Delta$ 23 and slope –4.0 may give reliable quantitative data only to a dilution of 1 in 10^3. The correlation coefficient of the standard curve should be as close as possible to 1.0 and values <0.98 should be considered unacceptable, highlighting defects in experimental technique in setting up the assay and/or variable degradation of the standards.

the relative level of MRD. RQ-PCR also permits identification of samples with poor quality RNA that could otherwise have led to "false-negative" results. A recent European Union funded initiative, Europe Against Cancer (EAC), involving over 25 laboratories including our own has led to the development and optimization of Taqman assays for the major leukemia-associated fusion genes, as well as control genes (***16,17***, further details of the EAC program may be found at http://194.214.97.12/scripts/defaultIE.asp). These assays have been shown to be relatively sensitive (1 in 10^4 to 10^5), highly reproducible, and readily standardized, facilitating quality control, thereby for the first time providing a really excellent opportunity for more reliable comparison of MRD data between national and international trial groups.

2. Materials

2.1. cDNA Synthesis

1. Sterile 0.5-mL capped tubes (Robbins Scientific Corporation).
2. Sterile nuclease-free water (Sigma).
3. 500 µ*M* stock solution of Random Hexamers (Pharmacia). Add 1.82 mL of sterile water to the lyophilized random hexamers in a laminar flow hood and store as 200-µL aliquots at –20°C.
4. 200 U/µL of Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) supplied with 0.1 *M* dithiothreitol (DTT) and 5X reaction buffer (GibcoBRL; store all at –20°C).
5. RNase inhibitor (Rnasin 40 U/µL, Promega; store at –20°C).
6. 10 m*M* stock of deoxynucleotide triphosphates (dNTPs)(Pharmacia Biotech). Prepare in a laminar flow hood and store as 100-µL aliquots at –20°C. dNTPs are labile and should not be freeze–thawed more than five times.
7. TE: 10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA.
8. Thermal cycler (Hybaid OmniGene).

2.2. Plasmid Standards

1. 0.2-mL thin-walled PCR tubes (Robbins Scientific Corporation).
2. 10X PCR buffer (Applied Biosystems).
3. 25 m*M* $MgCl_2$ (Applied Biosystems).
4. AmpliTaq Gold (Applied Biosystems).
5. 1.25 m*M* stock of dNTPs (Pharmacia Biotech).
6. PCR primers. In our laboratory, lyophilized pellets are resuspended in 1 mL of sterile nuclease-free water. These serve as the primary stocks from which 10 µ*M* working stocks are made for use in the PCR reaction. Both are stored at –20°C.
7. Sterile nuclease-free water (Sigma).
8. 50X TAE: 242 g of Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 *M* EDTA, pH 8.0.
9. 6X gel loading buffer: 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% v/v sterile glycerol.

10. LB medium: 1% w/v bactotryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl.
11. Ampicillin: 50 mg/mL in water. Sterilize by filtering through a 0.22-µm filter.
12. LB–ampicillin agar plates: Add 7.5 g of bacto-agar to 500 mL of LB medium, autoclave at 121°C and 121 psi, cool to approx 50°C, then add 500 µL of ampicillin and pour into 90-mm Petri dishes. Allow the plates to set and store at 4°C.
13. 50 mg/mL of X-gal (Promega).
14. 0.1 *M* Isopropyl-β-thio-galactoside (IPTG) in water. Sterilize by filtering through a 0.22-µm filter.
15. *E. coli* ribosomal 16S and 23S RNA (4 µg/µL, Roche)
16. Solution S: a 20 ng/µL solution of *E. coli* ribosomal 16S and 23S RNA made up in sterile nuclease-free water. Prepare in a laminar flow hood.
17. Qiagen PCR Purification kit.
18. Qiagen plasmid midi-prep kit.
19. TOPO TA cloning kit (Invitrogen).
20. Thermal cycler (MJ Research, PTC-200).
21. Sterile inoculating loops.
22. Ultraviolet (UV) spectrophotometer.

2.3. Real-Time Quantitative PCR (RQ-PCR)

1. MicroAmp Optical 96-well reaction plate (Applied Biosystems).
2. MicroAmp Optical Caps (Applied Biosystems).
3. ABI Prism7700 Sequence Detection System (Applied Biosystems).
4. 1.5 mL O-ring screw cap tubes (Sarstedt).
5. Solution S.
6. TE: 10 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.0.
7. RQ-PCR primers and probes (Applied Biosystems) (*see* **Note 2**).
8. 2X Taqman Universal PCR Mastermix (Applied Biosystems).

3. Methods

3.1. cDNA Synthesis

Full details of methods for sample preparation and RNA extraction have been published previously ***(18)***. For cDNA synthesis, our laboratory has adopted a method based on the protocol evaluated by the European Biomed-1 Concerted Action ***(19)***. All reactions are prepared in a laminar flow hood using sterile 0.5-mL capped tubes.

1. Add 1 µg of total RNA to 1 µL of 500 µ*M* random hexamers. Add sterile nuclease-free water to give a total volume of 6 µL.
2. Transfer the tubes to a thermal cycler and heat the mixture at 65°C for 10 min. Snap cool the tubes on ice for 1 min and then centrifuge for 1 min at 11,600*g* in a benchtop microcentrifuge to return all droplets to the bottom of the tube.
3. Add the following reagents to the mixture: 2 µL of 0.1 *M* DTT, 4 µL of 5X reaction buffer, 2 µL of dNTP (10 m*M* each), 0.5 µL of Rnasin, 0.5 µL of MMLV, and sterile nuclease-free water to give a final volume of 20 µL.

4. Gently flick-mix the tubes and centrifuge at 11,600*g* for 30 s to collect reactants to the bottom of the tube; then incubate for 1 h at 37°C followed by 3 min at 99°C, to stop the reaction, in a thermal cycler.
5. Following incubation, collect all reactants to the bottom of the tube by centrifugation at 11,600*g* for 1 min and then add 30 µL of TE.
6. Transfer the reactions to ice during real-time PCR setup. Alternatively, the samples may be stored at –20°C until required (up to a maximum of 1 mo).

3.2. RQ-PCR Primers and Probes

PCR primers and fluorogenic probes for all genes under analysis are designed using the computer program Primer Express (Applied Biosystems) following strict parameters (*see* **Note 3**). The Oligo 6.0 program (Molecular Biology Insights) is also helpful for primer design. The positions of primers and Taqman probes for detection of *PML–RARα*, *AML1–ETO*, and *CBFβ–MYH11* fusion transcripts in relation to the genomic breakpoints generated by t(15;17), t(8;21) and inv(16)/t(16;16) are shown in **Fig. 4**.

3.3. Construction of Plasmid Standards

To obtain standard curves for absolute quantitation of target gene expression levels, plasmids containing a cDNA fragment of the gene(s) under analysis may be constructed by PCR cloning (*see* **Notes 4,5**).

3.3.1. cDNA Synthesis

This is the same as described in **Subheading 3.1.** As a control for genomic DNA contamination, run a parallel reaction with all components except for the RT enzyme ("No RT control"; *see* **Note 3**).

3.3.2. PCR

Perform PCR using the cDNA from **Subheading 3.3.1.** and primers designed to flank the Taqman primers. Although it is feasible to use Taqman primers to generate a PCR product for cloning, it is not recommended as these amplicons are designed to be small. As a consequence, it may be difficult to ascertain the success of the PCR on an agarose gel as the product could be masked by the primer–dimer artefact.

Given below is a protocol for the amplification of cDNA using primers designed in our laboratory.

1. Add 5 µL of the cDNA from **Subheading 3.3.1.** to 0.2-mL thin-walled PCR tubes containing 2.5 µL of 10X PCR buffer, 1.5 µL of 1.5 m*M* $MgCl_2$, 1.25 µL of 10 µ*M* forward and reverse primers, 5.0 µL of 1.25 m*M* dNTP mix, 0.2 µL of 5 U/µL of AmpliTaq gold. Add sterile nuclease-free water to 25 µL.

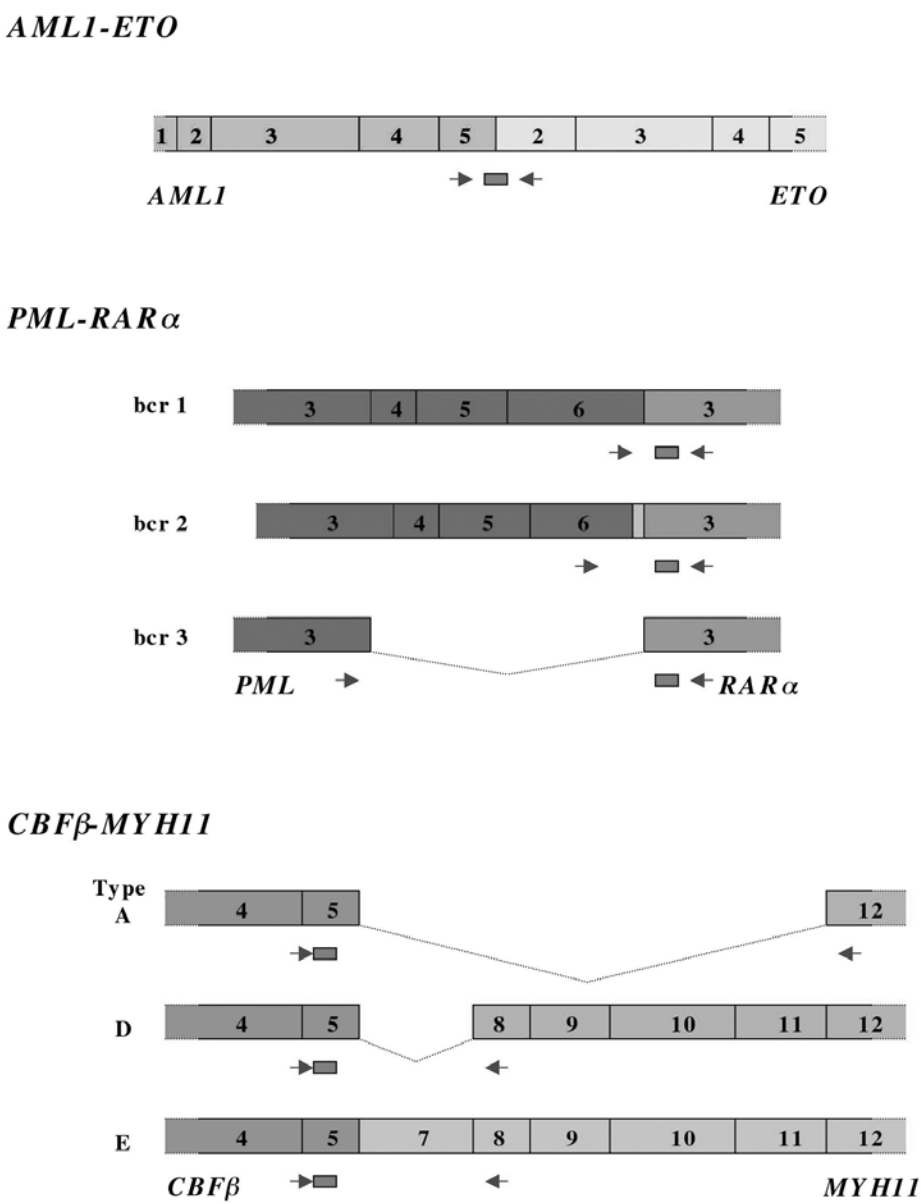


Fig. 4. Location of primers and probes relative to *AML1–ETO*, *PML–RARα*, and *CBFβ–MYH11* fusion genes generated by t(8;21), t(15;17), and inv(16)/t(16;16) chromosomal rearrangements associated with AML. Details of the primer and probe sequences may be found in **ref. *16*** and the EAC Web site (*see* **Note 3**).

2. Transfer the reactions to the thermal cycler and amplify the cDNA with the following conditions:
   a. 1 cycle at 95°C, 12 min.
   b. 30 cycles at 95°C, 30 s, 57°C for 45 s, 72°C for 1 min.
   c. 1 cycle at 72°C, 10 min.
3. Following amplification, to 10-µL aliquots of each PCR reaction add 2.0 µL of 6X gel loading buffer. Analyze the aliquots by electrophoresis through a 1% TAE/agarose gel running alongside the PCR reactions a 100-bp ladder to assess product sizes.

3.3.3. Cloning

Although expensive, our laboratory has found the use of commercially available kits ideal for quick and easy cloning of PCR products and subsequent purification of plasmid DNA.

1. Purify the remaining PCR reaction from **Subheading 3.3.2.**, **step 3** using the Qiagen PCR purification kit according to the manufacturer's instructions.
2. Clone the purified PCR product using the TOPO TA cloning kit according to the manufacturer's instructions.
3. Prepare plasmid DNA using the Qiagen midi-prep kit according to the manufacturer's instructions.
4. Quantify plasmid DNA by assessing absorbance at 260 nm and 280 nm. A ratio of the readings taken at 260 nm and 280 nm will give an indication as to the purity of the plasmid DNA. Ratios between 1.8 and 2.0 are deemed satisfactory.
5. Check for the presence of the PCR insert by amplifying 100 ng of plasmid DNA and analyzing the resultant product as for **Subheading 3.3.2.**, **steps 1–3**. The cloned insert should also be sequenced to check for any mutations that may interfere with annealing of Taqman primers and probes.

3.3.4. Plasmid Dilutions

1. Add 10-µg of plasmid DNA to 1.5-mL O-ring screw cap tubes containing 10 U of restriction enzyme and 2 µL of the appropriate restriction enzyme buffer. Make up to 20 µL with sterile nuclease-free water. Be sure to use a restriction enzyme that will only linearize the plasmid and not cut the insert (*see* **Note 5**).
2. Gently flick-mix the digests and centrifuge at 11,600*g* for 30 s in a benchtop centrifuge to collect all the reactants to the bottom of the tube. Transfer the digests to a 37°C water bath and incubate for 2 h.
3. Following incubation, inactivate the reactions by heating to 80°C for 5 min and then centrifuge the tubes at 11,600*g* for 1 min to gather all reactants to the bottom of the tube.
4. Analyze 5-µL aliquots of each digest by adding 1 µL of 6X gel loading buffer to the aliquots and electrophoresing through a 1% TAE–agarose gel. Run alongside the digests a 1-kb ladder to assess plasmid size. A single band should be visible.

5. Calculate the volume of digest that contains 1×10^{12} copies. It is given by the following formula:

$$V\ (\mu L) = 20 \times \frac{1 \times 10^{12} \times 309 \times \left[\text{plasmid size} + \text{insert size (bp)}\right]}{10 \times 1 \times 10^{-6} \times 6.02 \times 10^{23}}$$

6. Aliquot this volume from each of the remaining digests from **step 4** into O-ring 1.5-mL screw cap tubes and make up to 500 µL with solution S. This dilution now contains 2×10^9 plasmid copies/µL and should be designated arbitrarily as the 10^{-1} plasmid standard.
7. Make 1:10 (to minimize pipeting error) serial dilutions from the 10^{-1} standard, using solution S as the dilution factor, until a standard of dilution of 10^{-10} (this will contain two plasmid copies/µL) is achieved. At every point make sure that the contents of each tube are thoroughly mixed. Store plasmid standards 10^{-4}–10^{-10} as 50-µL aliquots at –20°C or –80°C until required for real-time PCR.

3.4. Quantitative Real-Time PCR

Real-time PCR is carried out in a 96-well reaction plate. For full details of use of the ABI PRISM® 7700 Sequence Detection System, readers are referred to the Manufacturer's User Manual. Typically in our laboratory plasmid standards ranging over 6 orders of magnitude (i.e., 10^{-5}–10^{-10}) for each gene are amplified to generate standard curves for absolute quantitation of gene expression levels (**Figs. 2** and **3**). However, once the range of gene expression detected in patient samples has been established, the number of data points used to construct standard curves may be reduced accordingly to permit increased sample throughput. In addition, to be sure of specific amplification for each gene, Positive controls using cell line RNA, No Amplification Controls (NAC) (water in place of RNA in the RT step), and No Template Controls (NTC) (TE in place of cDNA template, solution S alone as a negative control for plasmid dilutions) are run on the same plate. Should problems with contamination arise, NAC and NTC may indicate whether this relates to RT or PCR steps. Given the large number of wells lost to standards and negative controls we have found it useful therefore to amplify all standards and negative controls in duplicate and samples in triplicate such that the maximum number of samples may be analyzed without compromising the reliability of the assay (*see* **Note 6**).

1. In a laminar flow hood thaw relevant Taqman primers and probes for genes under analysis and place on ice in an ice bucket. Cover with a lid to prevent the probe from being exposed to light.
2. Prepare mastermixes for each gene by pipeting into 1.5-mL O-ring screw cap tubes the following: 12.5 µL of 2X Taqman universal PCR master mix, *x* µL of forward primer (final conc. 300 n*M*), *y* µL of reverse primer (final conc. 300 n*M*),

z µL of probe (final conc. 200 n*M*) and sterile nuclease-free water to 20 µL (*see* **Note 7**). This is for a single PCR reaction and hence all volumes should be multiplied by $X + 5$, where X is the number of wells that will be occupied by a particular mastermix.

3. Vortex mastermixes briefly and centrifuge for 30 s at 11,600*g* in a benchtop centrifuge to collect the mix to the bottom of the tube. Place the mixes on ice in an ice bucket and cover with a lid.
4. Take a MicroAmp® Optical 96-well reaction plate (Applied Biosystems) and place in a rack. Pipet 20 µL of each mastermix to the bottom of each well in the plate according to the plate design that you are following.
5. Pipet 5 µL of sample cDNAs from **Subheading 3.1.**, **step 6** (equivalent to 100 ng of template); 5 µL of plasmid standards from **Subheading 3.3.4.**, **step 7**; 5 µL of solution S; and 5 µL of TE into the appropriate wells for each master mix.
6. Seal the wells with strip caps and centrifuge the plate at room temperature for 1 min at 169*g* to gather all reactants to the bottom of the wells. Wrap the plate in aluminum foil to shield from light.
7. Before loading the plate into the machine click on "Thermal Cycling" and ensure the following amplification conditions are set:
   a. 1 cycle at 50°C for 2 min (activates UNG enzyme; *see* **Note 5**).
   b. 1 cycle at 95°C for 10 min (activates AmpliTaq).
   c. 50 cycles at 95°C for 15 s; 60°C for 1 min and that the appropriate reaction volume (25 µL) is selected.
8. Type in the schematic of the plate (includes details of sample name, type, other attributes, and reporter dye), save and click on "show analysis"; then remove the aluminum foil, place the plate into the 7700 Sequence Detector such that well A1 is located upper left, close the lid, and click on "Run." Always ensure that communication ("file sharing") between the ABI 7700 associated computer is prevented during the PCR run; otherwise data may be lost. The computer should also be "rebooted" between runs so as to clear the internal memory and to avoid loss of data.
9. Following completion of the PCR, save the data and analyze (*see* **Notes 8** and **9**).

4. Notes

1. Glossary of terminology:

   ***R*n**: normalized reporter signal, representing the fluorescent signal of the reporter dye (e.g., FAM) divided by the fluorescent signal of the reference dye (ROX).

   Δ*R*n: represents the normalized reporter signal minus the baseline signal established in the first few cycles of PCR. During the PCR, R_n and ΔR_n increase as the amplicon copy number rises until the reaction approaches a plateau.

   ***C*t**: threshold cycle, represents the PCR cycle at which an increase in reporter fluorescence above a baseline level is first detected (i.e., crosses the threshold). For a PCR with 100% efficiency, the C_t value decreases by one cycle as

the concentration of template doubles; that is, 1 log difference in the initial template concentration is equivalent to a C_t difference of 3.3.

Threshold: To distinguish background fluorescence (detected in wells lacking target template throughout the PCR reaction or in wells that contain the target sequence during early PCR cycles) from specific amplification signals, a threshold is set. This is typically set automatically at 10X standard deviation over the baseline region from cycle 3–15, but can be readjusted (*see* **Note 8**).

Baseline: Background level of fluorescence recorded during early PCR cycles (typically cycles 3–15 are selected; *see also* **Note 8**, **Fig. 2**).

2. In our laboratory lyophilized primers are resuspended in 1 mL of sterile nuclease-free water in a laminar flow hood and stored as 50-µL aliquots at –20°C. Probes are supplied ready-diluted and are also stored as 25 to 50 µL aliquots at –20°C. This is recommended as a drop in the sensitivity of the RQ-PCR reaction may be observed when using primers and probes that have undergone more than five freeze–thaw cycles.
3. For details of these and guidelines as to how to use the Primer Express software the reader is referred to the Protocol booklet which is available directly from Applied Biosystems. Alternatively it may be accessed via the Internet (http://www.appliedbiosystems.com). Ideally assays should be designed to generate amplicons of 50–150 bp to achieve maximal efficiency (and hence sensitivity); furthermore, for the purposes of MRD detection of leukemia-associated fusion genes, all assays should be RNA specific. To achieve the latter aim it is important to take into account the genomic structure of the target gene, designing primers and/or probes to hybridize to regions of the cDNA that straddle exon boundaries or by siting primers within adjacent exons separated by introns of at least 2 kb. Location of primers and probes should also take into account the possibility of alternative splicing which could lead to simultaneous amplification of a number of specific PCR products, thereby confounding reliable quantification of the intended target amplicon. Design of RNA specific assays eliminates the need for a DNase step prior to cDNA synthesis, an ideal situation given the labile nature of RNA and the frequently small amounts of RNA obtained from samples. RNA specificity for all assays should be confirmed by demonstrating that no amplification signals are generated using 100 ng of genomic DNA or "no RT controls" (i.e., RNA is subjected to RT protocol; *see* **Subheading 3.1.**, but with RT substituted with water) for the PCR. In designing assays to detect fusion gene transcripts, it is important that they are robust (achieving $\Delta R_n > 1$; *see* **Figs. 2** and **5**), thereby ensuring maximal sensitivity for MRD detection ***(16)***. Leukemic fusion gene assays designed by the EAC group typically achieve sensitivities of 1 in 10^4–10^5 as determined by serial dilution of RNA derived from fusion gene positive cell lines or diagnostic bone marrow in peripheral blood lymphocyte (PBL) or fusion gene negative cell line (e.g., HL60) RNA. EAC fusion gene primer sets reliably detect at least 10 plasmid copies (*see* **Figs. 2** and **5**). For the design of assays to amplify leukemic fusion gene cDNA junctional regions, the choice of suitable primers and probes may be somewhat limited. In this situation, use of

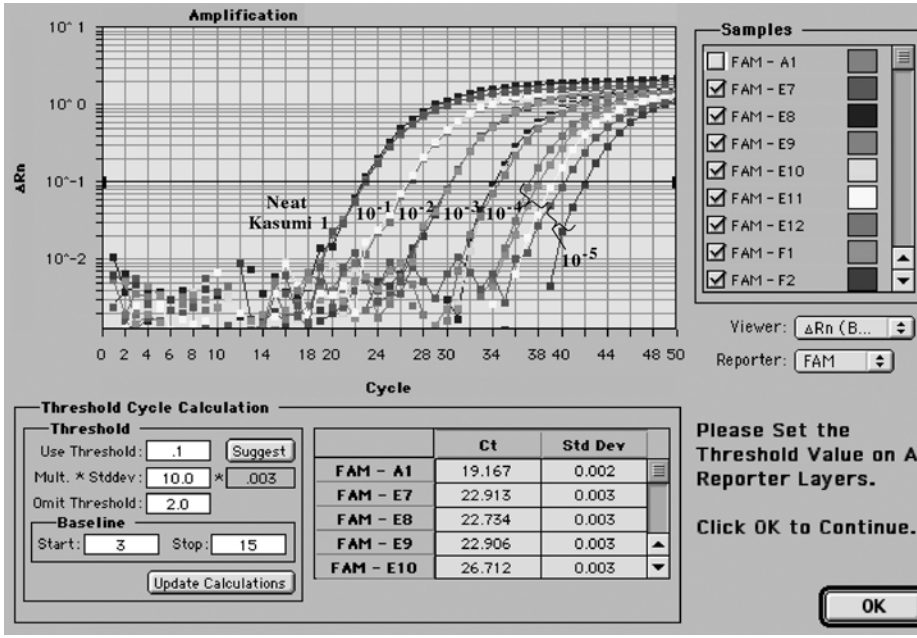


Fig. 5. Sensitivity of RQ-PCR assays for the detection of leukemia-associated fusion genes, as shown here for *AML1–ETO*, determined by serial dilution of the Kasumi 1 cell line in HL60 RNA. In this experiment, the detection limit of the *AML1–ETO* assay was the 10^{-5} dilution (equivalent to 1 pg of Kasumi 1 RNA). However, there was evident loss of reproducibility between replicates for 10^{-4} and 10^{-5} dilutions, with lack of amplification of one of the three replicates of the 10^{-5} dilution.

minor groove binding (MGB) probes (Applied Biosystems) may in some instances be advantageous in comparison to conventional TAMRA-labeled probes. The shorter MGB probes are associated with a relatively higher melting temperature (T_m), which may be particularly helpful in AT-rich regions. Details of primers and probes suitable for detection of AML-associated fusion genes are provided in **ref. *16*** and are available from the EAC Web site http://194.214.97.12/scripts/defaultIE.asp. For an extensive review of variables that need to be optimized and validated in the establishment of RQ-PCR assays for MRD detection in leukemia, see **ref. *20***. A number of factors should also be taken into account when considering design of assays for endogenous control gene transcripts, to serve as a reference for fusion gene quantitation. An ideal control gene assay should fulfil the following criteria: (1) The control gene lacks pseudogenes and the assay is RNA specific (*see* earlier comments regarding assay design). (2) The transcript level reflects RNA quantity and quality (thereby providing an indication of the MRD detection limit [sensitivity level] on an individual sample basis). (3) The assay measures the efficiency of the RT reaction. (4) Expression

levels are equivalent in leukemic blasts derived from bone marrow (BM) and peripheral blood (PB), and also in BM and PB during and after therapy. (5) The stability of control gene mRNA is equivalent to that of leukemic fusion gene transcripts. (6) Expression of the control gene should give a C_t value of 15–25 for reliable quantitation. The EAC group has undertaken an extensive evaluation of a series of potential endogenous control genes, determining their suitability for normalization of fusion gene transcript levels for MRD analyses in leukemia patients ***(17)***.

4. Full details of approaches to relative and absolute quantitation are provided in Applied Biosystems User Bulletin No. 2. Comparison of fusion gene and endogenous control gene transcript levels between samples may be derived from standard curves. For relative quantitation, cell line or diagnostic patient BM RNA may be serially diluted in PBL or fusion gene negative cell line (e.g., HL60) RNA (to bring total RNA for each dilution to 1 µg). Each dilution standard RNA is converted to cDNA (*see* **Subheading 3.1.**) and RQ-PCR performed (*see* **Subheading 3.4.**). An alternative approach is to initially generate cDNA from the cell line or primary leukemia RNA, from which serial dilutions in HL60 or PBL cDNA are made for subsequent PCR analysis. Use of PBL or cell line as a source of fusion gene negative nucleic acid is preferred to *E. coli* RNA; as, if a primer set amplifies a nonrelevant cDNA in human samples, primers and other reagents will be consumed, resulting in a reduction in reaction efficiency and impaired detection of fusion gene transcripts in samples with few copies. This may be accompanied by a decrease in the ΔR_n plateau as samples become more diluted; which would not be detected using *E. coli* RNA. However, *E. coli* RNA is of value for the generation of standard curves for endogenous control genes. Overall, the relative quantitation approach has a number of advantages: (1) It avoids the use of plasmid standards that could lead to problems with contamination. (2) It can be used to relate levels of MRD detected following treatment to fusion gene expression at the time of diagnosis on an individual patient basis (cell-line dilutions could be used as a standard reference for patients lacking available diagnostic samples). (3) All aspects of the RT and PCR steps are evaluated. However, there are a number of potential disadvantages as well: (1) RNA is relatively unstable, hampering the use of such dilutions as reliable internal and external quality control materials to control for interassay variability. (2) Fusion gene and control gene expression may differ between subclones of the same cell line and also from diagnostic patient samples. An alternative relative quantitative approach involves comparison of C_t values obtained for the target and the endogenous reference gene in each particular sample. This has the advantage of eliminating the need for running standard curves, increasing sample throughput, reducing reagent costs, and reducing risks of contamination if plasmids were to have been used as standards. However, this comparative C_t method is dependent on the target and endogenous reference PCR assays being of comparable efficiency, necessitating performance of a "Validation Experiment" (*see* User Bulletin No. 2). Provided the RQ-PCR reactions are confirmed to be of comparable

efficiency, the relative expression of different genes may be derived from the difference in the C_t values obtained ($\Delta\Delta C_t$ method; *see* Manufacturer's booklet for further details). If the efficiencies of the reactions are not comparable, a standard curve-based method for reporting of gene expression should be used. Plasmid standards have a number of advantages. They are (1) valuable for assay optimization and evaluation of assay specificity; (2) permit determination of absolute gene expression levels in unknown samples; and (3) provide relatively robust quality control materials, suitable for internal and external quality control, facilitating standardization within multicenter studies and between international trial groups. Ideally plasmid standards should not be used more than once to avoid degradation caused by freeze–thaw cycles. Prolonged storage should also be avoided; in some instances a decrease in plasmid copies has been associated with absorption of DNA into the plastic storage tubes. Any deterioration in plasmid standards is most apparent for the lowest dilutions, potentially leading to a fall in the standard curve correlation coefficient. Contamination remains a major concern with the use of plasmids, leading to the recent development of degradable dU-based DNA template as an alternative standard for real-time PCR quantitation ***(21)***.

5. A number of routine measures are taken to avoid problems of PCR contamination (*see* **ref. 18**). Separate areas provided with dedicated equipment, reagents, lab coats, and gloves are maintained for (1) sample preparation and RNA extraction, (2) preparation of RT and PCR reaction mixtures, (3) reaction plate setup, and (4) Taqman analysis. Plugged tips are used for all steps of RQ-PCR. Gloves are routinely changed each time a new sample is handled. All cDNA samples are centrifuged at 11,600*g* for 1 min prior to addition to wells, to reduce the possibility of false-positive results due to cDNA aerosols. Because of the latter problem, wells are covered with strip caps as soon as the template is added, with wells for remaining samples covered temporarily (e.g., with a pipet box lid). The inclusion of uracil-*N*-glycosylase (UNG) in conjunction with dUTP which replaces dTTP in the Taqman Universal PCR Mastermix serves to reduce significantly the likelihood of contamination due to carryover PCR products from previous Taqman assays. However, UNG does not protect against contamination derived from dT-containing DNA relating to plasmid controls or conventional PCR products. Rigorous steps must be taken to avoid cross-contamination between plasmid standards during their purification and dilution. All are prepared individually, rather than together in parallel, in a laminar flow hood distinct from that used for cDNA synthesis and Taqman mastermix preparation with fresh gloves being worn each time purification of a different standard has begun. For plasmid dilutions, gloves are changed at every dilution point, with all dilutions made into 1.5-mL O-ring screw cap tubes rather than 1.5-mL flick cap Eppendorf tubes to eliminate aerosol effects. Such effects are known to add to the risk of contamination and hence may result in a dilution of erroneous copy number. The UV light in the laminar flow hood is routinely switched on after completion of plasmid work. Sterile plugged tips are employed at all times. During real-time PCR setup, fresh gloves

are worn when aliquoting each dilution of every standard. All plasmid standards are added to wells in a separate laboratory after all other sample cDNAs have been added to the plate and their respective wells capped. Although time consuming, we have found this the most reliable way to prevent contamination of sample, NAC, and NTC wells by plasmid standards and cross contamination of standard wells.

6. Owing to the high reproducibility of RQ-PCR, all standards are run in duplicate, as performance of a higher number of replicates would significantly compromise the number of patient samples that could be analyzed on each plate. However, all patient samples should be analyzed at least in triplicate wells. This occasionally reveals an "outlying" well with a markedly different C_t value from the other two; in this situation the "outlier" is disregarded and gene expression evaluated from the mean of the remaining two replicates with comparable C_t. In situations in which only one of the triplicates gives an amplification signal, the "multicomponent view" should be checked to determine whether this is an artefact or a true amplification signal. If it is the latter, the C_t value is typically >35 and is likely to reflect unreliable amplification of a low level of target present in the sample or the result of amplification of contaminating cDNA introduced during setup of the reaction plate. Single-well "positives" associated with C_t values equivalent to less than one target copy (provided by the *y*-intercept of plasmid standard curve; *see* **Fig. 3**) can effectively be reported as "negative." However in clinical practice, PCR assays associated with "single-well" positivity for the leukemia associated fusion gene transcript should be repeated using a fresh patient sample. Control gene expression levels detected in individual samples should be compared with normal values obtained in bone marrow and peripheral blood. Consistently poorer than expected C_t values for all samples analyzed on a plate could indicate deficiencies in the RT step which would need to be addressed, whereas poorer C_t values for individual samples could be indicative of insufficient and/or poor quality RNA. For sensitive detection of MRD, a repeat sample collection for MRD analysis would be required.
7. Prior to evaluation of unknown samples, primer and probe concentrations are optimized (*see* ABI 7700 User's Manual), such that the minimum concentrations of primer and probe that give the maximum ΔR_n and minimum C_t for each Taqman assay are used. In our experience, 300 n*M* primer and 200 n*M* probe achieves optimal results for the majority of assays. Extensive testing of assays for a wide range of leukemic fusion gene targets has revealed that reduction of reaction volume to 25 µL from the standard 50-µL reaction volume is not associated with any fall in sensitivity ***(16)*** and hence the lower reaction volume has been adopted in our laboratory.
8. For full details of steps involved in data analysis please refer to protocols provided by the manufacturer. The baseline fluorescence recording is usually set between PCR cycles 3 and 15. In situations in which target genes are highly expressed, it may be necessary to shift the upper baseline limit such that it is at least three C_t values lower than the C_t associated with the well containing the

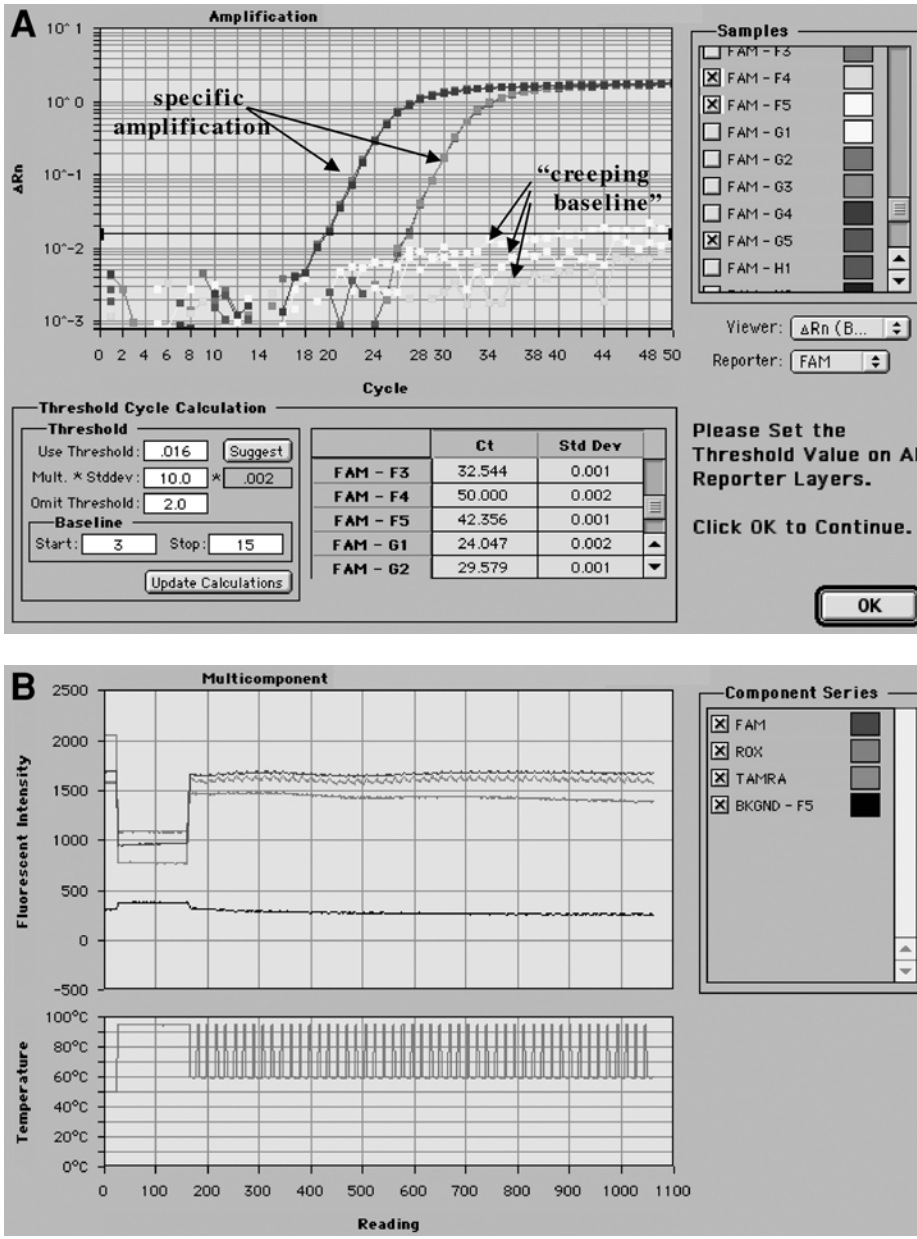


Fig. 6. Troubleshooting: distinguishing true amplification from nonspecific "baseline creeping." For some Taqman assays a steady rise in fluorescence may occasionally be noted over the time course of the PCR reaction in samples lacking the target sequence including NTC and NAC **(A)**, *(Caption continued on next page.)*

most target. The threshold setting is adjusted such that it lies above background fluorescence levels and any "creeping curves" (*see* **Fig. 6**), but still crosses the amplification curves in the part corresponding to the exponential phase of the PCR reaction.

9. There are a number of potential ways in which MRD data derived from RQ-PCR assays may be reported. This will clearly be determined by the method of quantitation adopted (e.g., absolute quantitation, relative quantitation using standard curves, or comparative C_t) and has been discussed in detail elsewhere ***(16,17,20)***. For comparison of C_t data for the same target gene between Taqman runs on an intra- or interlaboratory basis it is essential that all data are analyzed using an identical threshold value for each respective fusion gene target and the endogenous control gene used for normalization.

Acknowledgments

R. F. and D. G. are supported by the Leukaemia Research Fund of Great Britain. RQ-PCR analyses in our laboratory have been supported by the Leukaemia Research Fund and Special Trustees of Guy's Hospital. We are grateful to Joeri Aerts, Adam Corner, Jean Gabert, Niels Pallisgaard and Vincent van der Velden for helpful discussions and critical reading of the manuscript.

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Fig. 6. *(Caption continued)* which may in some instances cross the threshold (hence giving a C_t value of <50). The causes for this phenomenon are unclear; it may in some cases be associated with short baselines reducing the efficiency of the integral baseline subtraction algorithm (ΔR_n generation). It is important to distinguish "baseline creeping" from the presence of low levels of target sequence, which can occasionally give rise to comparable C_t values. This is apparent from the distinct shapes of the respective amplification plots **(A)** and also from examination of the corresponding "multicomponent view." This reveals a rise in reporter signal and fall in TAMRA signal in the presence of the target sequence of interest (*see* **Fig. 1A**), whereas the "multicomponent view" in the presence of "baseline creeping" reveals no evidence of specific amplification **(B)**.

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Real-Time PCR to Detect Minimal Residual Disease in Childhood ALL

Cornelia Eckert and Olfert Landt

1. Introduction

Measurement of minimal residual leukemia at early time points during therapy has now been shown to predict reliably outcome in childhood acute lymphoblastic leukemia (ALL) ***(1–3)***. Consequently, ongoing European treatment protocols are using this technology to stratify therapy. Currently the most widely applicable and sensitive method for the detection of minimal residual disease (MRD) in ALL is polymerase chain reaction (PCR) of antigen receptor gene rearrangements. This technique relies on identification of the leukemia specific junctional sequence from amplification of diagnostic DNA. The sequence is then used either as a clone-specific probe or allele specific primer to detect MRD in DNA taken at times of remission.

The advent of real-time quantitative (RQ) PCR technologies has meant that it is now feasible to generate truly quantitative MRD results within a clinically acceptable time frame. A fuller discussion of the principles of RQ-PCR is presented in the chapter by Grimwade, this volume. Although strategies based on junctional sequence specific Taqman probes have been described ***(4)***, this chapter concentrates on a method involving germline Taqman probes in which specificity is provided by the use of a specific primer that is located within the clone-specific junctional region. The economy of this method is such that it is used in most laboratories, providing results for ongoing clinical trials.

2. Materials

1. Platinum *Taq* polymerase (Invitrogen; Karlsruhe, Germany) (*see* **Note 1**).
2. 50 m*M* $MgCl_2$ (Invitrogen; Karlsruhe, Germany).

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

3. 10X PCR buffer minus Mg^{2+} (Invitrogen; Karlsruhe, Germany).
4. dNTPs (Invitrogen; Karlsruhe, Germany).
5. 5,6-Carboxy-X-rhodamin (ROX) (TIB MOLBIOL; Berlin, Germany).
6. Primers and Taqman probes (TIB MOLBIOL; Berlin, Germany) (*see* **Note 2**).
7. AmplyFly (Diagnostic Research; Athens, Greece).
8. Buffy coat (BC) DNA (derived from equivalent mixtures of blood mononuclear cells of 5–10 healthy volunteers).
9. Dimethyl sulfoxide (DMSO) (Sigma; St. Louis, MO, USA).
10. ABI 7700 real-time PCR machine (Applied Biosystems; Foster City, CT, USA).

3. Methods

The method described here aims to generate clone-specific primers with a sensitivity of at least 10^{-4}. The first step involves identification and sequencing of clone-specific antigen receptor gene rearrangements (*see* **Note 3**). This is followed by a primer design in which the clone-specific sequence is examined to identify potential allele-specific oligonucleotides. The specificity and sensitivity of these primers are then tested. Remission DNA is then subject to allele-specific priming and the results compared with those produced from a standard curve derived from dilution of leukemic DNA into BC DNA. Controlled for variation in amplifiability of individual DNA samples is achieved using a control PCR, in this case β-globin.

3.1. Design of Clone-Specific Primer

Clone-specific forward primer may be designed using the OLIGO 6.0 (Molecular Biology Insights, Cascade, USA) and the JAVA OLIGO program (TIB MOLBIOL). The following rules are useful:

1. The melting temperature of the primer should be approx 55–60°C; calculated with JAVA OLIGO or Primer Express (Applied Biosystems; Foster City, CT, USA).
2. The primer should have a length between 19 and 25 bp.
3. The 3' end of the primer should be located in the junctional region (V-N-J/D-N-J/V-N-D/D-N-D).
4. No more than 4 (or 3 GC) bases of the 3' end of the primer should overlap to the next D- or J- region.
5. For IgH, when possible the 3' end of the primer should be located in the junctional region of D-N-J instead of V-N-J (*see* **Note 4**).
6. No more than two C and/or G residues at the 3' end.
7. Avoid identities between primer and probes, especially to the 3' termini of the primers.
8. Avoid oligonucleotides that are likely to form secondary structures.

3.2. Testing Primer Specificity

The specificity test is performed with 50 ng of BC DNA (in at least triplicate) and 50 ng of DNA from sample of the patient leukemic DNA at diagnosis and a control without DNA. The primer is specific when the C_t value of the signal in the leukaemic sample lies between 23.0 and 28.0 cycles (*see* **Note 5**) and the C_t of the BC is greater than the cycle after additional 13 cycles.

3.3. Testing Primer Sensitivity and Generation of a Standard Curve

Sensitivity is tested using serial dilutions of leukemic DNA in BC. Start with leukemic DNA and the BC DNA at a concentration of 100 ng/µL (*see* **Note 6**). The dilution is done in 5 log steps from 10^{-1} to 10^{-5} and is now used to create a standard curve. The following samples should be analyzed to construct the standard curve:

1. Duplicate samples of 500 ng of DNA from each dilution point.
2. Four samples of 500 ng of BC DNA.
3. Two nontemplate controls.

The standard curve is delineated by plotting the logarithm of the initial template copy number on the *x*-axis and the cycle number at the crossing point (C_t) on the *y*-axis. The slope of this line is the negative reciprocal of the log of the efficiency. The optimal efficiency is –3.3 (*see* **Note 7**). The standard curve must have a correlation coefficient of at least 0.95.

The reproducible range of a given primer is the range over which the maximal difference in C_t values of the duplicate dilution samples is less than 1.5 cycles. Reproducible sensitivity of the primer refers to the lowest dilution within the reproducible range at which the C_t is 3 cycles less than the highest C_t seen in the BC DNA. The maximal sensitivity of the primer is defined as that at which the C_t of at least one of the duplicate dilution samples is at least 1.5 cycles less than that of the lowest unspecific amplification as seen in BC DNA (**Figs. 1** and **2**).

3.4. Real-Time PCR

3.4.1. PCR Mastermix

Each 20 µL of PCR should contain:

1. $MgCl_2$: 5 m*M* for TCR-δ, Igκ, IgH, β-globin PCR or 7.5 m*M* for TCR-γ PCR.
2. 1 U Platinum DNA *Taq* polymerase.
3. 2 µL 10X PCR buffer 20 µL.

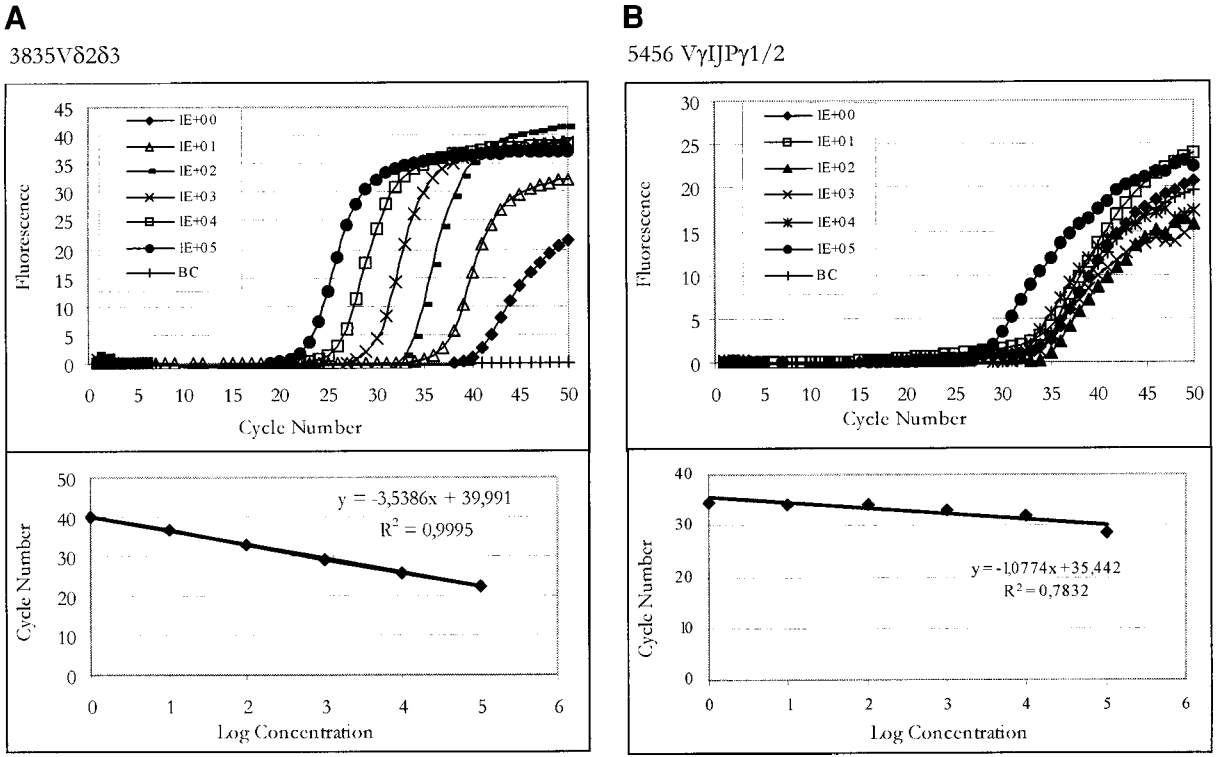


Fig.1. (**A**) PCR curves of a 10-fold dilution series with a BC as negative control and a standard curve are shown. No unspecific amplification is seen in the BC. A sensitivity of 10^{-5} (1 leukemic cell in 100,000 normal cells) could be achieved. The slope of the standard curve is –3.5, and the correlation coefficient is –1. (**B**) The amplification curves of a dilution series and the standard curve shown here demonstrate a poor target. Because a standard curve cannot be created, and unspecific amplification still occurs (after optimization), another PCR target should be used for MRD quantification.

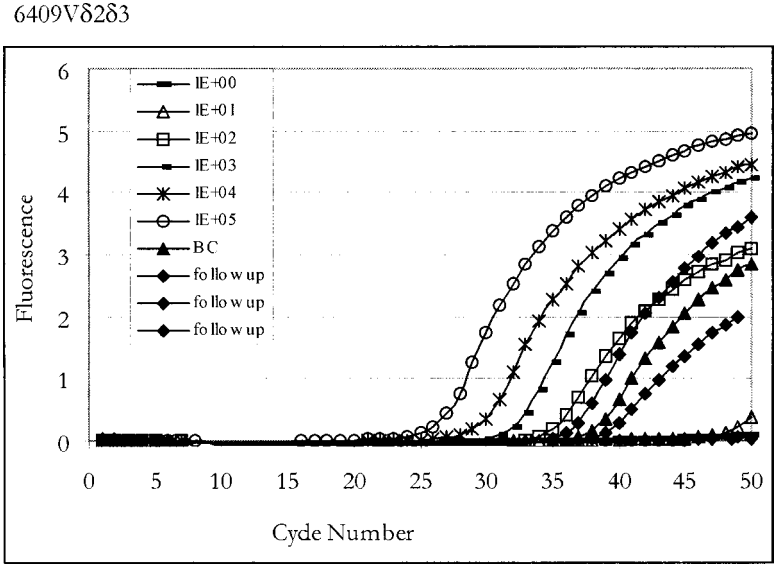


Fig. 2. These amplification curves should illustrate how the sensitivity determines the detection limit. An unspecific signal of the BC DNA sample is seen after the fourth dilution step 100 leukemic cells in 100,000 normal cells (10^{-3}). Therefore it is not possible to quantify the follow up sample of the patient. Moreover, the differences in C_t values of each from the triplicate of the follow-up sample are very high.

4. 0.2 m*M* dNTPs.
5. 1.5 µ*M* ROX.
6. AmplyFly.
7. 0.5 µ*M* Primer.
8. 0.1 µ*M* Hydrolyzation probe (*see* **Note 8**).
9. 500 ng of DNA (100 ng/µL).

3.4.2. PCR Cycle Conditions

The amplification protocol is

1. Predenaturation at 94°C, 5min, and then
2. 50 cycles of denaturation at 94°C for 45 s, annealing/elongation at 60–69°C for 1 min (*see* **Note 9**).

3.5. Quantification

Each remission DNA follow-up sample should be measured in triplicate. The C_t values of a sample measured in duplicate or in triplicate should have an

intra- and interassay SD between 0.0 and 0.5. Calculation of the amount of MRD present is performed directly using the real-time instrument. However, it can also simply calculated by plotting the C_t through the "experimental report" and respective calculation using Microsoft® Excel.

3.5.1. Normalization

To correct for differences in quantity and amplification quality of the DNA samples a reference gene should be used. In our case, the β-globin was used. As previously mentioned, a correction for the values obtained for a reference gene for the same DNA samples that were measured for the target gene is necessary.

A β-globin standard curve has to be established using BC DNA diluted in water. Corrections on target genes quantified in follow-up samples were performed following. In brief, the expected quantity of cells per tube (100,000 cells about 500 ng DNA) is divided by the concentration of the reference gene measured in the sample. This factor has to be multiplied by the concentration of the target gene.

4. Notes

1. Several *Taq* polymerases are suitable for RQ-PCR. However, long-range polymerases are associated with a single-strand specific nuclease activity, thus destroying Taqman probes and should be avoided ***(5)***.
2. Sequences of all germline primers and probes for quantification of clone specific Igκ, TCRδ, IgH, and TCRγ gene rearrangements:

Igκ: κde (Vκ κde):

Reverse (κdeR):	5'-AGA CCC TTC AGG CAC ATG CTT-3'
TM-probe (igκTM):	5'-6-FAM-TCC TCA GAG GTC AGA GCA GGT TGT CC XT ph-3'

TCRδ: Dδ3 (Vδ2Dδ3, Dδ2Dδ3):

Reverse (Dδ3R):	5'-CTG CTT GCT GTG TTT GTC TCC T-3'
TM-probe (Dδ3TM):	5'-6FAM-ATA TCC TCA CCC TGG GTC CCA TGC CXT ph-3'

TCRδ: Jδ1 (Vδ1Jδ1, Vδ2Jδ1, Vδ3Jδ1, Dδ2Jδ1):

Reverse (Jδ1R):	5'-TGCCTTAACCTTAAACTTCAGA-3'
TM-probe (Jδ1TM):	5'-6FAM-AACCCGTGTGACTGTGGAACCAA GT XT p-3'

IgH: JH1 - 6 (VHJH)

Reverse:

R-JH1-CONS1	5'-CGCTATCCCCAGACAGCAGA-3'
R-JH2-CONS1	5'-GGTGCCTGGACAGAGAAGACT-3'
R-JH3-CONS1	5'-AGGCAGAAGGAAAGCCATCTTAC-3'
R-JH4-CONS2	5'-CAGAGTTAAAGCAGGAGAGAGGTTGT-3'
R-JH5-CONS1	5'-AGAGAGGGGGTGGTGAGGACT-3'

R-JH6-CONS2	5'-GCAGAAAACAAAGGCCCTAGAGT-3'
TM-probes:	
T-JH1.2.4.5.-CONS1	5'-CCCTGGTCACCGTCTCCTCAGGTG-3'
T-JH3-CONS1	5'-CAAGGGACAATGGTCACCGTCTCTTCA-3'
T-JH6-CONS2	5'-CACGGTCACCGTCTCCTCAGGTAAGAA-3'
TCR-γ: Jγ1/2 (VγJγ1/2) (VγJγ1.3, 2.3):	
Reverse (Jγ1/2R):	5'-GTTTAATAATTTCCTGCTTTCCCTCTAT-3'
TM-probe (Jγ1/2TM):	5'-6FAM-TCCGATACTTACCTGTGACAACAA GTGTXT ph-3'
TCR-γ: JPγ1/2 (VγJPγ1/2) (VγJγ1.1, 2.1):	
Reverse (JPγ1/2R):	5'-ACCCTGAAAAATTGCTGTTCGT-3'
TM-probe (JPγ1/2TM):	5'-6FAM-TACTGAGGCCAGGAATGTGACAT ATXTCAG ph-3'
Reference gene: β-globin:	
Forward:	5'-TATTGGTCTCCTTAAACCTGTCTTG-3'
Reverse:	5'-CTGACACAACTGTGTTCACTAGC-3'
TM-probe:	5'-6FAM-CCCACAGGGCAGTAACGGCAGA CXT ph-3'

X, Modified; ph, 3'-phosphate.

The sequences of the IgH RQ PCR were published by Verhagen et. al ***(6)***; the other primers and probes were designed and tested in our own laboratory. All Taqman probes used are FAM (6-carboxyfluorescein)/TAMRA(6-carboxytetramethyl rhodamine) labeled. FAM was chosen as reporter dye at the 5' end of the probe and TAMRA as the quencher dye at the 3' end. The annealing temperature can be in the range between 60°C and 69°C.

3. A large number of primer systems have now been described for the amplification of antigen receptor gene rearrangements in ALL. Those described by the Biomed group are the most widely applicable and are currently used in many centers across Europe ***(7–9)***.
4. The impact of clonal instability at the IgH locus can be minimized by designing primers directed to the D-N-J junction in preference to the V-N-D sequence.
5. The C_t value is the threshold cycle number; that is, the cycle at which emitted fluorescence exceeds the 10 times SD of baseline emission—simply the C_t value is that cycle where PCR product is first detected.
6. It is very important to consider the percentage of blasts at diagnosis. In the cases where the bone marrow contains fewer than 85% blasts, then the amount of DNA used to construct the standard curve should be adapted. When the initial percentage of blast is too low or insufficient diagnostic DNA is available, it is possible to clone the PCR product and to prepare a dilution series from plasmid in a background of BC DNA.
7. A range of a slope from –3.0 to –3.9 can be tolerated. Slopes outside this may be caused by erroneous primer concentration, $MgCl_2$ concentration, and PCR inhibitors.

8. Fluorophores are highly sensitive to light and must be stored appropriately.
9. Raising the annealing temperature may improve specificity and thus sensitivity of the primer. Increments of 3°C to a maximum of 69°C are suggested. In addition, adding of DMSO could be helpful.

Acknowledgment

This study was kindly supported by Deutsche Kinderkrebsstiftung, Germany, and by TIB MOLBIOL, Germany.

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13

Oligonucleotide Microarray Analysis of Gene Expression in Leukemia

Frederik W. van Delft and Louise K. Jones

1. Introduction

Analysis of gene expression is used to appreciate gene function, as the pool of messenger RNA (mRNA) determines, at least partly, the physiological status of the cell. Until now it has been possible to measure single gene function only by Northern assays or reverse transcriptase-polymerase chain reaction (RT-PCR). With the development of microarrays it is possible today to analyze global gene expression patterns in a quantitative fashion. This can be used to understand biological processes ***(1)*** and to identify new disease classes ***(2–7)*** or drug targets ***(8)***. Other uses include genotyping ***(9)***, comparative genomic hybridization ***(10)***, and analysis of DNA–protein interaction ***(11)***. The amount of data produced by these experiments is formidable, and application of bioinformatics is integral to this technology.

Microarray chips differ in the type of nucleic acid "target" molecules used and method of application. Oligonucleotide chips such as those made by Affymetrix (Santa Clara, CA, USA) are produced by photolithographic synthesis of 25-mers, linked to a quartz wafer. Each probe (analogous to probe used in Northern assays) is constructed *in situ* by adding a specific nucleotide on top of another according to the sequence derived from databases (e.g., Unigene). The majority of probes represent known and fully sequenced genes, but expressed sequence tag (EST) sequences are also synthesized. Each gene is represented by 11–16 different oligonucleotides. These probes are placed on different locations on the chip surface. In addition, mismatch nucleotides are represented, to allow normalization for nonspecific cross-hybridization. The latest generation of Affymetrix chips contains nucleotides for 33,000 unique human genes and ESTs. The oligonucleotide array has a high specific-

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

ity and is capable of distinguishing 90% homology between genes. The Affymetrix technology, while currently expensive and fairly inflexible, is very robust, reproducible, and allows the detection of single nucleotide polymorphisms (SNPs) and other small features in the DNA. As the Affymetrix produces only an absolute intensity of expression rather than ratios, it is very important to normalize between the experimental and control genes.

Although protocols for the use of cDNA chips will not be presented here, they are briefly discussed for completeness. cDNA chips are made by robotically spotting of PCR products onto a poly-L-lysine coated glass slide (e.g., The Sanger Institute, Hinxton, UK). These PCR products range in size from 100 basepairs to several kilobases. The information for the generation of the cDNA clones is derived from the EST database. ESTs are partial sequences of cDNA clones, widely used for gene discovery and mapping and represent >70% of all Genbank entries. One assumption is that as many unique transcripts, representing as many genes as possible, are used. Analysis of these clones can be done through a number of programs including UniGene at the National Centre for Biotechnology (http://www.ncbi.nlm.nih.gov/UniGene. Currently 8000–10,000 unique human genes are represented on this chip, but a new chip with 15,000–20,000 genes is planned for the summer of 2002 ***(12)***. More limited customized chips can be easily designed and produced. It is important to note that the spotting process is inherently variable. In addition, the length of the cDNAs used as targets makes it impossible to discriminate between genes with >80% homology.

Successful microarray experiments require the extraction of high-quality RNA. Gloves and lab coat should always be worn; diethylpyrocarbonate (DEPC)-treated water and other solutions should come from separate compartment stocks and kept in smaller aliquots, clearly marked "RNA only." Filter tips should be used and bench pipets cleaned with RNase zap periodically. In our lab RNA is extracted with TRIzol. cDNA is then synthesized using a T7-oligo (dT) primer. In vitro transcription labels the cDNA with biotin, which is subsequently chemically fragmented. The biotinylated ss RNA is then hybridized to the probes on the microarray chip. The chips are washed and stained. The Agilent Gene Array (Palo Alto, CA, USA) laser scanner is used to scan the chip. The Micro Array Suite (MAS) software analyzes the raw scanning data and measures the signal intensity of all probe sets on a chip. The relative signal intensity is calculated for each probe set separately, by dividing the measured intensity by the mean intensity of all probe sets on one chip. Each probe set will have a value (signal intensity) and a flag (Absent or Present call, depending on the signal intensity compared with the background intensity). These data can then be exported as an Excel or Text file and are now ready to be analyzed by gene expression analysis software, for example, Affymetrix,

GeneSpring, Kensington. Many other packages are available on the World Wide Web.

2. Materials

1. TRIzol Reagent (Invitrogen Life Technologies, P/N 15596-018).
2. Chloroform.
3. Propan-2-ol.
4. RNase-free water.
5. 0.1% DEPC (Sigma D-5758).
6. Phosphate-buffered saline (PBS).
7. Ethanol 75% (diluted with RNase-free water).
8. Qiagen RNeasy Total RNA isolation kit (optional)(P/N 741104).
9. 1.5-mL Polypropylene disposable homogenizer (k-749520-0000).
10. 1.5-mL and 2-mL sterile Eppendorf tubes.
11. Sterile, filter p1000, p200, p20, p10 tips.
12. 3 *M* Sodium acetate, pH 5.2 (Sigma, P/N S7899).
13. 80%, 100% Ethanol (diluted with RNase-free water).
14. 5 mg/mL of Glycogen (Ambion, P/N 9510).
15. Superscript ds-cDNA Synthesis Kit (Gibco, P/N 11917-010).
16. T7-$(dT)_{24}$ oligomer, high-performance liquid chromatography (HPLC) purified. 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3'
17. 0.5 *M* EDTA, pH 8.0 (Sigma Chemical, P/N E7889).
18. 2 mL of Phase Lock Gel Light, (Eppendorf; P/N 0032005 101).
19. Phenol–chloroform–isoamyl alcohol (25:24:1), alkaline buffer (10 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA) (Ambion, P/N 9732).
20. 7.5 *M* Ammonium acetate (Sigma, P/N A2706).
21. BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem, NY, USA, P/N 900182).
22. RNeasy Mini Kit (Qiagen, P/N 74104).
23. Fragmentation buffer Trizma base (Sigma Chemical, P/N T 1503), MgOAc (Sigma Chemical, P/N M 2545), KOAc (Sigma Chemical, P/N P 5708), glacial acetic acid (Sigma Chemical, P/N A 628).
24. Gene Chip Eukaryotic Hybridization Control Kit (Enzo, Affymetrix; 900299).
25. 10 mg/mL of herring sperm DNA (Promega/Fisher Scientific, P/N D1811).
26. 50 mg/L of acetylated bovine serum albumin (BSA) (GIBCO BRL Life Technologies, P/N 15561-020).
27. 2-Morpholinoethanesulfonic acid (MES) free acid monohydrate SigmaUltra (Sigma Chemical, P/N M5287).
28. MES sodium salt (Sigma Chemical, P/N M3058).
29. 1X, 2X Hybridization buffer.
30. Probe array (Affymetrix) 24 μm × 24 μm probe cells.

31. R-phycoerythrin streptavidin (SAPE), Molecular Probes (P/N S-866).
32. 5 *M* NaCl RNase-free, DNase-free (Ambion, P/N 9760).
33. PBS, pH 7.2 (GIBCO BRL, P/N 20012-027).
34. 20X SSPE (3 *M* NaCl, 0.2 *M* NaH_2PO_4, 0.02 *M* EDTA) (BioWhittaker, P/N 16-010Y).
35. Goat IgG, reagent grade (Sigma Chemical, P/B I 5256).
36. Anti-streptavidin antibody (goat), biotinylated (Vector Laboratories, P/N BA-0500).
37. Antifoam 0-30 (Sigma Chemical, P/N A-8082).
38. 10% Tween 20 (Pierce Chemical, P/N 28320).
39. Bleach (5.25% sodium hypoclorite) (VWR, P/N 21899-504).
40. Water, molecular biology grade (BioWhittaker, P/N 16-001Y).
41. 1000 mL of stringent wash buffer B: 100 m*M* MES, 0.1 *M* (Na^+), 0.01% Tween 20 (83.3 mL of 12X MES stock buffer, 5.2 mL of 5 *M* NaCl, 1.0 mL of 10% Tween 20, 910.5 mL of water). Filter through a 0.2-µm filter.
42. 1000 mL of nonstringent wash buffer A: 6X SSPE, 0.01% Tween 20, 0.005% Antifoam (300 mL of 20X SSPE, 1.0 mL of 10% Tween 20, 698 mL of water). Filter through a 0.2-µm filter, After filtering, add 1.0 mL of 5% Antifoam.
43. 5% (w/v) Antifoam stock solution. Prepare a 5% (w/v) solution of Antifoam in water. Do not filter.
44. 250 mL of 2X stain buffer (41.7 mL of 12X MES stock buffer, 92.5 mL of 5 *M* NaCl, 2.5 mL of 10% Tween 20, 112.8 mL of water). Filter through a 0.2-µm filter. After filtering, add 0.5 mL of 5% Antifoam.
45. 10 mg/mL of goat IgG stock. Resuspend 50 mg in 5 mL of PBS. Store at 4°C.
46. 12X MES stock buffer: 70.4 g of MES free acid monohydrate, 193.3 g of MES sodium salt, 800 mL of molecular biology grade water. Mix well. Make volume up to 1000 mL. Aim for pH between 6.5 and 6.7. Filter through a 0.2-µm filter. Store in a dark room (light sensitive) at 4°C. Discard solution if yellow.
47. 1200 µL of SAPE staining solution (600 µL of 2X stain buffer 540 µL of water, 48 µL of 50 mg/mL acetylated BSA, 12 µL of 1 mg/mL of SAPE. Mix well, and divide into two aliquots of 600 µL. Keep in the dark.
48. 600 µL of antibody solution: 300 µL of 2X stain buffer, 266.4 µL of water, 24 µL of 50 mg/mL of acetylated BSA, 6 µL of 10 mg/mL normal goat IgG, 3.6 µL of 0.5 mg/mL biotinylated antibody.
49. 50 mL of 2X hybridization buffer: 8.3 mL of 12X MES stock, 17.7 mL of 5 *M* NaCl, 4.0 mL of 0.5 *M* EDTA, 0.1 mL of 10% Tween 20, 19.9 mL of water.
50. 20 mL of cRNA fragmentation buffer: 200 m*M* Tris-acetate, pH 8.1; 500 m*M* KOAc; 150 m*M* MgOAc (4.0 mL of 1 *M* Tris-acetate, pH 8.1 [adjust pH with glacial acetic acid], 0.64 g of MgOAc; 0.98 g of KOAc). Use RNase-free water to make up to 20 mL. Mix well and filter with a 0.2-µm vacuum filter unit. Divide into smaller tubes. These can be stored at room temperature.

3. Methods

3.1. RNA Extraction

RNA can be extracted from fresh material or material stored in the freezer at –70°C or in liquid nitrogen (*see* **Note 1**). Several products for total RNA extraction are available on the market (TRIzol Reagent, Gibco BRL Life Technologies; RNeasy Total RNA Isolation Kit, Qiagen). Alternatively, protocols for selective extraction of poly(A) mRNA are available.

1. If the sample is frozen, make sure it is completely thawed and washed in PBS to remove remnants of dimethyl sulfoxide (DMSO) or tissue culture media.
2. Homogenize 50–100 mg of tissue in 1 mL of TRIzol or use 1 mL of TRIzol reagent per 5–10 × 10^6 cells in suspension. Tissue volume should not exceed approx 10% of the TRIzol (*see* **Note 2**).
3. Leave the homogenized sample for 5 min at room temperature (RT) and add 200 µL of Chloroform per milliliter of TRIzol used (*see* **Note 3**).
4. Shake vigorously for 15 s and leave for 2–3 min at RT.
5. Centrifuge at 12,000*g* for 15 min at 4°C (*see* **Note 4**).
6. Transfer the upper layer—all the RNA is contained in this aqueous phase—into a fresh Eppendorf tube. Make sure to change tips between samples and do not disturb the middle white layer or bottom organic layer. The organic layer can be stored for the extraction of protein or DNA if required.
7. Add 500 µL of propan-2-ol to the aqueous phase and mix by inversion. Do not vortex-mix. Incubate for 10 min at RT and centrifuge for 10 min at 12,000*g* at 4°C. A pellet is visible at the bottom in most cases.
8. Remove the supernatant and wash once with 1 mL of 75% ethanol for every milliliter of TRIzol used, by pipeting up and down.
9. Centrifuge for 5 min at 7500*g* at 4°C.
10. Remove the ethanol and air dry for 5 min. Dissolve in RNase-free water. Place at 55–60°C to dissolve if necessary (*see* **Note 5**).

3.2. RNA Precipitation

1. Add 1/10 volume of 3 *M* NaAc, pH 5.2; and 2.5 volumes of 100% ethanol to the RNA. Use 0.5–1 µL of glycogen, as this may improve recovery of RNA and also aids in visualization of the pellet.
2. Mix and incubate for at least 1 h at –20°C.
3. Centrifuge for 20 min at 4°C at ≥ 12,000*g*.
4. Wash the pellet twice with ethanol 80%.
5. Air-dry, and dissolve in RNase-free water and incubate for 10 min at 55–60°C.

3.3. Quality Check of RNA

RNA quality and concentration should now be determined. Spectrophotometric analysis of the sample will determine the absorbance at 260 and 280 nm. The A_{260}/A_{280} ratio should be at least 1.8 in order to continue with the protocol. RNA concentration is measured. Protein absence can be confirmed. Integrity of RNA and presence of intact 18S and 28S (and sometimes 5S) ribosomal bands can be checked by running 2 µg using a 1% agarose gel with ethidium bromide staining. Ideally, agarose, gel tank, and comb should be kept for RNA work only, or washed thoroughly with DEPC-treated water before use. The size and brightness of 28S band should be double that of the 18S band, with little or no evidence of smearing, which would indicate degradation.

A more accurate approach to determine the quality and quantity of your total RNA is to use the Agilent Bioanalyzer. As little as 50–500 ng of total RNA is sufficient to calculate the 28S/18S ratio (1.5–2.5) and detect any degradation (**Fig. 1**). At least 5 µg of good quality total RNA is essential to proceed to the next step. Proceed to cDNA synthesis or store at –80°C (*see* **Note 7**).

3.4. Synthesis of First-Strand cDNA from Total RNA

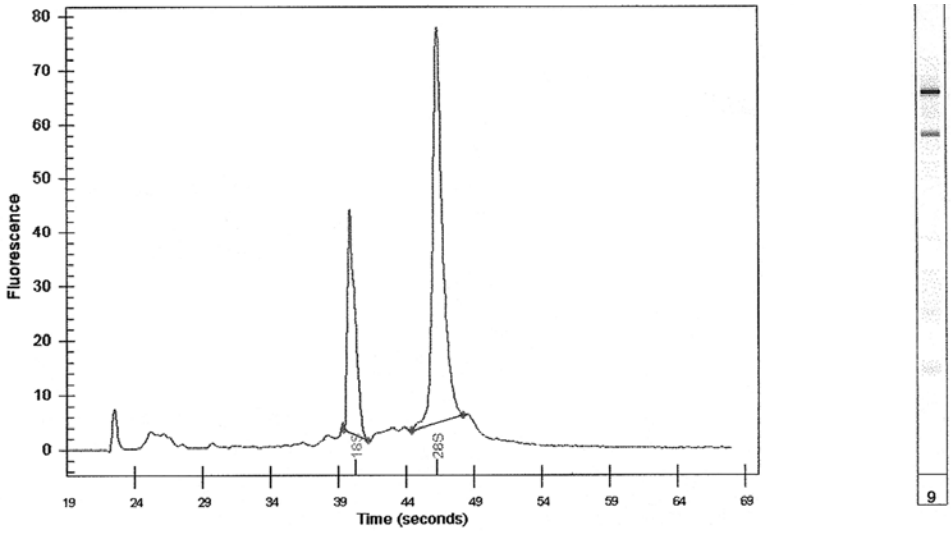
For the synthesis of double-stranded cDNA, we use the Superscript ds-cDNA Synthesis kit (Invitrogen Life Technologies, P/N 11917-010) as follows:

1. Take RNA (5–16 µg) in *x* µL, 1 µL T7-$(dT)_{24}$ primer. Add *y* µL RNase-free water to make volume up to 11 µL (*see* **Note 8**). Mix by pipeting.
2. Incubate at 70°C or 10 min and place on ice.
3. Then add 4 µL of 5X first-strand cDNA buffer, 2 µL of 0.1 *M* dithiothreitol (DTT), and 1 µL of 10 m*M* dNTP. Mix by pipeting.
4. Incubate at 42°C for 2 min.
5. Then add Superscript II Reverse Transcriptase (SS II RT) (*see* **Notes 9** and **10**). End volume is 20 µL.
6. Incubate at 42°C for 60 min. Place on ice.

3.5. Second-Strand Synthesis

1. The reaction mix is made up as follows (*see* **Note 11**):
   a. 91 µL of RNase free water.
   b. 30 µL of 5X Second-strand reaction buffer.
   c. 3 µL of 10 m*M* dNTP.
   d. 1 µL of DNA ligase (10 U/µL).
   e. 4 µL of DNA polymerase I (10 U/µL).
   f. 1 µL of RNase H (2 U/µL).

   Total 130 µL.
2. Mix by pipeting.



Corrected RNA Area: 361.76 rRNA Ratio [28S / 18S]: 2.08
RNA Concentration: 442.32 ng/ul

#	Name	Start Time (s)	End Time (s)	Corr.Area	% of total Area
1	18S	39.40	41.20	65.97	18.24
2	28S	44.40	48.20	137.47	38.00

Fig. 1. RNA analyzed with the Agilent Bioanalyser. The three peaks in the diagram represent a marker, introduced in the sample, and 18S and 28S ribosomal bands. The area under the curve corresponds to the RNA concentration. The surface area of 28S divided by 18S results in the rRNA ratio. The figure on the right is a graphic representation of a gel image.

3. Centrifuge briefly and incubate at 16°C for 2 h in a water bath.
4. Add 2 µL of T4 DNA polymerase (5 U/µL).
5. Incubate for a further 5 min at 16°C. Add 10 µL of 0.5 *M* EDTA to stop the reaction.
6. At this stage you can start cleanup of ds cDNA or store at –80°C.

3.6. Cleanup of Double-Stranded cDNA

1. To purify the cDNA, we combine the use of Phase Lock Gel (Eppendorf) and phenol–chloroform extraction (*see* **Note 12**).
2. Centrifuge the Phase Lock Gel at maximum speed for 30 s.
3. Add 162 µL of (25:24:1) phenol–chloroform–isoamyl alcohol (saturated with 10 m*M* Tris-HCl, pH 8.0–1 m*M* EDTA) with the total volume of your cDNA synthesis reaction (162 µL) and vortex-mix (*see* **Note 13**).

4. Transfer the final volume of 324 µL to the Phase Lock Gel tube.
5. Centrifuge for 2 min at maximum.
6. Transfer the aqueous phase (layer on top of gel) to a fresh 1.5-mL tube ± 150 µL.
7. Add 75 µL of 7.5 *M* NH_4Ac and 375 µL of 100% cold ethanol. Glycogen can be added to increase the yield (4 µL, 5 mg/mL) (*see* **Note 14**). Vortex.
8. Centrifuge for 20 min at room temperature at maximum to precipitate the cDNA
9. Remove supernatant.
10. Wash twice with 0.5 mL of 80% ethanol. Be careful not to dislodge the pellet.
11. Remove all the ethanol, air-dry for 5 min, and redissolve in 12 µL of RNase-free water. Proceed to in vitro transcription or store at –80°C.

3.7. In Vitro Transcription

In the following reaction the T7 RNA polymerase will produce biotin-labeled single-stranded RNA molecules (*see* **Note 15**). The ENZO Bioarray High Yield RNA Transcript Labeling Kit provides all the components of this reaction. Make sure all the ingredients are at room temperature before starting.

1. Reaction mix is made up as follows
   a. 12 µL of cDNA.
   b. 10 µL of RNase-free water.
   c. 4 µL of 10X HY reaction buffer.
   d. 4 µL of 10X Biotin-labeled ribonucleotides.
   e. 4 µL of 10X DTT.
   f. 4 µL of 10X RNase inhibitor mix.
   g. 2 µL of 20X T7 RNA polymerase.

   Total, 40 µL.
2. Incubate the reaction in a 37°C water bath for 4–5 h.
3. Tap the tube every 30–60 min to mix the enzymes.
4. Store a 1-µL aliquot for gel electrophoresis.

3.8. Cleanup of cRNA

1. Use RNeasy spin columns from Qiagen (using the provided protocol).
2. 60 µL of RNase-free water is added to the sample to make the volume up to 100 µL.
3. Add 350 µL of RLT buffer and mix thoroughly. Do not add β-mercaptoethanol to the buffer (*see* **Note 16**).
4. 250 µL of 100% ethanol is added and mixed by pipeting.
5. The sample is then transferred to a centrifuge column and centrifuged for 15 s at 8000*g*.
6. Place filter in a fresh tube and add 500 µL of RPE buffer. Centrifuge for 15 s at 8000*g*.
7. Discard flow-through and reuse tube.
8. Add 500 µL of RPE buffer and centrifuge for 2 min at maximum.
9. Place filter in a fresh tube and centrifuge for 1 min at ≥ 12,000*g* to dry the filter.

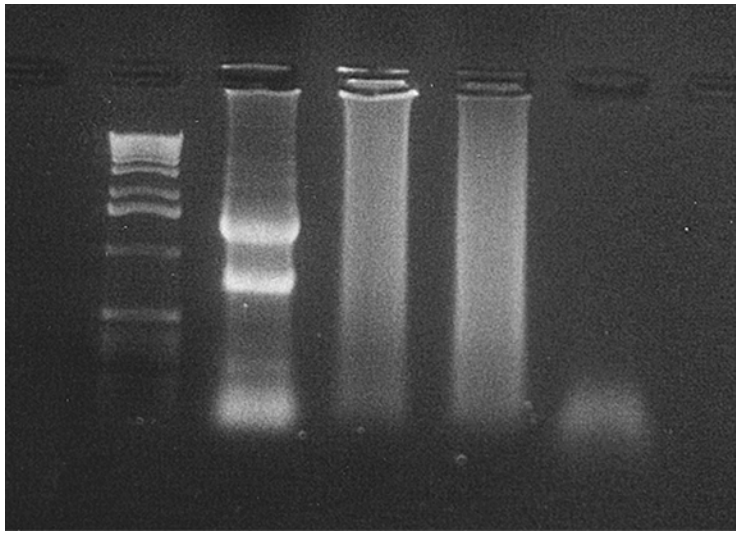


Fig. 2. 1% agarose gel. *Lane 1*, 1-kb ladder; *lane 2*, total RNA with 18S and 28S bands; *lanes 3* and *4*; cRNA before and after cleanup; *lane 5*, fragmented cRNA.

10. Place filter into a second fresh tube and add 30–50 µL of RNase-free water.
11. Wait 4 min to allow the RNA to dissolve in the water and centrifuge for 1 min at 8000*g* to elute cRNA.
12. Store a 1-µL aliquot for gel electrophoresis. Proceed to fragmentation or store at –80°C.
13. Before the cRNA can be fragmented, quality and quantity needs to be established. You can use Gene Quant or Bioanalyzer. The minimum concentration of cRNA required is 0.6 µg/µL (*see* **Note 17**).

3.9. Fragmentation of cRNA

For fragmentation 20 µg of cRNA is needed.

1. Pipet the cRNA into a fresh tube and add RNase-free water to a volume of 32 µL.
2. Add 8 µL of 5X fragmentation buffer to make up to a total of 40 µL and incubate for 35 min at 94°C, then quench on ice.
3. Remove an aliquot for gel electrophoresis (1–2 µL).
4. Run a 1% agarose gel for 30–45 min at 100 V comparing the cRNA before and after cleanup and the size of the fragmented cRNA (35–200 bp) (**Fig. 2**).

3.10. Preparing the Hybridization Cocktail

The total reaction volume is based on use for both a standard array (200 µL) and a test chip (80 µL).

1. Defrost the Control Oligonucleotide B2 and 20X hybridization control.
2. Heat the vials at 65°C for 5 min and briefly centrifuge them before use (*see* **Note 18**).
3. The reaction mix is made up as follows:
    a. 30 µL (15 µg) of Fragmented RNA.
    b. 5 µL of Control oligonucleotide B2.
    c. 15 µL of 20X Eucaryotic hybridization control.
    d. 3 µL of Herring sperm DNA.
    e. 3 µL of Acetylated BSA.
    f. 150 µL of 2X Hybridization buffer.
    g. 94 µL of RNase-free water.

    Total, 300 µL.

3.11. Hybridization to Chip

The Affymetrix GeneChips™ should be stored at 4°C protected from light.

1. While we prepare the target, the chips should be allowed to reach RT.
2. They can then be filled with 200 µL 1X hybridization buffer and placed in the rotating hybridization oven for 10 min at 45°C.
3. The hybridization cocktail is heated at 99°C for 5 min. After heating the cocktail at 99°C, it needs to be cooled to 45°C for 5 min.
4. Centrifuge the tube for 5 min at ≥ 12,000*g* to precipitate any insoluble material.
5. Take the 1X hybridization buffer out the chip and fill with the hybridization cocktail (80 µL for a test chip and 200 µL for a standard array).
6. Place the chip in the GeneChip Hybridization Oven 640 (Affymetrix, P/N 800139). Balance and rotate the chips for ±16 h at 45°C.
7. Following hybridization, the hybridization cocktail is removed and replaced by nonstringent wash buffer A and placed in the GeneChip Fluidics Station 400 (Affymetrix, Santa Clara, CA, USA).
8. In the fluidics station, the chip is washed with nonstringent buffer A and stringent buffer B to remove nonhybridized targets and nonspecific binding. Streptavidin phycoerythrin is then used to stain the biotinylated nucleotides and this fluorescent signal is amplified by using goat IgG and anti-streptavidin biotinylated antibodies, followed by a second staining procedure.

3.12. Data Analysis

Microarray experiments create huge sets of data, which are virtually uninterpretable without additional tools. The main concept of data mining programs is to detect patterns in these expression profiles. GeneSpring™ is one of the leading datamining software packages available (http://www.silicongenetics.com/) It is capable of recognizing data files from many commercially available formats, or can be programmed to recognize a certain format if necessary. The first step is to load all data obtained from the scanner. GeneSpring will assume this to be the raw data. Some scanned data may come in already

normalized. If not, the next step is to apply normalization, to allocate the necessary parameters to your experiment and adjust interpretation settings. Normalization is used to standardize microarray data, to allow "true" biological variations in gene expression levels to be differentiated from measurement variables. Experimental parameters can be set to include data such as drug concentration, time course setting, cell line—in fact any other additional information, which will allow grouping of data in many different permutations. Experimental Interpretation is set to determine precisely how the experiment is displayed. One can, for example, indicate whether data are derived from a continuous time-course experiment or whether data are replicates of each other. The data are now ready for further analysis. You will first need to confirm the quality of your data. Compare the overall profiles of your experiments in the Graph view and as a Scatterplot. Consider removing experiments with aberrant profiles. Second, there are more complex ways of discovering similarities and differences in the profiles, briefly summarized as follows:

1. Filtering allows a restriction to be applied to the whole data set in order to create a smaller gene list, which can then be used in nonhierachical or hierachical cluster analysis.
2. K-Means clusters genes together with a similar expression profile. Self Organizing Map clustering has an identical function, but also demonstrates the similarity between clusters. Tree clustering will organize your data in a dendrogram reflecting similarities in expression profile.
3. Class prediction finds the genes that most discriminate two or more defined classes and applies these to allocate a class to an unknown class set.
4. Once a list of interesting genes has been identified, hyperlinks provide a source of information for further in-depth study.

4. Notes

1. RNA extraction. To minimize enzymatic degradation of RNA the sample should be well mixed in a cell lysis solution (e.g., TRIzol) immediately after reception or be snap frozen in liquid nitrogen. The quality of the RNA will remain optimal if the number of freeze–thaw procedures is minimal. Where possible or stated keep on ice or store at –80°C.
2. If the resulting homogenate appears too "gloopy," more TRIzol may be added. Insufficient TRIzol will result in impure RNA.
3. The yield of RNA will be decreased by fat in bone marrow. Use of chloroform in extraction will remove the lipids.
4. Following centrifugation, the homogenate separates into two distinct layers, an upper (clear) aqueous phase and a lower (pink-TRIzol) organic phase. A third phase at the interface may also be seen.
5. When taking off the aqueous phase, avoid disturbing the interface to avoid protein contamination of RNA. When washing the pellet after ethanol precipitation,

make sure the pellet comes loose from the tube in order to remove all remaining salt. Centrifuge to collect your RNA again.

6. The sample may be stored at this point, without detrimental effects to the RNA integrity. Tubes should be labeled with the sample date and stored at –70°C. Storage of RNA in RNase-free water at –80°C is adequate for a short period (several months), but preferably the pellet should be stored under three volumes of ethanol. Aliquotting of RNA minimizes thawing and the risk of RNase contamination. RNA is easier to redissolve in water if the pellet is not overdried.
7. To check concentration and quality of RNA, Affymetrix suggests spectrophotometric and agarose gel analysis. Alternatively, a denaturing gel can give you a better idea of the RNA size distribution. The Agilent Bioanalyzer may prove to be more accurate than our current protocol. In our experience this device provides more accurate information on quality and quantity of RNA, cRNA, and fragmented RNA.
8. This step requires the HPLC-purified T7-(dT)24 primer, at a concentration of 100 pmol/μL. The sequence for the T7-$(dT)_{24}$ primer: 5'-GGCCAGTGAATT GTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3'.
9. The volume of SS II RT is dependent on the amount of RNA used in this reaction. 10–15 μg of total RNA is optimal to provide sufficient cRNA for hybridization. Use 1 μL of SS II RT (200 U/μL) for 5.0–8.0 μg of total RNA, and 2 μL for 8.0–16.0 μg.
10. cDNA synthesis. The T7-oligo (dT) primer carries the promotor sequence for the bacteriophage T7 derived RNA polymerase, used in the in vitro transcription. This primer will hybridize with the poly(A) tail and secure selective amplification of the mRNA. Consequently, if the messengers are very long, amplification might be restricted to the 3' end. Superscript is an enzyme with an enhanced polymerase but without RNase H activity.
11. *E. coli* DNA polymerase has 5' -> 3' polymerase activity and 3' -> 5' and 5' -> 3' exonuclease activity. It will fill in 3' overhanging strand of DNA. The exonuclease enables synthesis from nicks in duplex DNA. This process is called nick translation and works optimally at 15°C; at higher temperatures, the DNA can "snap back/reanneal." The ligase does ligate second strand fragments but not blunt ends. RNase H will remove RNA from the RNA–DNA hybrid. T4 DNA polymerase has a strong 3' -> 5' exonuclease activity. It removes 3' protruding ends and extends 3' if the 5' end protrudes.
12. Phase Lock Gel will improve the recovery of cDNA by reducing loss of sample at the interface. The gel will separate the aqueous and organic phases. The denatured proteins in the interface will migrate into the gel.
13. To prepare the phenol-saturated buffer, add 65 μL of alkaline buffer (10 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA) to 1 mL of phenol–chloroform–isoamyl alcohol, 25:24:1. Vortex-mix for 2 min and centrifuge briefly.
14. The use of NH_4Ac instead of NaAc allows preferential precipitation of longer DNA molecules.

15. In vitro transcription (IVT). The T7 RNA polymerase recognizes only the T7 promotor and as such produces a high level of specific transcripts. It is quick and does not dissociate from the template. It produces a homogeneous pool of uniformly labeled ssRNA.
16. Similarly, we recommend not adding β-mercaptoethanol when using the RNeasy spin column to clean the cRNA.
17. You can typically expect 10 µg of total RNA to produce 60 µg of cRNA. If cDNA production seems to be adequate, but your IVT yield low, check the quality of your T7 primer.
18. It is important not to freeze–thaw the control mixtures more then 10 times, as this might lower the quality.

Acknowledgment

Frederik W. van Delft is the recipient of a grant from the Joint Research Board of Barts and The London School of Medicine, Queen Mary University of London. Louise K. Jones is supported by the CR-UK.

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14

HLA Low-Resolution Genotyping for Hematopoietic Stem Cell Transplantation

John Harvey, Jenny Price, and Ahna Stanton

1. Introduction

Hemopoietic stem cell transplantation from a fully matched sibling donor has been successfully carried out since the mid-1960s. Unfortunately only a third of potential transplant recipients have a fully matched sibling. In addition in the early 1990s it became apparent that the serological tissue typing methods developed for sibling pairs were imprecise when assessing unrelated donors. Molecular techniques capable of discrimination between more than 1500 HLA class I and class II alleles were therefore developed ***(1)***. Currently no single method is capable of offering genotyping to the allelic level for all possible heterozygous HLA combinations; it is common to combine more than one method to achieve allelic typing or confirm homozygosity.

Our current first line typing method for clinical samples is the polymerase chain reaction–sequence-specific primer method (PCR–SSP) for low-resolution typing followed by sequence-based typing (SBT) in which allelic typing is required (as described in Chapter 15). By using both PCR–SSP and SBT discrimination of all heterozygous combinations to the allelic level is possible.

Using PCR–SSP ***(2)*** as the first line test on families and selected unrelated donors a primary selection can rapidly be made on likely donor/recipient HLA matches prior to high resolution SBT typing. This combination of methods speeds the donor selection process within a busy transplant program by reducing number of labor-intensive high-resolution types performed.

2. Materials

1. Stock oligonucleotide primers at 500 μ*M* concentration (Cruachem).
2. Gilson Pipetman: P2, P10, P20, P200, P1000, P5000 (Anachem).

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

3. Pipet tips: up to 10 µL, up to 200 µL, up to 1000 µL, up to 5000 µL (Anachem).
4. 30-mL universal sterile containers (Sterlin).
5. 1X TBE buffer: 0.089 *M* Tris, 0.089 *M* Borate, 0.002 *M* EDTA (Amresco).
6. Agarose (Bioline).
7. Ethidium bromide (Sigma).
8. UV transilluminator (Flowgen).
9. Electrophoresis power packs (Bio-Rad).
10. Microwave oven.
11. Magnetic mixing plate.
12. Glycerol (Sigma).
13. Sucrose (Sigma).
14. Cresol red (Sigma).
15. 96-Well PCR plates (ABgene).
16. Electronic thermometer (Hanna).
17. Electrophoresis tank plus gel combs (Flowgen).
18. Biohit 100 multichannel pipet (Biohit).
19. Molecular biology grade H_2O (Sigma).
20. Thermoprime plus DNA polymerase (ABgene).
21. 10X Reaction buffer IV and $MgCl_2$ (ABgene).
22. 100 m*M* dATP, dCTP, dGTP, dTTP nucleotides (Bioline).
23. Linbro liquisystem tubes (ABgene).
24. Gell imaging system or polaroid camera (Genetic Research Instruments).
25. Hydra 96 dispenser (Robbins; optional extra).
26. Tetrad thermal cycler (Genetic Research Instruments).
27. Contamination testing primer mix. (Genovision).

3. Methods

3.1. Preparation of Primer Mix

1. On receipt from the supplier reconstitute all primers to a concentration of 500 µ*M* for use as stock primer solutions and store them at –40°C until use.
2. Remove the stock primers from the –40°C freezer (*see* **Note 1**).
3. Thaw at room temperature. Vortex to mix each primer.
4. Label a 30-mL sterile tube with the primer mix identifier (i.e., mix a1), the batch number, date prepared, and expiration date (18 mo). Place the tubes in suitable racks in rows of eight.
5. Using a new pipet tip for each addition, make up the reaction mixes as defined in the primer combination tables. **Tables 1–5** detail the primer volumes to be added to each primer mix. **Tables 6** and **7** detail the primer sequences. All mixes are reconstituted to a 15-mL volume using molecular biology grade H_2O.
6. Positive control primers are added to each primer mix as detailed in **Tables 1–5**. The control primers in each mix are chosen to yield an amplicon size distinct from the HLA specific amplicon (*see* **Fig. 1**).

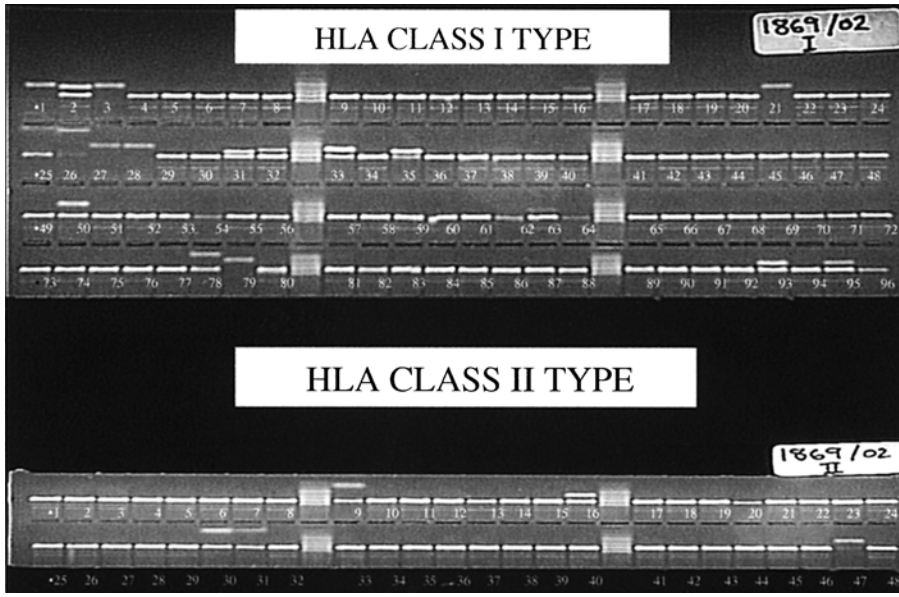


Fig. 1. Photograph of PCR-SSP gel results for HLA type. HLA-A*0201-22/25-27/29-54,A*2901-2906; B*4402-05/07/11/13/19N-30,B*4501/03/04/05; Cw0602/03/07,1601; DRB1*0701/03/05/06,-; DRB4*01011-0106,-; DQB1*0202,-. Note that HLA class I, lanes 1, 3, 21, 27, 28, and 79, have the faster moving HGH control primers added. Every ninth lane has molecular weight markers added as a control of product size. Class II, lane 47, has the faster moving HGH control primers amplicon. Class II, lane 16, is an example of a false-positive band recognized as a wrong-sized product. Positive bands for interpretation are: Class I: lanes 2, 16, 25, 26, 31, 32, 33, 35, 50, 78, 93, 95; Class II: lanes 9, 30, and 31.

3.2. Quality Control of Primer Mixes

The primer mixes listed have been extensively tested with a range of HLA alleles and shown to amplify correctly alleles for an excess of 15,000 HLA types as performed in our laboratory. When a new batch of primers is prepared it is important to show that the newly prepared primer mix will specifically amplify the correct allelic group (*see* **Note 2**).

1. HLA-typed DNA samples put aside for quality control testing of freshly prepared primers are removed from the –30°C freezer and allowed to thaw.
2. Dispense 10 complete sets of primers to be tested in 5-µL aliquots into 96-well thermal cycler trays. This is best performed using a Hydra 96 or a Biohit 100 multichannel pipet.

3. Each primer mix is tested against two DNA samples known to be positive and two negative for the specificities listed for the HLA primer mix, creating a composite type for all primer mixes. These tests are performed in duplicate (*see* **Note 3**).
4. Vortex each DNA sample before use.
5. Using a Gilson pipet (P2) add 0.5 µL of control DNA (concentration 0.1 µg/µL) directly to the primer mix to be tested. Mix by expelling and aspirating with the pipet and then discard the pipet tip, taking a new tip for the next sample.
6. Make up four volumes of PCR "mastermix" and set up the PCR–SSP reaction. (*see* **Subheading 3.4.** and **Note 4**).

The final quality control before putting the primer mixes into use is to test the new batch against 30 different DNA samples of known specificities.

3.3. Plating Out of Primer Mixes

1. Label 15 Linbro liquisystem tubes (ABgene) for each of the primer mixes with the primer mix and batch number and subbatch identifier (e.g., a1: 1/02:1).
2. On passing quality control steps dispense the primer mixes into 15 × 1 mL subbatches for classes I and II (96 mixes in total for class I and 48 mixes for class II).
3. Vortex-mix each universal thoroughly, and dispense into the corresponding labeled tubes (*see* **Fig. 2**)
4. Arrange the tubes numerically in Linbro boxes in the same order they are to be presented on the HLA typing test plate and interpretation sheets (15 boxes for class I and 8 boxes for class II—two class II typing sets per tray).
5. When a row of eight mixes is complete, seal the tubes with a capband-8 strip. Label the tabs of the capband-8 strip with the first tube number and the last tube number to allow the caps to be reoriented when they have been removed.
6. Transfer 14 of the boxes to the –40°C freezer and label with a shelf life of 18 mo. The remaining box is the current subbatch and should be stored at –30°C until required.
7. Label 200, 96-well thermalcycle plates for HLA-A, B, C typing; similarly for HLA-DR, DQ typing when required.
8. The remaining subbatch can then be transferred in 5-µL aliquots to 200 labeled 96-well PCR plates for class I and a further 200 for class II. This yields typing trays for 200 class I and 400 class II types.
9. The labeled trays can then be transferred to a –30°C freezer until required.

Table 1
Primer Combination Table for the HLA-A Locus (Mixes a1 to a24)

Mix and lane no.	Specific primer conc. (μ*M*)	5' Primer	Volume added (μL)	3' Primer	Volume added (μL)	Add 300 μL of 15 μ*M* control primer	Add 300 μL of 15 μ*M* control primer
a1	0.5	5a1	15	3a1	15	HGH I	HGH II
a2	0.5	5a2	15	3a2	15	63	64
a3	0.5	5a3	15	3a3	15	HGH I	HGH II
a4	0.5	5a1	15	3a4	15	63	64
a5	1.0	5a4	19	3a2	30	63	64
a6	0.5	5a5	15	3b10	15	63	64
a7	1.0	5b2	30	3a6	30	63	64
a8	0.5	5a6	15	3a7	15	63	64
			15	3a8	15		
a9	0.5	5a7	15	3a9	15	63	64
a10	0.5	5a8	15	3a9	15	63	64
a11	0.5	5a9	15	3a9	15	63	64
a12	0.5	5a10	15	3a9	15	63	64
a13	0.5	5a11	15	3a4	15	63	64
		5a12	15			6	
a14	0.5	5a13	15	3a9	15	63	64
		5a14	15		15		
a15	0.5	5a13	15	3a7	15	63	64
a16	0.5	5a10	15	3a10	15	63	64
a17	0.5	5a15	15	3a11	15	63	64
a18	2.0	5a16	60	3a12	60	63	64
a19	5.0	5a17	150	3a13	150	63	64
a20	2.0	5a16	60	3a14	60	63	64
a21	2.0	5a17	60	3a10	60	HGH I	HGH II
a22	0.5	5a7	15	3a2	15	63	64
a23	0.5	5a13	15	3a15	15	63	64
a24	1.0	5a18	30	3a16	30	63	64

Table 2
Primer Combination Table for the HLA-B Locus (Mixes b25-b72)

Mix and lane no.	Specific primer conc. (µ*M*)	5' Primer	Volume added (µL)	3' Primer	Volume added (µL)	Add 300 µL of 15 µ*M* control primer	Add 300 µL of 15 µ*M* control primer
b25	2	5b32	60.0	3b36	60	63	64
—	—	5b33	60.0	3b37	60	—	—
—	—	5b34	60.0	3b38	60	—	—
b26	2	5b32	60.0	3b39	60	63	64
—	—	5b33	60.0	—	—	—	—
—	—	5b34	60.0	—	—	—	—
b27	0.5	5b6	15.0	3b2	15	HGH I	HGH II
b28	2	5b27	60	3b4	60	HGH I	HGH II
b29	0.5	5b9	15	3b5	15	63	64
b30	1	5b2	30	3b6	30	63	64
b31	1.5	5b10	45	3b6	45	63	64
b32	0.5	5b11	15	3b7	15	63	64
b33	1	5b9	30	3b8	30	63	64
—	—	5b12	30	3b9	30	—	—
b34	1	5b14	30	3b4	30	63	64
b35	0.5	5b13	15	3b10	15	63	64
b36	0.5	5b14	15	3b11	15	63	64
b37	0.5	5b13	15	3b12	15	63	64
b38	0.5	5b9	15	3b13	15	63	64
b39	1	5b15	30	3b6	30	63	64
b40	0.5	5b16	15	3b14	15	63	64
b41	1	5b17	30	3b15	30	63	64
b42	0.5	5b11	15	3b16	15	63	64
b43	0.5	5b22	15	3b16	15	63	64
b44	1	5b1	30	3b16	30	63	64
—	—	5b2	30	—	—	—	—

b45	2	5b2	60	3b17	60	63	64
b46	1	5b20	30	3b18	30	63	64
b47	1	5b20	30	3b19	30	63	64
b48	2	5b21	60	3b21	60	63	64
b49	2	5b19	60	3b22	60	63	64
b50	0.5	5b5	15	3b6	15	63	64
b51	0.5	5b22	15	3b23	15	63	64
b52	1	5b23	30	3b22	30	63	64
—	—	5b25	30	—	—	—	—
b53	0.5	5b9	15	3b24	15	63	64
—	—	—	—	3b25	15	—	—
b54	2	5b27	60	3b37	60	63	64
b55	0.5	5b13	15	3b27	15	63	64
b56	0.5	5b22	15	3b4	15	63	64
b57	0.5	5b19	15	3b13	15	63	64
b58	1	5b20	30	3b5	30	63	64
b59	1	5b26	30	3b28	30	63	64
b60	0.5	5b15	15	3b29	15	63	64
b61	2	5b14	60	3b21	60	63	64
b62	0.5	5b27	15	3b17	15	63	64
b63	2	5b19	60	3b21	60	63	64
b64	0.5	5b28	15	3b23	15	63	64
b65	0.5	5b15	15	3b7	15	63	64
—	—	—	—	3b30	15	—	—
b66	0.5	5b6	15	3b31	15	63	64
b67	1	5b6	30.0	3b17	30	63	64
b68	1	5b24	30	3b32	30	63	64
b69	1	5b8	30	3b18	30	63	64
—	—	—	—	3b33	30	—	—
b70	0.5	5b11	7.7	3b34	15.0	63	64
b71	2	5b6	60.0	3b34	60.0	63	64

Table 3
Primer Combination Table for the HLA-C Locus (Mixes C73-C96)

Mix and lane no.	Specific primer conc. (μ*M*)	5' Primer	Volume added (μL)	3' Primer	Volume added (μL)	Add 300 μL of 15 μ*M* control primer	Add 300 μL of 15 μ*M* control primer
c73	0.5	5c1	15	3c1	15	63	64
c74	0.5	5c2	15	3c2 (3a8)	15	63	64
c75	1.0	5c1	30	3c3	30	63	64
c76	1.0	5c2	30	3c4	30	63	64
c77	0.5	5c2	15	3c5	15	63	64
c78	1.0	5c3 (5a18)	30	3c6 (3b14)	30	63	64
c79	1.0	5c4	30	3c7	30	HGH I	HGH II 300
c80	1.0	5c5	30	3c8	30	63	64
c81	1.0	5c3 (5a18)	30	3c9	30	63	64
c82	1.0	5c3 (5a18)	30	3c10 (3b23)	30	63	64
c83	2.0	5c3 (5a18)	60	3c5	60	63	64
c84	0.5	5c6	15	3c11	15	63	64
—	—	—	—	3c12	15	—	—
c85	0.5	5c7	15	3c3	15	63	64
c86	0.5	5c8	15	3c3	15	63	64
c87	2.0	5c1	60	3c13	60	63	64
c88	0.5	5c9	15	3c13	15	63	64
c89	3.0	5c1	90	3c14	90	63	64
c90	3.0	5c10	90	3c15	90	63	64
c91	1.0	5c2	30	3c16(3b17)	30	63	64
c92	1.0	5c2	30	3c17	30	63	64
c93	1.0	5c1	30	3c18	30	63	64
c94	1.0	5c2	30	3c18	30	63	64
c95	0.5	5c2	15	3c19 (3b31)	15	63	64
c96	1.0	5b27	30	3c4	30	63	64

Table 4
Primer Combination Table for the HLA-DR Locus (Mixes DR1-DR30)

Mix and lane no.	Specific primer conc. (μ*M*)	5' Primer	Volume added (μL)	3' Primer	Volume added (μL)	Add 300 μL of 15 μ*M* control primer	Add 300 μL of 15 μ*M* control primer
dr1	1.0	5dr01	30	3dr15	30	63	64
dr2	0.5	5dr01	15	3dr10	15	63	64
dr3	1.0	5dr02	30	3dr252	30	63	64
dr4	1.0	5dr02	30	3dr491	30	63	64
dr5	0.5	5dr05	15	3dr255	15	63	64
dr6	0.5	5dr06	15	3dr048	15	63	64
dr7	1.0	5dr03	30	3dr047	30	63	64
dr8	1.0	5dr04	30	3dr047	30	63	64
—	—	—	—	3dr048	30	—	—
dr9	1.0	5dr07	30	3dr079	30	63	64
dr10	2.5	5dr08	75	3dr045	75	63	64
—	—	—	—	3dr05	75	—	—
dr11	1.0	5dr06	30	3dr079	30	63	64
dr12	0.5	5dr10	15	3dr047	15	63	64
dr13	1.5	5dr05	45	3dr06	45	63	64
—	—	5dr273	45	—	—	—	—
dr14	1.0	5dr12A	30	3dr048	30	63	64
dr15	1.0	5dr05	30	3dr259	30	63	64
—	—	5dr273	30	—	—	—	—
dr16	1.5	5dr263	45	3dr259	45	63	64
—	—	—	—	3dr252	45	—	—
dr17	1.5	5dr03	45	3dr10	45	63	64
—	—	—	—	3dr485	45	—	—
dr18	1.5	5dr03	45	3dr258	45	63	64

continued

Table 4 *(Continued)*
Primer Combination Table for the HLA-DR Locus (Mixes DR1-DR30)

Mix and lane no.	Specific primer conc. (μ*M*)	5' Primer	Volume added (μL)	3' Primer	Volume added (μL)	Add 300 μL of 15 μ*M* control primer	Add 300 μL of 15 μ*M* control primer
—	—	—	—	3dr485	45	—	—
dr19	1.0	5dr05	30	3dr485	30	63	64
dr20	0.5	5dr05	15	3dr045	15	63	64
dr21	1.0	5dr05	30	3dr17	30	63	64
dr22	0.5	5dr05	15	3dr261	15	63	64
dr23	1.0	5dr05	30	3dr047	30	63	64
dr24	1.5	5dr263	45	3dr11	45	63	64
—	—	5dr264	45	3dr05	45	—	—
dr25	1.0	5dr05	30	3dr19	30	63	64
dr26	0.5	5dr03	15	3dr05	15	63	64
—	—	—	—	3dr15	15	—	—
dr27	1.0	5dr03	30	3dr048	30	63	64
dr28	0.5	5dr51	15	3dr01	15	63	64
—	—	—	—	3dr16	15	—	—
dr29	0.5	5dr52.1	15	3dr13	15	63	64
—	—	5dr52.2	15	3dr14	15	—	—
dr30	0.5	5dr53	15	3dr048	15	63	64

Table 5
Primer Combination Table for the HLA-DQ Locus (Mixes DQ31-DQ45)

Mix and lane no.	Specific primer conc. (μ*M*)	5' Primer	Volume added (μL)	3' Primer	Volume added (μL)	Add 300 μL of 15 μ*M* control primer	Add 300 μL of 15 μ*M* control primer
dq31	0.5	5dq2l	15	3dq2r	15	63	64
dq 32	0.5	5dq4l	15	3dq4r	15	63	64
dq 33	0.5	5dq5l	15	3dq5r	15	63	64
dq 34	0.5	5dq6a (1-3)lh	15	3dq6(1-3)r	15	63	64
dq 35	0.5	5dq8l	15	3dq3'05	15	63	64
dq 36	0.5	5dq6b(1-3)	15	3dq6(1-3)r	15	63	64
dq 37	0.5	5dq6(3-8)l	15	3dq350	15	63	64
—	—	—	—	3dq6b(3-8)	—	—	—
dq 38	0.5	5dq7l	15	3dq7r	15	63	64
dq 39	1.0	5dq8l	30	3dq8r	30	63	64
dq 40	1.0	5dq9b	30	3dq9r	30	63	64
dq 41	0.5	5dq5'10	15	3dq4r	15	63	64
dq 42	0.5	5dq5'11	15	3dq4r	15	63	64
dq 43	1.0	5dq5'11	30	3dq8r	30	63	64
dq 44	1.0	5dq9bl	30	3dq4r	30	63	64
dq 45	0.5	5dq7l	15	3dq9r	15	63	64
dq 46	0.5	5dq851	15	3dq630	15	63	64
dq 47	1.0	5dq988	30	3dq990	30	63	64
dq 48	0.5	5dq5l	15	3dq3'01	15	63	64

Table 6
HLA Class-I Primer Details

Primer	Sequence	Location	Length
HLA-A*	**3' Primers**		
3a1	AGC CCG TCC ACG CAC CG	EX3 216-232	17
3a2	CCA AGA GCG CAG GTC CTC T	EX3 110-129	19
3a3	CAC TCC ACG CAC GTG CCA	EX3 213-231	18
3a4	GAG CCA CTC CAC GCA CGT	EX3 216-234	18
3a6	CCT CCA GGT AGG CTC TCT G	EX3 195-214	19
3a7	GAG CCA CTC CAC GCA CCG	EX3 216-234	18
3a8	GAG CCA CTC CAC GCA CTC	EX3 216-233	18
3a9	ATG TAA TCC TTG CCG TCG TAA	EX3 80-101	21
3a10	AGC GCA GGT CCT CGT TCA A	EX3~100	19
3a11	CCG TCG TAG GCG TGC TGT	EX3 71-89	18
3a12	CTT CAC ATT CCG TGT CTC CT	257-276	20
3a13	CGC TGT GGT TGT AGT AGC G	EX2 246-264	19
3a14	GCC TTC ACA TTC CGT GTG TT	EX2 186-205	20
3a15	CCG CGG AGG AAG CGC CA	EX3 50-70	17
3a16	GAG CCC GTC CAC GCA CTC	EX3 216-234	18
HLA-A*	**5' Primers**		
5a1	CGA CGC CGC GAG CCA GAA	EX2 11-128	18
5a2	GTG GAT AGA GCA GGA GGG T	EX2 149-167	19
5a3	AGC GAC GCC GCG AGC CA	EX2 111-127	17
5a4	TAT TGG GAC GAG GAG ACA G	EX2 174-192	19
5a5	GGC CGG AGT ATT GGG ACG A	EX2 168-186	19
5a6 (5a11)	GGG TAC CAG CAG GAC GCT	EX3 63-80	18
5a7 (5b6)	GGA GTA TTG GGA CCG GAA C	EX2 170-188	19
5a8	TCA CAG ACT GAC CGA GAG AG	EX2 210-229	20
5a9	TCA CAG ACT GAC CGA GCG AA	EX2 210-229	19
5a10	CCG GAG TAT TGG GAC CTG C	EX2 166-184	19
5a11(5a6)	GGG TAC CAG CAG GAC GCT	EX3 63-80	18
5a12	GGG TAC CGG CAG GAC GCT	EX3 63-80	18
5a13	ACG GAA TGT GAA GGC CCA G	EX2 191-209	19
5a14	ACG GAA AGT GAA GGC CCA G	264 - 282	19
5a15	CCC GGC CCG GCA GTG GA	EX2 36-53	17
5a16	CCA CTC CAT GAG GTA TTT CAC	EX2 5-25	21
5a17	CCA CTC CAT GAG GTA TTT CTT	EX2 4-25	21
5a18	TAC TAC AAC CAG AGC GAG GA	EX2 249-268	20
HLA-B *	**3' Primers**		
3b2	TACCAGCGCGCTCCAGCT	EX3 262-279	18
3b4	CCGCGCGCTCCAGCGTG	EX3 260-276	17
3b5	CTCTCAGCTGCTCCGCCT	EX3 184-201	18
3b6	ATCCTTGCCGTCGTAGGCT	EX3 77-95	19
3b7	CCAGGTATCTGCGGAGCG	EX3 229-246	18

3b8	GTCGTAGGCGTCCTGGTC	EX3 ~69	18
3b9	CGTCGTAGGCGTACTGGTC	EX3 69-87	19
3b10	TCCCACTTGCGCTGGGT	EX3 156-172	17
3b11	GCGGCGGTCCAGGAGCG	EX3 120-136	17
3b12	GAGCCACTCCACGCACTC	EX3 216-233	18
3b13	CTCCAACTTGCGCTGGGA	EX3 156-173	18
3b14	CGTCGCAGCCATACATCCA	EX3 18-36	19
3b15	GTGTGTTCCGGTCCCAATAT	EX2 173-192	20
3b16	CGTGCCCTCCAGGTAGGT	EX3 201-218	18
3b17	GCCATACATCCTCTGGATGA	EX3 10-29	20
3b18	GAGGAGGCGCCCGTCG	EX3 44-59	16
3b19	CGTCGCAGCCATACATCAC	EX3 18-36	19
3b21	CTTGCCGTCGTAGGCGG	EX3 76-92	17
3b22	CCATACATCGTCTGCCAA	EX3 11-28	18
3b23	GAGCCACTCCACGCACAG	EX3 216-233	18
3b24	CTCGGTCAGTCTGTGCCTT	EX2 207-225	19
3b25	TCTCGGTAAGTCTGTGCCTT	EX2 207-226	20
3b27	TCGTAGGCGTCCTGGTGG	EX3 68-85	18
3b28	GCCGCGGTCCAGGAGCT	EX3 120-136	17
3b29	GCAGGTTCCGCAGGCTCT	EX2 225-242	18
3b30	CAGGTATCTGCGGAGCCC	EX3 228-245	18
3b31	CCTCCAGGTAGGCTCTCCA	EX3 196-214	19
3b32	GCGCAGGTTCCGCAGGC	EX2 229-245	17
3b33	GGAGGAAGCGCCCGTCG	EX3 44-60	17
3b34	CGT TCA GGG CGA TGT AAT CT		20
3b36	GTT GTA GTA GCG GAG CGC GA	311-330	20
3b37	TTG TAG TAG CGG AGC AGG G	311-329	19
3b38	TGT AGT AGC GGA GCG CGG	311-328	18
3b39	TTG TAG TAG CCG CGC AGG T	311-329	19

HLA-B 5' Primers

5b1	CCA CTCCAT GAG GTATTT CAC	78-98	21
5b2	ACCGAGAGAACCTGCGGAT	EX2 220-238	19
5b5	CGAGAGAGCCTGCGGAAC	EX2 222-239	18
5b6 (5a7)	GGAGTATTGGGACCGGAAC	EX2 170-188	19
5b8	GACCGGAACACACAGATCTT	EX2 180-199	20
5b9	GCTACGTGGACGACACGCT	EX2 76-94	19
5b10	CCACTCCATGAGGTATTTCC	EX2 5-24	20
5b11	CCGAGAGAGCCTGCGGAA	EX2 221-238	18
5b12	GGGGAGCCCCGCTTCATT	EX2 52-68	18
5b13	CGCCACGAGTCCGAGGAA	EX2 116-133	
5b14	ACCGGGAGACACAGATCTC	EX2 116-133	18
5b15	CGCGAGTCCGAGGATGGC	EX2 119-136	18
5b16	AGCAGGAGGGGCCGGAA	EX2 157-173	17
5b17	CCATGAGGTATTTCTACACCG	EX2 5-30	21
5b19	CGCCGCGAGTCCGAGAGA	EX2 116-133	18
5b20	AACATGAAGGCCTCCGCG	EX2 195-212	18

continued

Table 6 *(Continued)*
HLA Class-I Primer Details

Primer	Sequence	Location	Length
5b21	GCGCCGTGGATAGAGCAA	EX2 144-161	18
5b22	CAGATCTACAAGGCCCAGG	EX2 192-210	19
5b23	GCCGCGAGTTCGAGAGG	EX2 190-206	17
5b24	GCCGCGAGTCCGAGGAC	EX2 117-133	17
5b25 (5a18)	TACTACAACCAGAGCGAGGA	EX2 249-268	20
5b26	GAGACACAGAAGTACAAGCG	EX2 187-205	20
5b27	GGGAGCCCCGCTTCATCT	EX2 52-69	18
5b28	ACCGGAACACACAGATCTG	EX2 181-199	19
5b32	GACGACACCCAGTTCGTGA	EX2 157-175	19
5b33	GACGACACGCTGTTCGTGA	EX2 157-175	19
5b34	GACGACACGCAGTTCGTGA	EX2 157-175	19
HLA-C*	**3' Primers**		
3c1	CCCCAGGTCGCAGCCAC	EX3 25-41	17
3c2 (3a8)	GAGCCACTCCACGCACTC	EX3 216-233	18
3c3	AGCGTCTCCTTCCCATTCTT	EX3 244-264	20
3c4	GCCCCAGGTCGCAGCCAA	EX3 25-42	18
3c5	CGCGCGCTGCAGCGTCTT	EX3 258-275	18
3c6 (3b14)	GGTCGCAGCCATACATCCA	EX3 18-36	19
3c7	CAGCCCCTCGTGCTGCAT	EX4 234-252	18
3c8	CGCACGGGCCGCCTCCA	EX3 169-186	17
3c9	CCCCAGGTCGCAGCCAG	EX3 25-42	17
3c10 (3b23)	GAGCCACTCCACGCACAG	EX3 216-233	18
3c11	GCGCAGGTTCCGCAGGC	EX2 229-246	17
3c12	TCTCAGCTGCTCCGCCGT	EX3 183-200	18
3c13	TGAGCCGCCGTGTCCGCA	EX3 134-151	18
3c14	CCGCCGTGTCCGCGGCA	EX3 131-148	17
3c15	GGTCGCAGCCAAACATCCA	EX3 18-36	19
3c16 (3b17)	GCC ATA CAT CCT CTG GAT GA	EX3 10-29	20
3c17	CCTCCAGGTAGGCTCTCAG	EX3 195-213	19
3c18	CCCTCCAGGTAGGCTCTCT	EX3 196-214	19
3c19 (3b31)	CCTCCAGGTAGGCTCTCCA	EX3 196-214	19
HLA-C*	**5' Primers**		
5c1	CACAGACTGACCGAGTGAG	EX2 211-229	19
5c2	CCGAGTGAACCTGCGGAAA	EX2 221-239	19
5c3 (5a18)	TACTACAACCAGAGCGAGGA	EX2 249-268	20
5c4	CCGCGGGTATGACCAGTC	EX3 59-76	18
5c5	GGACCGGGAGACACAGAAC	EX2 179-197	19
5c6	ACGACACGCAGTTCGTGCA	EX2 84-103	19
5c7	TACAACCAGAGCGAGGCCA	EX2 252-270	19
5c8	ACAACCAGAGCGAGGCCG	EX2 252-270	18
5c9	AGTCCAAGAGGGGAGCCG	EX2 123-140	18
5c10	CCACTCCATGAGGTATTTCTC	EX2 5-25	21

Table 7
HLA Class-II Primer and Control Primer Details

Primer	Sequence	Location	Length
HLA-DRB1*	**3' Primers**		
3'dr1	CCGCGCCTGCTCCAGGAT	199-216	18
3'dr045	TGTTCCAGTACTCGGCGCT	169-187	19
3'dr047	CTGCACTGTGAAGCTCTCAC	257-276	20
3'dr048	CTGCACTGTGAAGCTCA	257-276	20
3'dr05	CTGCAGTAGGTGTCCACCAG	220-239	20
3'dr06	CTGGCTGTTCCAGTACTCCT	173-192	20
3'dr079	CCCGTAGTTGTGTCTGCACAC	232-252	21
3'dr10	CCCGCTCGTCTTCCAGGAT	199-217	19
3'dr11	TCTGCAATAGGTGTCCACCT	221-240	20
3'dr13	CTGTTCCAGGACTCGGCGA	170-188	19
3'dr14	GCTGTTCCAGTACTCGGCAT	170-189	20
3'dr15	CCGCCTCTGCTCCAGGAG	199-216	18
3'dr17	CCCGCCTGTCTTCCAGGAA	199-217	19
3'dr19	CTGTTCCAGTGCTCCGCAG	170-188	19
3'dr252	CCACCGCGGCCCGCGC	211-226	16
3'dr255	GTCCACCCGGCCCCGCT	212-228	17
3'dr258	ACCGCGGCCCGCCTGTC	208-224	17
3'dr259	TCCACCGCGGCCCGCTC	211-227	17
3'dr261	CTGTTCCAGTACTCGGCATC	169-188	20
3'dr485	TCCACCGCGGCCCGCTT	211-227	17
3'dr491	CTCCGTCACCGCCCGGT	140-156	17
HLA-DRB1*	**5' Primers**		
DR 5'01	TTG TGG CAG CTT AAG TTT GAA T	22-43	22
DRB 5'02	TCC TGT GGC AGC CTA AGA G	20-38	19
DRB 5'03	TAC TTC CAT AAC CAG GAG GAG A	88-109	22
DRB 5'04	GTT TCT TGG AGC AGG TTA AAC A	17-38	22
DR 5'05	GTT TCT TGG AGT ACT CTA CGT C	17-38	22
DRB 5'06	GAC GGA GCG GGT GCG GTA	60-77	18
DR 5'07	CCT GTG GCA GGG TAA GTA TA	21-40	20
DR 5'08	AGT ACT CTA CGG GTG AGT GTT	26-46	21
DR 5'10	CGG TTG CTG GAA AGA CGC G	73-91	19
DR 5'51	GTT TCT TGC AGC AGG ATA AGT A	17-38	22
DRB 5'52.1	TTT CTT GGA GCT GCG TAA GTC	18-38	21
DRB 5'52.2	GTT TCT TGG AGC TGC TTA AGT C	17-38	22
DRB 5'53	GAG CGA GTG TGG AAC CTG A	64-82	19
5' DR12A	CAT AAC CAG GAG GAG CTC C	94-112	19
DRB 263	GTT CCT GGA CAG ATA CTT CC	75-94	20
DRB 264	GTT CCT GGA GAG ATA CTT CC	75-94	20
DRB 273	TTC TTG GAG TAC TCT ACG GG	19-38	20

continued

Table 7 *(Continued)*
HLA Class-II Primer and Control Primer Details

Primer	Sequence	Location	Length
HLA - DQB1*	**3' Primers**		
3'dq2R	GTA GTT GTG TCT GCA CAC CC	230-249	20
3'dq4R	TGG TAG TTG TGT CTG CAT ACG	231-251	21
dq 3'05	GCT GTT CCA GTA CTC GGC AT	170-189	20
3'dq5R	CCC GCG GTA CGC CAC CTC	250-267	18
3'dq6 (1-3) R	CCG CGG AAC GCC ACC TC	250-266	17
3'dq6B (3-8) R	TGC ACA CCC TGT CCA CCG	221-238	18
3'dq7 R	CGT GCG GAG CTC CAA CTG	250-267	18
3'dq8 R	CTG TTC CAG TAC TCG GCG G	170-188	19
3'dq9R	CTG TTC CAG TAC TCG GCG T	170-188	19
350	TGC ACA CCG TGT CCA ACT C	220-238	19
HLA - DQB1*	**5' Primers**		
5'dq2 L	GTG CGT CTT GTG AGC AGA AG	70-89	20
5'dq4 L	CTA CTT CAC CAA CGG GAC C	44-63	19
5'dq5 L	ACG GAG CGC GTG CGG GG	61-77	17
5'dq6A LH(1-3)	TTT CGT GCT CCA GTT TAA GGC	18-38	21
5'dq6B (1-3)	GAC GTG GGG GTG TAC CGC	127-144	18
5'dq6(3-8) L	GGA GCG CGT GCG TCT TGT A	63-81	19
5'dq7 L	ATT TCG TGT ACC AGT TTA AGG C	17-38	22
5' DQ8 L	GTG CGT CTT GTG ACC AGA TA	70-89	20
5' DQ9B L	GAC GGA GCG CGT GCG TCT	60-77	18
DQ 5'10	CAC CAA CGG GAC CGA GCT	51-68	18
DQ 5'11	CAC CAA CGG GAC CGA GCG	51-68	18
Control primers			
63	CAA GTG GAG CAC CCA A		
64	GCA TCT TGC TCT GTG CAG AT		
HGH I	CAG TGC CTT CCC AAC CAT TCC CTT A		
HGH II	ATC CAC TCA CGG ATT TCT GTT GTG TTT C		

3.4. PCR–SSP Setup

Prepare the following stock solutions:

3.4.1. Preparation of Stock Solutions

3.4.1.1. 100 m*M* Cresol Red

1. Label a 5-mL tube with 100 m*M* cresol red.
2. Add 0.08 g of cresol red to 2 mL of molecular grade H_2O.

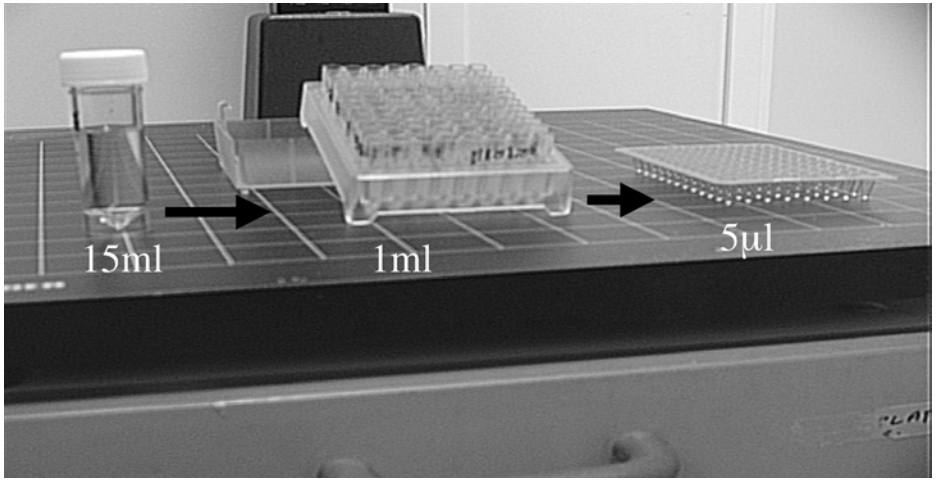


Fig. 2. Primer preparation and dispensing. Primers are prepared in 15-mL volumes. Subaliquot to 15 × 1 mL volumes in 96-well format, then dispense in 5-µL volumes to 96-well thermal cycler trays for use.

3. Vortex-mix to dissolve.
4. Once fully dissolved this may be stored as a stock solution at –30°C and used when required.

3.4.1.2. 60% Sucrose/1 m*M* Cresol Red

1. In a 30-mL tube dissolve 6 g of sucrose in 10 mL of autoclaved deionized H_2O using a vortex.
2. Add 100 µL of 100 m*M* cresol red.
3. Vortex to mix and store at 4°C.
4. Fill in the appropriate sheet in the reagent records file.
5. Prepare label with batch no., expiration date, and date prepared, and affix to a 30-mL tube.

3.4.1.3. 1 m*M* dNTP Stock

1. Remove one dNTP set (dATP, dCTP, dGTP, and dTTP) from the –30°C freezer and thaw at room temperature.
2. Label 5 × 5-mL tubes with the batch number and an aliquot number from 1 to 5 and expiration date of 6 mo.
3. To each 5-mL tube add 4800 µL of molecular biology quality H_2O. Add 50 µL of dATP, 50 µL of dCTP, 50 µL of dGTP, and 50 µL of dTTP to each tube.
4. Vortex to mix.
5. Store the first aliquot at 4°C until required and the other aliquots at –30°C until needed.

3.4.2. Preparation of PCR Reaction

1. Remove the 1 m*M* dNTP mix, 10X reaction buffer, $MgCl_2$ (ensure the $MgCl_2$ is compatible with the current lot number of 10X reaction buffer), 60% sucrose–1 m*M* cresol red, and sample DNA from 4°C storage.
2. Remove the required number of prepared primer mix trays from the –30°C freezer and allow to thaw at room temperature.
3. Label the primer tray and a 1.5-mL tube with the DNA sample number.
4. Using a clean filtered tip for each addition add 150 µL of the 1 m*M* dNTP mix, 150 µL of 10X buffer IV solution, 111 µL of $MgCl_2$ solution, 256 µL of the 60% sucrose–1 m*M* cresol red solution, and 11.25 µL of *Taq* polymerase (Abgene) to a labeled 1.5-mL tube.
5. Take 5 µL of the prepared mix and add to 5 µL of negative control primer mix (Genovision) in a PCR well to be tested for contamination (contamination surveillance).
6. Add 75 µL of DNA at a concentration of 0.3 µg/µL to the labeled 1.5-mL tube
7. Take the labeled tube containing the test DNA and vortex-mix. (This is enough sample mix for a single HLA class I and II type.)
8. Set a single-channel repeat dispensing pipet to dispense 5 µL. Add 5 µL of the sample mix to each of the primer mixes in the test plates (*see* **Note 6**).
9. The test plate must be sealed prior to thermal cycling. A plate can be sealed using foil strips with a heat sealer. (If using caps to seal the plates, ensure that the caps are firmly fitted.)
10. The typing plates should be transferred to a thermal cycler equipped with a heated bonnet, able to accommodate 96 well PCR trays and run using the following cycling parameters.
    a. 1 cycle: 96°C, 2mins
    b. 10 cycles: 96°C, 25 s
       65°C, 45 s
       72°C, 30 s
    c. 20 cycles: 96°C, 10 s
       61°C, 50 s
       72°C, 30 s
    d. 4°C hold

3.5. Gel Electrophoresis

PCR products can be visualized using submerged agarose gel electropheresis.

1. For analysis of 138–312 products use a 24 × 32 cm gel tray (Flowgen). Electrophoresis tank for above and 12 × 26 slot combs.
2. Dissolve 85 g of TBE buffer dry powder in 650 mL of deionized H_2O on the magnetic stirrer.
3. Make up to 1 L with deionized H_2O to give 5X normal concentration TBE solution.

4. To a 1 L volume heat/microwave protective bottle add 8 g of molecular biology grade agarose, 40 mL of the 5X normal TBE buffer, and 360 mL of deionized H_2O.
5. Heat the bottle and its contents in a microwave oven for 2 min, and mix the gel by swirling wearing heat protective gloves. Repeat in 2-min heating steps until the agarose is totally dissolved.
6. Remove the hot bottle from the microwave wearing protective gloves, and using an electronic thermometer probe to measure temperature cool the gel to 60°C. Avoid the creation of air bubbles in gel.
7. Add 25 µL of ethidium bromide (from a 10 mg/mL stock solution). Swirl the bottle to mix the contents (*see* **Note 5**).
8. Seal the ends of the tray with autoclave tape and pour the agarose solution into the gel tray.
9. Insert the combs into the gel tray, creating the sample troughs when the gel solidifies.
10. Allow the gel to cool for at least 30 min.
11. Using 5X normal TBE buffer and deionized H_2O, prepare 2500 mL of 0.5 *N* TBE buffer and add this to the electrophoresis tank prior to submerging the prepared gel.
12. Carefully remove the gel combs, avoiding damage to the sample wells, and remove the autoclave tape from the tray ends. Ensure the gel is oriented correctly.
13. Immerse the gel into the buffer. Using the end of a clean pipet tip, dislodge any trapped air bubbles from the sample troughs.
14. Carefully remove the sealing foil or caps from the PCR plates.
15. Set a Gilson pipet to 10 µL and fit a clean pipet tip, then remove the PCR product from the first reaction well and load into the first gel sample trough by placing the pipet above the mouth of the trough and slowly dispense the mix
16. Rinse the tip in the electrophoresis buffer by aspirating and dispensing several times.
17. Repeat **step 15** for all products, making sure they are dispensed in the same order they are in the PCR tray.
18. To each row of the agarose gel a product size ladder (100-bp ladder) should be added; products interpreted should be compared with the product size ladder, ensuring correct product size.
19. Run the gel at 200 V until the cresol red present migrates to the next row of sample troughs, being careful that it does not run into the next row of sample wells.

3.6. Gel Visualization

1. Wear UV protective face mask when inspecting the gel.
2. Remove the tray containing the test gel from electrophoresis tank and place on the UV transilluminator.
3. Switch on the UV transilluminator; the PCR products are visible if present as bright bands of white light.
4. Record the gel image with a gel imaging system or a polaroid photograph.

3.7. Interpretation of Results

It is recommended that interpretive software such as Score™ or SSP manager *(3,4)* is used to assist the interpretation of HLA types from the results obtained.

1. Attach the image of the gel obtained to the report sheets, filling in the sheet with reagent data information and DNA sample identification (*see* **Figs. 1, 3,** and **4**).
2. The image should show a line of PCR product bands of the same migration speed produced by the control primers. Where the alternative sized control primer is used this is detailed on the report sheet. An additional band in a lane indicates an HLA-specific PCR product (the size of which is given on the report sheet). Mark a positive result on the report sheet with a cross in the appropriate lane box. If no HLA-specific band is present and the control bands are visible then the lane is negative.
3. By matching the positive lanes to the alleles they contain, it is possible to determine an HLA type. As previously stated we recommend the use of interpretational software for this. The report sheets (*see* **Figs. 3** and **4**) permit manual interpretation of HLA types to the first two digits of an HLA type.
4. Occasionally blank lanes will appear. This means a reaction mix has failed. If a heterozygous type is still obtained, that is, two allele strings can be positively identified, then the result can still be reported. If not then the tests should be repeated and the failed lane must show that the control primers are working on repeat.
5. False-positive bands also occasionally appear (check that the band is the correct size). If no assignment of type is possible repeat the test and if the band is still visible repeat using an alternative typing method (unexplained bands sometimes indicate novel alleles).

4. Notes

1. Wear protective gloves and use Gilson pipets designated only for PCR setup work when preparing primers working in a dedicated "clean" area for PCR setup where post-amplification products are not permitted.
2. When performing quality control work on primer mixes it is advisable to maintain record sheets on each batch detailing the work performed.
3. Panels of HLA-typed DNA for the quality control of HLA typing methods are available from the National Institute of Biological Standards and Controls (http://nibsc.ac.uk). Failed amplification of control bands may be due to failure to add the control primers: add control primers to the original primer mix according to the relevant primer combination table and repeat the quality control. If test bands fail on both positive controls but the control bands amplify, remake the primer mix and quality control the new mix. If the test bands are very weak (may lead to false-negative results during their interpretation), add the same volume of stock primers to the mix and quality control the strengthened mix. If both the control and the test bands fail on both positive controls, repeat the quality control with an

NAME **LAB No.** **THERMALCYCLER**

Primer Batch No: **Exp. Date:** **EnzymeLot.No:** **Exp. Date:**
$MgCl_2$Lot.No: **Exp. Date:** **10X Buffer Lot.No:** **Exp. Date:**
Sucrose/cresol red Batch No: **Exp. Date:** **dNTP Batch No:** **Tube No:** **Exp. Date:**

Attach gel image here

HGH Control in lanes:
1 3 21 27 28 79

A*	A*	B*	B*	Cw*	Cw*

RESULT																							
LANE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Product Size	629	489	628	630	464	516	503	170	440	400	400	442	170	419	552	465	561	198	259	200	628	468	383	494
AMPLIFIED ALLELES	A ***01**	A ***02**	A ***03** *2418 *3204	A ***3601**	A ***23** ***24** *0246	A ***23** *2405 *2416 *3007 B *5804	A ***24**	A ***25** ***26** *4301 *66	A ***25** ***26** *34 *66	A ***25**	A ***26** *4301	A ***4301**	A ***34** *2609 *3103 *3104	A ***34** ***66** *2502 *2613	A ***11** ***6601** *2502 *2613	A ***29**	A ***30**	A ***31**	A ***32** *2503	A ***33**	A ***32** *74	A ***68** *2424 *2610 *6901	A ***6901**	A ***8001**

RESULT																								
LANE	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
PRODUCT SIZE	172	172	619	734	635	385	600	535	520 545	605	566	465	627	607	486	389	182	507	536	498 508	319	374	351	458
AMPLIFIED ALLELES	Bw4	Bw6	B ***07** *4025 *4806 *8101	B ***08** ***42**	B ***27** ***4901** ***50** *40	B ***49** *4418 *5115 *5901	B ***45** ***49** ***50** *4415 *4418 *5402	B ***45** *0720 *1514 *4409 *5002 *82 *8301	B ***44** *1553 *1804 *2706 *2721 *2722 *40 *4104 *5306	B ***41**	B ***4002** ***41** ***44** ***45** ***47** ***49** ***50** *1546 *1553 *3519	B ***40** ***41** *1533 *48	B ***40** *47	B ***4001** *40	B ***13** *1542	B ***1401** ***1402** *14	B ***1402** *14 *3526 *3805 *3904	B ***39** ***6701** *1548 *1569 *3535	B ***6701**	B ***38** *5119	B ***53** ***58** *1513 *4406 *5104 *5705	B ***58** *5705	B ***57**	B ***18**

Fig. 3. Report sheet for interpreting HLA types.

RESULT																								
LANE	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
PRODUCT SIZE	422	383	551	289 421	150	204	479	594	567	516	460	124	421	487	486	562	636	553	369	128	389	400	451	440
AMPLIFIED ALLELES	B ***3906** ***55** ***56** *0809 *2714 *5901 *7301	B ***45** ***50** ***54** ***55** ***56** *82 *1542 *3917 Cw *1507	B ***56** *5508	B ***54** *5507 *7301	B ***27**	B ***37** *0727	B ***47**	B ***42**	B ***48** *4012 *8101	B *1516 *1517 *1567 *5806	B ***46** *6701	B ***15** *1309 *4021	B ***15** *1304 *1812 *3528 *4003 *4017 *4020 *4438 *4802	B ***4802** ***8301** *0712 *0714 *0718 *1562 *3525 Cw *0311 *0710	B ***15** *0709 *0711 *0717 *3525 *3526 *3907 *4802 *5603	B ***15** *3526 *5122 *5125 *7803	B *1512 *1514 *1519 *4408 *5707	B ***15** *3514 *5603 A ***25** ***26** *3401 ***66** ***68**	B ***35** *53 *0712 *0714 *0718 *15 *4406 *4412 *5104 *8301	B ***35** ***18** *1522 *1559 *3919 *5606 *78	B ***35** *53	B ***78** *15012 *1509 *4026 *4028 *5605 *5606	B ***51** ***78** *1509 *5605 *5606	B ***52** *15012 *4026 *4028

RESULT																								
LANE	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
PRODUCT SIZE	340	522	564	331	564	297	1056	516	302	494	536	160 625	523	522	450	538	446	541	318	502	513	503	502	500
AMPLIFIED ALLELES	Cw *0102 *0103 *0104	Cw *02 *17	Cw *0302 *03031 *03	Cw *04 *18	Cw *0501	Cw *06	Cw *07	Cw *0701 *07	Cw *0702 *0703 *07	Cw *0703 A *2604	Cw *0704 *07	Cw *08	Cw *03031 *03032	Cw *0302 *0304 *03	Cw *0104 *12 *1301	Cw *12021 *12022	Cw *1203 *1206	Cw *14	Cw *0307 *15	Cw *0203 *0307 *0404 *0406 *0604 *0707 *0709 *15 *17	Cw *1601	Cw *1602	Cw *0202 *0602 *0603 *12	Cw *0708 *1801 *1802 B *8201 *8202

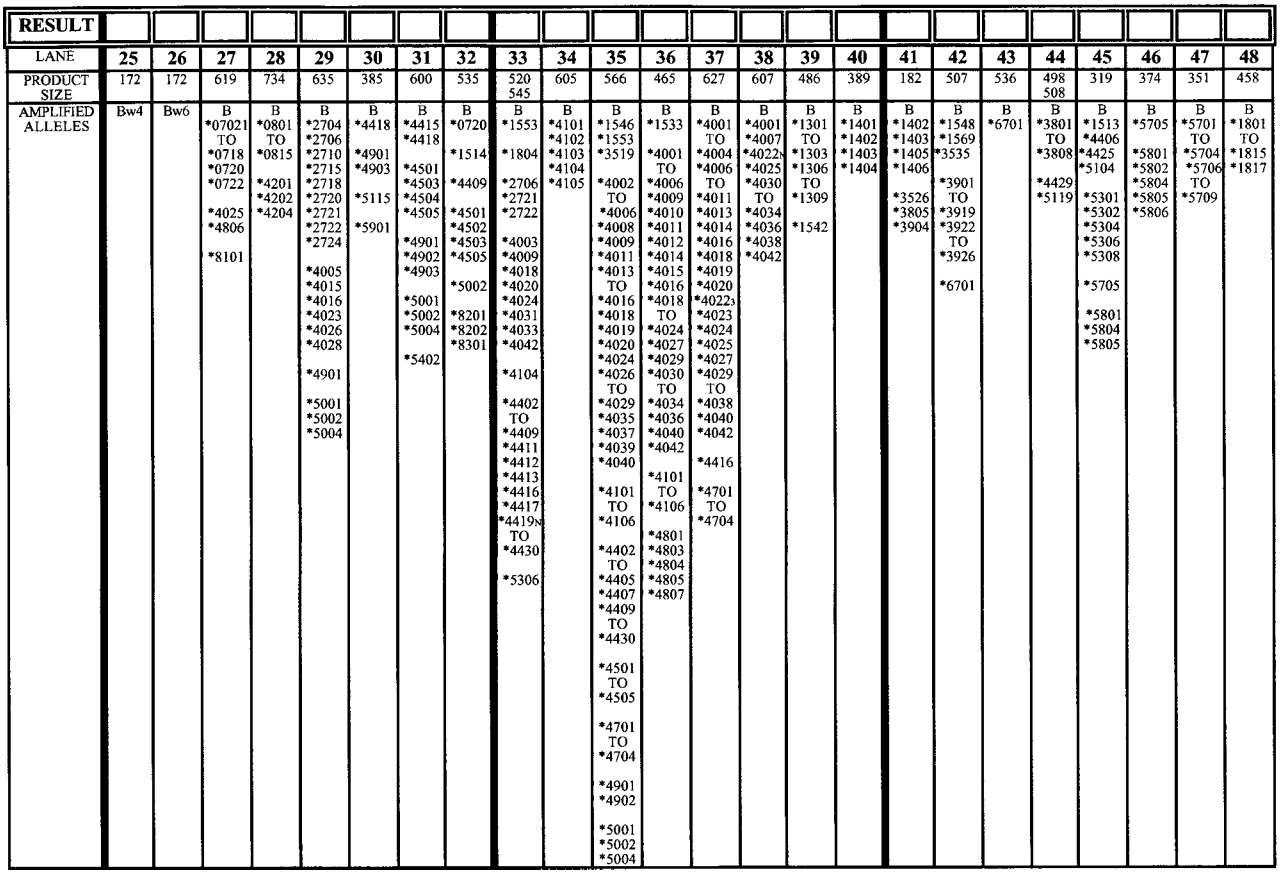
PERFORMED BY **DATE** **CHECKED BY** **DATE**

Fig. 3. *(cont.)* Report sheet for interpreting HLA types.

PCR-SSP HLA Class I Results 2002 update

RESULT																								
LANE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Product Size	629	489	628	630	464	516	503	170	440	400	400	442	170	419	552	465	561	198	259	200	628	468	383	494
AMPLIFIED ALLELES	A *01011 *01012 *0102 *0103 *0104N *0105N *0106 *0107 *0109	A *02011 TO *0222 *0225 TO *0227 *0229 TO *0254	A *03011 *03012 *03013 *0302 *0303N *0304 *0307 *0308 *0309 *2418 *3204 *6819	A *3601 *3602 *3603	A *0246 *0248 *2301 *2302 *2304 TO *2308N *24021 *24022 *24031 *24032 TO *2407 *2409N *2410 *2411N *2413 *2414 *2415 *2417 TO *2420 *2422 *2423 *2425 TO *2428 *2430 *2432 TO *2434	A *2301 TO *2308 *2405 *2416 *3007 B *5804	A *24021 *24022 *24031 *24032 *2405 *2407 *2408 *2409N *2410 *2411N *2414 *2415 *2417 *2420 *2421 *2423 *2425 *2426 *2427 *2429 *2430 *2431 *2433 *2434	A *2501 TO *2504 *2601 *2603 *2605 *2607 *2608 *2610 TO *2618 *4301 *6601 TO *6604	A *2501 *2502 *2503 *2504 *2601 *2602 *2603 *2604 *2605 *2606 *2608 TO *2618 *3401 *3402 *3403 *3404 *6601 *6602 *6603 *6604	A *2501 *2502 *2503 *2504	A *2601 *2602 *2604 *2607 *2608 *2609 *2610 *2611N *2618 *4301	A *4301	A *2609 *3103 *3104 *3401 *3402 *3403 *3404	A *2502 *2613 *3401 *3402 *3403 *3404 *6601 *6602 *6604	A *11011 *11012 *1102 *1103 *1105 TO *1112 *2502 *2613 *6601 *6604	A *2901 *2902 *2903 *2904 *2905 *2906	A *3001 *3002 *3003 *3004 *3006 *3007 *3008 *3009 *3010 *3011	A *31012 *3103 *3104 *3105 *3106	A *2503 *3201 *3202 *3203 *3204 *3205 *3206	A *3301 *3303 *3304 *3305 *3306	A *3201 *3202 *3203 *3205 *3206 *7401 TO *7408	A *2424 *2610 *68011 *68012 *6802 *68031 *68032 *6804 *6805 *6806 *6807 *6808 *6809 *6811N *6812 *6815 *6816 *6817 *6818N *6819 *6820 *6901	A *6901	A *8001

Fig. 3. *(cont.)* Report sheet for interpreting HLA types.



RESULT																								
LANE	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
PRODUCT SIZE	172	172	619	734	635	385	600	535	520 545	605	566	465	627	607	486	389	182	507	536	498 508	319	374	351	458
AMPLIFIED ALLELES	Bw4	Bw6	B *07021 TO *0718 *0720 *0722 *4025 *4806 *8101	B *0801 TO *0815 *4201 *4202 *4204	B *2704 *2706 *2710 *2715 *2718 *2720 *2721 *2722 *2724 *4005 *4015 *4016 *4023 *4026 *4028 *4901 *5001 *5002 *5004	B *4418 *4901 *4903 *5115 *5901	B *4415 *4418 *4501 *4503 *4504 *4505 *4901 *4902 *4903 *5001 *5002 *5004 *5402	B *0720 *1514 *4409 *4501 *4502 *4503 *4505 *5002 *8201 *8202 *8301	B *1553 *1804 *2706 *2721 *2722 *4003 *4009 *4018 *4020 *4024 *4031 *4033 *4042 *4104 *4402 TO *4409 *4411 *4412 *4413 *4416 *4417 *4419N TO *4430 *5306	B *4101 *4102 *4103 *4104 *4105	B *1546 *1553 *3519 *4002 TO *4006 *4008 *4009 *4011 *4013 TO *4016 *4018 *4019 *4020 *4024 *4026 TO *4029 *4035 *4037 *4039 *4040 *4101 TO *4106 *4402 TO *4405 *4407 *4409 TO *4430 *4501 TO *4505 *4701 TO *4704 *4901 *4902 *5001 *5002 *5004	B *1533 *4001 TO *4006 *4009 *4010 *4011 *4012 *4014 *4015 *4016 *4018 TO *4024 *4027 *4029 *4030 TO *4034 *4036 *4040 *4042 *4101 TO *4106 *4801 *4803 *4804 *4805 *4807	B *4001 TO *4004 *4006 TO *4011 *4013 *4014 *4016 *4018 *4019 *4020 *4022N *4023 *4024 *4025 *4027 *4029 TO *4038 *4040 *4042 *4416 *4701 TO *4704	B *4001 *4007 *4022N *4025 *4030 TO *4034 *4036 *4038 *4042	B *1301 TO *1303 *1306 TO *1309 *1542	B *1401 *1402 *1403 *1404	B *1402 *1403 *1405 *1406 *3526 *3805 *3904	B *1548 *1569 *3535 *3901 TO *3919 *3922 TO *3926 *6701	B *6701	B *3801 TO *3808 *4429 *5119	B *1513 *4406 *4425 *5104 *5301 *5302 *5304 *5306 *5308 *5705 *5801 *5804 *5805	B *5705 *5801 *5802 *5804 *5805 *5806	B *5701 TO *5704 *5706 TO *5709	B *1801 TO *1815 *1817

Fig. 3. *(cont.)* Report sheet for interpreting HLA types.

PCR-SSP HLA Class I Results 2002 update

RESULT																								
LANE	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
PRODUCT SIZE	422	383	551	289 421	150	204	479	594	567	516	460	124	421	487	486	562	636	553	369	128	389	400	451	440
AMPLIFIED ALLELES	B *0809 *2714 *3906 *5501 *5502 *5503 *5505 *5509 TO *5512 *5601 *5605 *5901 *7301	B *1309 *1542 *3917 *4501 *4503 *4504 *4505 *5001 *5002 *5004 *5401 *5402 *5501 *5502 *5503 *5505 *5507 *5509 TO *5512 *5601 *5602 *5604 *8201 *8202 Cw *0312 *1507	B *0724 *5508 *5601 TO *5607	B *5401 *5402 *5507 *7301	B *2701 TO *2715 *2717 *2719 *2720 *2721 *2722 *2724 *2725	B *0727 *3701 TO *3704	B *4701 TO *4704	B *4201 *4202 *4202	B 4012 *4801 *4803 *4804 *4806 *4807 *8101	B *1516 *1517 *1567 *5806	B *4601 *4602 *6702	B *1309 *1501 *1502 *1504 TO *1508 *1511 *1512 *1514 *1515 *1519 *1520 *1521 *1525 TO *1528 *1530 TO *1535 *1538 *1539 *1540 *1542 *1544 *1545 *1548 *1550 *1555 *1556 *1558 *1560 *1563 *1565 *1566 *1570 *1571 *4021	B *1304 *1501 *1503 TO *1507 *1512 *1514 *1519 *1520 *1524 *1525 *1526N *1527 *1532 *1533 *1534 *1535 *1536 *1538 *1539 *1540 *1543 *1546 *1547 *1549 *1550 *1553 *1554 *1557 *1560 *1561 *1562 *1565 *1568 TO *1571 *1812 *3528 *4003 *4020 *4038 *4417 *4802	B *0712 *0714 *0718 *1562 *3525 *4802 *8301 Cw *0311 *0710	B *0709 *0711 *0717 *1503 *1518 *1523 *1529 *1547 *1549 *1551 *1552 *1554 *1561 *1562 *1564 *1568 *1569 *1572 *3525 *3526 *3907 *4802 *5603	B *1509 *1510 *1518 *1521 *1523 *1537 *1551 *3526 *5122 *5125 *7803	B *1512 *1514 *1519 *4408 *5707	B *1508 *1511 *1515 *1522 *1559 *3514 *5603 A *2501 TO *2618 *3401 *6601 TO *6604 *6801I TO *6807 *6811N *6812 *6815 TO *6820	B *0712 *0714 *0718 *1502 *1513 *1521 *1544 *3501 TO *3504 *3506 TO *3509 *3511 *3512 *3514 *3515 *3518 TO *3521 *3523 TO *3527 *3529 *3533 TO *3536 *3538 *3539 *3540n *4406 *4412 *5104 *5301 TO *5306 *5308 *5309 *8301	B *1522 *1559 *1801 TO *1808 *1810 TO *1813 *3501 TO *3518 *3520 TO *3524 *3528 TO *3540n *3919 *5606 *7801 TO *7805	B *3501 TO *3509 *3511 *3514 *3515 *3517 *3518 *3519 *3521 *3523 *3524 *3525 *3527 *3529 *3530 *3532 TO *3538 *3540n *5301 TO *5306 *5308	B *1501 *1509 *4026 *4028 *5605 *5606 *7801 TO *7805	B *1509 *5101I TO *5109 *5111n TO *5123 *5126 TO *5129 *5605 *5606 *7801 TO *7804	B *15012 *4026 *4028 *5201 TO *5203 *7805

Fig. 3. *(cont.)* Report sheet for interpreting HLA types.

PCR-SSP HLA Class I Results 2002 update

RESULT																								
LANE	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
PRODUCT SIZE	340	522	564	331	564	297	1056	516	302	494	536	160 625	523	522	450	538	446	541	318	502	513	503	502	500
AMPLIFIED ALLELES	Cw *0102 *0103 *0104	Cw *02021 *02022 *02023 *02024 *02025 *0203 *0204 *0205 *1701 *1702 *1703	Cw *03021 *03022 *03031 *03032 *03041 *03042 *0305 *0306 *0308 *0309 *0310 *0311 *0312 *0313 *0314	Cw *0401 *0403 *0404 *0405 *0406 *0407 *0408 *0409 *1801 *1802	Cw *0501 *0503 *0504	Cw *0602 *0603 *0604 *0605 *0606 *0607	Cw *07011 *07012 *0702 *0703 *0705 *0706 *0707 *0708 *0709 *0710 *0714	Cw *07011 *07012 *0706 *0707 *0709	Cw *0702 *0703 *0710 *0713 *0715	Cw *0703 A *2604	Cw *07041 *07042 *0711 *0712	Cw *0801 *0802 *0803 *0804 *0805 *0806 *0807 *0808 *0809	Cw *03031 *03032 *03033 *0311 *0312 *0313	Cw *03021 *03022 *03041 *03042 *0305 *0306 *0307 *0308 *0309 *0310 *0314	Cw *0104 *12021 *12022 *12023 *1208 *1301	Cw *12021 *12022 *12023	Cw *1203 *1206	Cw *14021 *14022 *1403 *1404	Cw *0307 *15021 *15022 *1503 *1504 *15051 *15052 *1506 *1508 *1509 *1510 *1511	Cw *0203 *0307 *0404 *0406 *0604 *0707 *0709 *15021 *15022 *1503 *1504 *15051 *15052 *1506 *1509 *1510 *1511 *1701 *1702 *1703	Cw *1601	Cw *1602	Cw *02021 *02022 *02023 *02024 *0602 *0603 *12041 *12042 *1205	Cw *0708 *1801 *1802 B *8201 *8202

Fig. 3. *(cont.)* Report sheet for interpreting HLA types.

NAME **LAB No.** **THERMALCYCLER**

Primer Batch No:	**Exp. Date:**	**EnzymeLot.No:**	**Exp. Date**
$MgCl_2$Lot.No:	**Exp. Date:**	**10X Buffer Lot.No:**	**Exp. Date**
Sucrose/cresol red Batch No:	**Exp. Date:**	**dNTP Batch No:**	**Tube No:** **Exp. Date:**

Attach gel image here

HGH control in mix 47

DRB1*	DRB1*	DRB *	DRB *	DQB1*	DQB1*
DR INTERPRETATION				DQ INTERPRETATION	
DR	DR	w	w	DQ	DQ

RESULT																								
LANE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
PRODUCT SIZE	194	195	206	136	211	216	188	259	231	161 213	235	203	175 173	182	210 208	151 152	129 139	136 139	210	170	200	171	259	164 165
AMPLIFIED ALLELES	DRB1 *0101 *01021 *01022 *0104 *0105 *0106	DRB1 *0103	DRB1 *15011 TO *1509	DRB1 *1507 *1601 TO *1605 *1607 *1608	DRB1 *03011 TO *0317 *1107	DRB1 *03011 *03012 *0304 *0306 *0308 *0310 TO *0313 *0315 *0316 *1327	DRB1 *03021 *03022 *0305 *0314 *1109 *1120 *1302 *13 *14 *1608 DRB3 *0206	DRB1 *04011 TO *0422 *0424 TO *0433 *0435 *0436 *1122 *1410	DRB1 *07011 *0703	DRB1 *0801 TO *0813 *0815 TO *0819 *1415	DRB1 *09012	DRB1 *10011 *10012	DRB1 *0308 *11011 *11 *1204 *1411	DRB1 *1201 TO *1204 *1206	DRB1 *11 *1301 *1302 *13 *1416	DRB1 *1116 *1120 *1301 *1302 *13 *1416	DRB1 *1116 *1120 *1301 *1302 *13 *1419 *1421	DRB1 *1109 *13 *14 *1608	DRB1 *13031 *13032 *1310 *1333 *1337 *1419 *1421	DRB1 *0312 *13031 *13032 *1304 *1312 *1313 *1321 *1330 *1332 *1333 *1338 *1413	DRB1 *0820 *11011 *11 *13 *1422 *1425 *1427	DRB1 *03011 *0302 *03 *0820 *1301 *1302 *13 *14	DRB1 *0302 *03 *11011 *11 *1302 *13031 *13032 *13 *14	DRB1 *0809 *0821 *1117 *1318 *14

Fig. 4. Report sheet for interpreting HLA types.

RESULT																								
LANE	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
PRODUCT SIZE	171	151 128	188	199 206	171	212	179	207	206	248	119	139	175	250	118	128	200	200	216	191	171	153	903	128
AMPLIFIED ALLELES	DRB1 *0310 *1343 *1345 *1401 *1407 *1416 *1422 *1425 *1426	DRB1 *03011 *0302 *03... *1318 *14 DRB3* 0206	DRB1 *03011 *03 *1116 *1301 *13 *14	DRB5*	DRB3*	DRB4*	DQB1 *0201 *0202 *0203	DQB1 *0401 *0402	DQB1 *05011 *05012 *0502 *05031 *05032 *0504	DQB1 *06011 *06012 *06013	DQB1 *0602 *06111 *0615	DQB1 *0602 *0603 *0608 *0610 *06111 *06112 *0613 *0614 *0616	DQB1 *0603 *06041 *06042 *06051 *06052 *0606 *0607 *0608 *0609 *06112 *0612 *0614 *0617	DQB1 *0301 *03012 *0304 *0309 *0310	DQB1 *0302 *0307 *0308	DQB1 *0203 *03032 *03033 *0306	DQB1 *0401	DQB1 *0402	DQB1 *0305 *0402	DQB1 *0306	DQB1 *03011 *03012 *0309 *0310 *06011 *06012 *06013	DQB1 *0602 *0610 *0613	DQB1 *0201	DQB1 *05011 *05012

PERFORMED BY **CHECKED BY**

DATE **DATE**

Shaded alleles are theoretically detected, but are usually only seen weakly when the allele is homozygous.

See **PCR-SSP Class II Primers file** for complete allelic strings.

Shaded alleles are theoretically detected, but are usually only seen weakly when the allele is homozygous.

Fig. 4. *(cont.)* Report sheet for interpreting HLA types.

PCR-SSP HLA Class II Results 2002 Update

LANE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	23
PRODUCT SIZE	194	195	206	136	211	216	188	259	231	161 213	235	203	175 173	182	210 208	151 152	129 139	136 139	210	170	200	171	259	259
AMPLIFIED ALLELES	DRB1 *0101 *01021 *01022 *0104 *0105 *0106	DRB1 *0103	DRB1 *15011 TO *1509	DRB1 *1507 *1601 TO *1605 *1607 *1608	DRB1 *03011 TO *0317 *1107	DRB1 *03011 *03012 *0304 *0306 *0308 *0310 TO *0313 *0315 *0316 *1327	DRB1 *03021 *03022 *0305 *0314 *1109 *1120 *13021 *13022 *1305 *1326 *1329 *1331 *1334 *1336 *1339 *1341 *1402 *1403 *1409 *1413 *1419 *1424 *1427 *1430 *1608 DRB3* 0206	DRB1 *04011 TO *0422 *0424 TO *0433 *0435 *0436 *1122 *1410	DRB1 *07011 *0703	DRB1 *0801 TO *0813 *0815 TO *0819 *1415	DRB1 *09012	DRB1 *10011 *10012	DRB1 *0308 *1101 TO *1121 *1123 TO *1129 *1132 *1134 *1137 *1139 *1204 *1411	DRB1 *1201 TO *1204 *1206	DRB1 *1102 *1103 *1111 *1114 *1116 *1120 *1121 *1301 *13021 *1304 *1308 *1315 *1316 *1317 *1319 *1320 *1322 *1323 *1324 *1327 *1328 *1329 *1331 *1332 *1334 *1335 *1336 *1338 *1339 *1340 *1341 *1343 *1345 *1416	DRB1 *1116 *1120 *1301 *13021 *1308 *1309 *1316 *1320 *1327 *1328 *1329 *1331 *1332 *1334 *1335 *1336 *1339 *1340 *1341 *1343 *1416	DRB1 *1116 *1120 *1301 *13021 *13022 *1306 *1310 *1315 *1316 *1327 *1328 *1331 *1332 *1334 *1335 *1336 *1339 *1340 *1341 *1419 *1421	DRB1 *1109 *1305 *1306 *1310 *1318 *1326 *1342 *1403 *1412 *1419 *1421 *1427 *1608	DRB1 *13031 *13032 *1310 *1333 *1337 *1419 *1421	DRB1 *0312 *13031 *13032 *1304 *1312 *1313 *1321 *1330 *1332 *1333 *1338 *1413	DRB1 *0820 *11011 *11012 *11013 *1103 *11041 *11042 *1106 *1109 TO *1112 *1115 *1123 *1124 *1125 *11271 *11272 *1128 *1129 *1132 *1133 *1135 *1137 *1138 *1139 *1305 *13071 *13072 *1311 *13141 *13142 *1318 *1321 *1324 *1326 *1342 *1422 *1425 *1427	DRB1 *03011 TO *0307 *0309 *0311 *0314 *0315 *0316 *0317 *0820 *1301 *13021 *13022 *1305 TO *1311 *13141 *1315 *1316 *1318 *1319 *1320 *1322 TO *1325 *1327 *1328 *1329 *1334 *1335 *1336 *1337 *1340 *1341 *1342 *1344 *1402 *1403 *1406 *1409 *1412 *1414 *1417 *1419 *1420 *1421 *1423 *1424 *1427 *1429 *1430 *1436	DRB1 *03021 *03022 *0305 *0309 *0314 *0317 *11011 *11012 *11013 *11081 *11082 *1109 TO *1112 *1114 *1115 *1119 *1120 *1123 *1124 *1126 TO *1129 *1131 *1132 *1133 *1137 *1139 *13021 *13022 *13031 *13032 *1305 *13071 *13072 *1312 *1313 *13141 *13142 *1321 *1323 *1325 *1326 *1329 *1330 *1331 *1333 *1334 *1336 TO *1339 *1341	*1345 *1402 *1403 *1407 *1409 *1413 *1414 *1419 *1422 *1424 *1425 *1427 *1430 *1436 24 164 165 DRB1 *0809 *0821 *1117 *1318 *1401 *1403 *1404 *1405 *1407 *1408 *1410 *1411 *1412 *1414 *1415 *1418 *1423 *1426 *1427 *1428 *1433 *1435 *1436

Fig. 4. *(cont.)* Report sheet for interpreting HLA types.

LANE	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
PRODUCT SIZE	171	151 128	188	199 206	171	212	179	207	206	248	119	139	175	250	118	128	200	200	216	191	171	153	903	128
AMPLIFIED ALLELES	DRB1 *0310 *1343 *1345 *1401 *1407 *1416 *1422 *1425 *1426	DRB1 *03011 *03012 *03021 *03022 *0303 *0305 *0306 *0307 *0308 *0310 TO *0316 *1318 *1402 *1403 *1406 *1409 *1412 *1413 *1417 *1418 *1419 *1421 *1427 *1429 *1430 *1433 DRB3* 0206	DRB1 *0301 *0303 *0306 *0307 *0308 *0310 TO *0313 *0315 *0316 *1116 *1301 *1306 *1309 *1310 *1315 *1318 *1320 *1327 *1328 *1332 *1335 *1340 *1342 *1406 *1412 *1417 *1418 *1421 *1429 *1433	DRB5 *01011 *0102 *0105 *0106 *0107 *0108 *0110N *0202 *0203	DRB3 *01011 *01012 *01014 *0103 *0104 *0105 *0106 *0107 *0201 TO *0206 *0209 *0210 *0211 *0213 *03011 *03012 *0302 *0303	DRB4 *01011 *0102 TO *0105	DQB1 *0201 *0202 *0203	DQB1 *0401 *0402	DQB1 *05011 *05012 *0502 *05031 *05032 *0504	DQB1 *06011 *06012 *06013	DQB1 *0602 *06111 *0615	DQB1 *0602 *0603 *0608 *0610 *06111 *06112 *06013 *06014 *0616	DQB1 *0603 *06041 *06042 *06051 *06052 *0606 *0607 *0608 *0609 *06112 *0612 *0614 *0617	DQB1 *03011 *03012 *0304 *0309 *0310	DQB1 *0302 *0307 *0308	DQB1 *0203 *03032 *03033 *0306	DQB1 *0401	DQB1 *0402	DQB1 *0305 *0402	DQB1 *0306	DQB1 *03011 *03012 *0309 *0310 *06011 *06012 *06013	DQB1 *0602 *0610 *0613	DQB1 *0201	DQB1 *05011 *05012

Fig. 4. *(cont.)* Report sheet for interpreting HLA types.

alternative DNA sample positive for an allele amplified by that primer mix. If the repeat fails remake the mix from stock solutions and repeat the quality control. If this fails contact the primer manufacturer for replacement primers. If any primer mix produces false-positive reactions during testing, repeat and see if it occurs again with the same sample and another sample of the same specificity. If it still produces a false-positive result, remake the mix from stock solutions and repeat the quality control.

4. Where there are no available positive DNA controls to test a primer mix, an individual primer should be paired with another primer resulting in detecting another specificity for which DNA is available, thus assuring that all primers function in a PCR reaction.
5. Ethidium bromide is a powerful mutagen. Protective measures should be taken when handling gels or solutions containing containing ethidium bromide.
6. We chose to use PCR–SSP as our first line test for clinical samples. The technique is suited to generate results quickly on single samples with the capacity to process more than eight types per day for HLA- A, B, Cw, DRB1, DRB3, DRB4, DRB5 and DQB1 using four 96-well heads on a thermal cycler.
7. A number of quality reviews have previously been published on PCR–SSP ***(2,5,6)***. The method used in our laboratory uses previously published primer sequences ***(2)*** as well as some that have been designed "in house." Commercially available HLA PCR–SSP typing kits are now readily available. However, retaining an "in house" method maintains an important skill base within an HLA typing laboratory for the design of primers and optimisation of PCR reactions.
8. Primer design: In a PCR reaction the specificity of the primer is assigned by incorporating the polymorphic residue to be amplified at the 3' end of the primer sequence. This rule applies for both sense and anti-sense primers, although the process of designing each differs. The sense primer is taken as a straight reading of the DNA sequence published for a given allele or group of alleles up to and including the polymorphic residue. It is the G/C and A/T composition of the primer sequence that determines the length of the designed primer. The target annealing temperature is standardised to be 60°C or 62°C using the formula $4(G + C) + 2(A + T)$ where A, C, G, and T are numeric values equating to their frequency in any given primer sequence. Thus in designing a sense primer, the length is adjusted at its 5' end to yield an annealing temperature of 60°C or 62°C. When designing the anti-sense primer using published sequence alignments, the polymorphic residue is first incorporated into the 5' end of the sequence and its length is adjusted at its 3' end to yield an annealing temperature of 60°C or 62°C. This sequence is then complemented to the anti-sense conformation by converting all As to Ts, Ts to As, Cs to Gs, and Gs to Cs. This yields a DNA sequence complementary to the published sequence. The sequence as complemented now when read left to right runs 3' to 5'; it is necessary to invert this sequence yielding an anti-sense primer reading 5' to 3' (*see* **Fig. 5**) with the polymorphic residue at the 3' end of the primer.

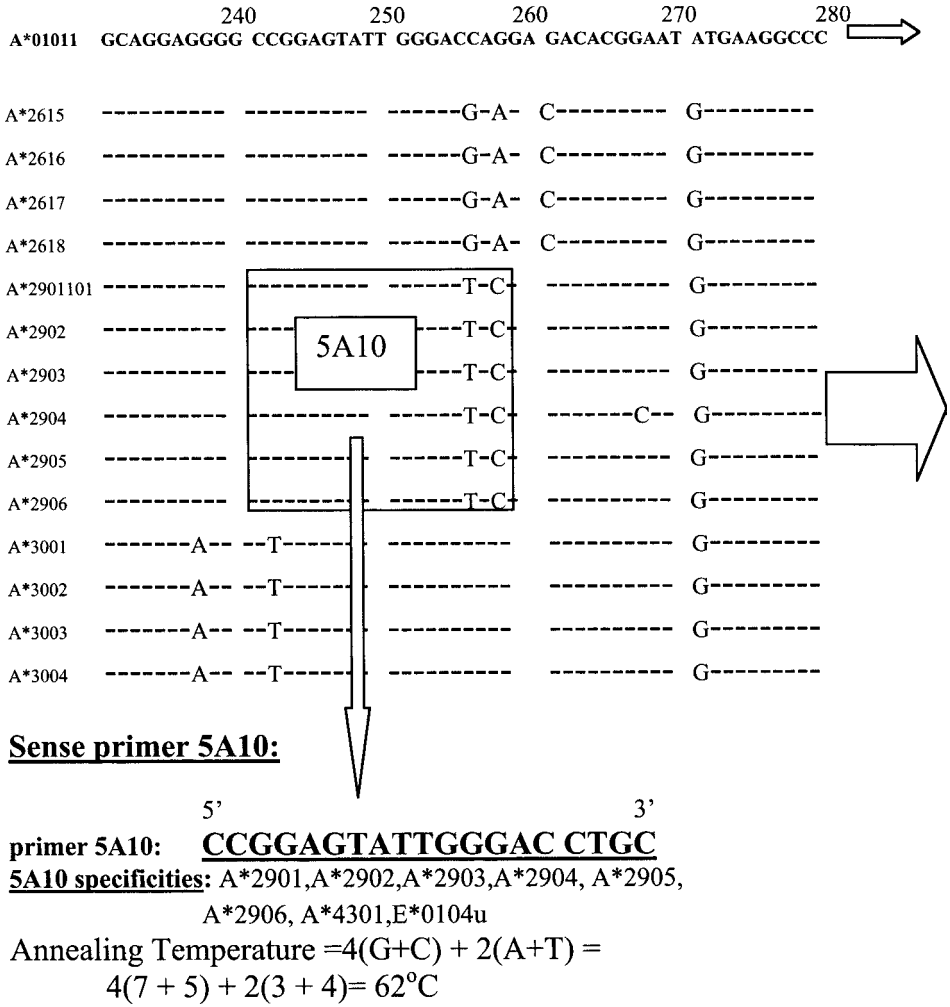


Fig. 5. Primer design using HLA sequence alignments.

9. Primer selection: To facilitate sample throughput, first round PCR–SSP typing is restricted to a maximum of 144 reaction mixes. A component's negative control as a monitor for nucleic acid contamination should be set up with each assay. Using 96 primer mixes we achieve high-quality "serological equivalence" for class I HLA-A* and B* loci and typing superior to serology for HLA-Cw*. Using 48 primer mixes, class II typing for DRB1*, 3*, 4*, 5*, and DQB1* superior to serological equivalence is obtained. The primers we design are selected on the basis of offering allelic group typing to the level of all well-defined serological HLA antigens and their splits while not further expanding

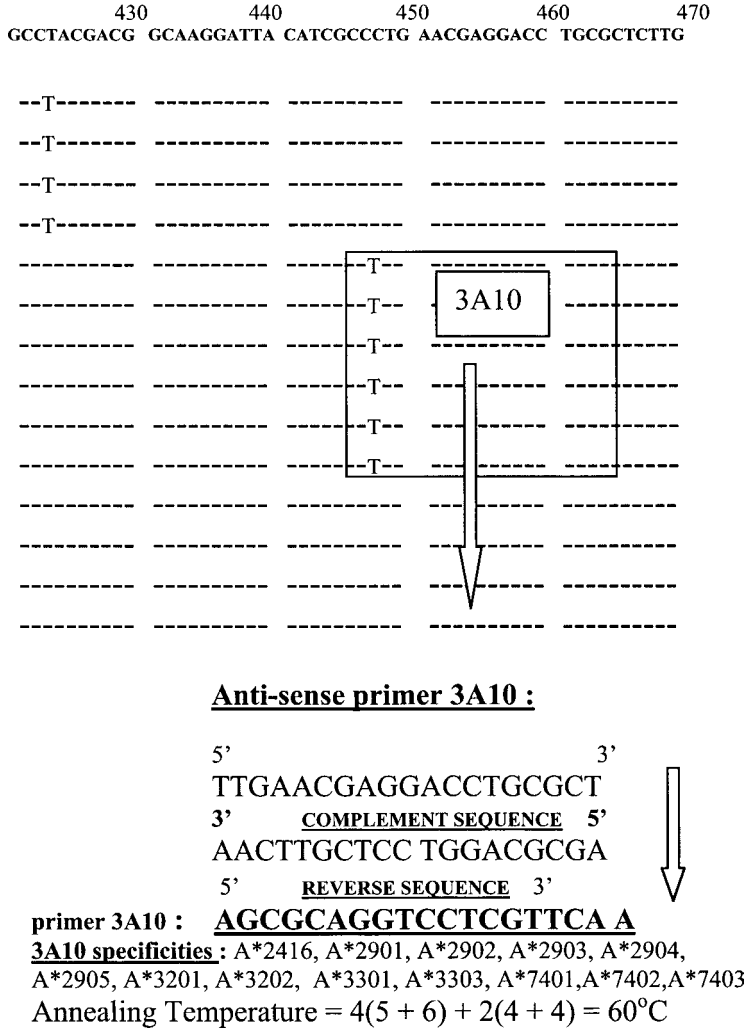


Fig. 5. *(Continued)*

the number of primers requires for our first round HLA type. The primer mix specificity is derived from the specificity overlap between the sense and anti-sense primers (*see* **Fig. 5**). If the sense primer was specific for HLA-A*2901, A*2902, A*2903, A*2904, A*2905, A*2906, A*4301, E*0104u and the anti-sense primer was specific for A*2416, A*2901, A*2902, A*2903, A*2904, A*2905, A*2906, A*31012, A*3201, A*3202, A*3301, A*3303, A*7401, A*7402, A*7403 then the specificity of the mix is A*2901, A*2902, A*2903, A*2904, A*2905, A*2906 commonly summarized as A2901/02/03/04/05/06 or A*29. Where more than two primers are used in a single primer mix then all

possible specificity overlaps between the sense and anti-sense primers present must be considered.

10. Sequence alignments and updating primer specificities: When designing new primers it is essential to access up-to-date HLA sequence alignments. The IMGT/HLA database ***(1,7)*** offers ready access to all sequences recognized by the WHO Nomenclature Committee for Factors of the HLA system and is accessible via the World Wide Web. This Web site also offers a number of tools and facilities to aid analysis of HLA sequences. It is recommended that other software tools available to assist with primer design, update primer mix specificities, management of primer mix sets for HLA typing, and assistance with HLA type assignment following PCR–SSP typing be used ***(3,4)***.

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DNA Sequencing as a Tissue-Typing Tool

Paul P. J. Dunn, Sarinder Day, Siaron Williams, and Nina Bendukidze

1. Introduction

The ever increasing numbers of reported HLA alleles pose a problem to the modern tissue typing laboratory (**Table 1**) ***(1–4)***. PCR-sequencing based typing (PCR-SBT) analyzes the complete nucleotide sequence of the polymorphic exons of HLA class I and II and, as such, provides the highest resolution available for HLA typing which includes the complete characterization of new alleles. Sequencing using *Taq* DNA polymerase, or "cycle sequencing," is essentially PCR in which one primer and the enzyme have been modified to ensure even incorporation of each ddNTP into the growing DNA strand. Such even incorporation is important for the detection of heterozygous bases where two different nucleotides are present. The methods described here have been developed and used on a slab gel automated DNA sequencer (Applied Biosystems 373) and they are now being used on a 16-capillary automated DNA sequencer (Applied Biosystems 3100 Genetic Analyzer).

PCR-SBT employed in our laboratory uses locus-specific amplification of the polymorphic exon(s) (exon 2 for class II; exons 2 and 3 or the whole gene for class I) followed by cycle sequencing using primers that are nested with respect to the amplification primers. Dedicated software is then used to analyze/edit the sequence and compare it with the appropriate library of alleles.

An advantage of our PCR-SBT technique is that amplification and sequencing primers anneal in introns which, compared to the adjacent polymorphic exons, are relatively invariant, so these primers do not need to be changed. This chapter describes methods for the amplification and sequencing of HLA class I (A, B, C) and class II (*DPB1*, *DQB1*, *DRB1*, *3*, *4*, *5*) genes.

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

Table 1
Comparison of HLA Alleles Reported in WHO Nomenclature Reports

Locus	No. of alleles 1996	No. of alleles 1998	No. of alleles 2002
HLA-A	78	119	246
HLA-B	173	245	481
HLA-C	42	74	118
HLA- DPB1	77	85	99
HLA-DQB1	31	39	53
HLA-DRB1	162	201	325

Data from **refs. *1–3***.

2. Materials

2.1. HLA Class I Locus-Specific Amplification

1. Primers (MWG Biotech).
2. Biotaq DNA polymerase (Bioline).
3. NH_4 reaction buffer, 10X (Bioline).
4. 0.2 m*M* dNTP mix (Roche).
5. 25 m*M* $MgCl_2$.
6. MilliQ pure H_2O.

2.2. HLA Class II Locus-Specific Amplification

1. Primers (MWG Biotech).
2. AmpliTaq Gold DNA polymerase (Applied Biosystems).
3. AmpliTaq Gold buffer (10X): 50 m*M* Tris-HCl, pH 8.3, 10 m*M* $(NH_4)_2SO_4$, 0.01% v/v Triton X-100. The components of this buffer are mixed together, sterile filtered, and stored in 1-mL aliquots at –20°C.
4. 5 *M* Betaine (Sigma).
5. 0.2 m*M* dNTP mix (Roche).
6. 25 m*M* $MgCl_2$.
7. MilliQ pure H_2O.
8. 0.2-mL PCR tubes, strips of eight (ABgene).

2.3. Whole-Gene Amplification

1. Primers (MWG Biotech).
2. Expand High Fidelity PCR system (Roche).
3. Expand High Fidelity PCR buffer, 10X (Roche).
4. Dimethyl sulfoxide (DMSO) (Sigma).
5. 0.2 m*M* dNTP mix (Roche).
6. 25 m*M* $MgCl_2$.
7. MilliQ pure H_2O.

2.4. Splitting HLA Class I Alleles Using Allele-Specific Primers

1. Primers (MWG Biotech).
2. Expand High Fidelity PCR system (Roche).
3. Expand High Fidelity PCR buffer, 10X (Roche).
4. DMSO (Sigma).
5. Biotaq DNA polymerase (Bioline).
6. NH_4 reaction buffer, 10X (Bioline).
7. 0.2 m*M* dNTP mix (Roche).
8. 25 m*M* $MgCl_2$.
9. MilliQ pure H_2O.

2.5. Cycle Sequencing and Separation of Sequenced Products

1. Sequencing primers (MWG Biotech).
2. Sequencing dilution buffer: 0.2 *M* Tris-HCl, pH 9.0, 5 m*M* $MgCl_2$.
3. BigDye™ terminator v2.0 (ABI 373 sequencer) or v3.0 (ABI 3100 Genetic Analyzer) cycle sequencing ready reaction kit (Applied Biosystems).
4. AGTC™ gel filtration cartridges or 96-well trays (VH Bio).
5. 3100 POP-6 polymer (Applied Biosystems).
6. Running buffer, 10X (Applied Biosystems).
7. Hi-Di formamide for ABI 3100 (Applied Biosystems).
8. Loading buffer for ABI 373 (5 parts formamide/1 part EDTA): deionized formamide/25 m*M* EDTA, pH 8.0/blue dextran (50 mg/mL).

2.6. HLA Sequencing Analysis

1. MatchTools™ Allele Identification software (Applied Biosystems).
2. MTNavigator™ (Applied Biosystems).

2.7. Equipment

1. Tetrad or Dyad thermal cycler (GRI).
2. Biofuge microcentrifuge (Heraeus).
3. Minifuge GL (Heraeus).
4. Centrifuge GP8 (IEC).
5. SpeedVac vacuum concentrator/drier (Savant).
6. 3100 Genetic Analyzer (Applied Biosystems).
7. 373 DNA sequencer (Applied Biosystems).

3. Methods

3.1. HLA Class I Locus-Specific Amplification

1. *See* **Note 1**. Set up class I PCRs in strip tubes in a rack on ice. Each PCR (50 µL) contains the following:
   a. 1 µL of 10 µ*M* each primer (*see* **Table 2**) ***(5,6)***.
   b. 5 µL of NH_4 reaction buffer.

Table 2
HLA Class I Amplification Primers

Locus	Forward primer	Sequence (5'→3')	Location
A	5AIn1–46	gAAACSgCCTCTgYggggAgAAgCAA	Intron 1, 21–46
B	5B48	AggCgggggCgCAggACCCgg	Intron 1, 46–77
B	5B49	AggCgggggCgCAggACCTgA	Intron 1, 46–77
C	5CIn1–61	AgCgAggKgCCCgCCCggCgA	Intron 1, 42–61
Locus	**Reverse primer**	**Sequence (5'→3')**	**Location**
A	3AIn3–66	TgTTggTCCCAATTgTCTCCCCTC	Intron 3, 66–89
B	3BIn3–37	ggAggCCATCCCCggCgACCTAT	Intron 3, 37–59
C	3BCIn3–12	ggAgATggggAAggCTCCCCACT	Intron 3, 12–35

Data from **refs. *5*,*6***.

c. 3 µL of $MgCl_2$.
d. 0.4 µL of dNTP mix.
e. 0.2 µL of Biotaq DNA polymerase.
f. 5 µL of genomic DNA (50 ng/µL).
g. 34.4 µL of MilliQ H_2O.
See **Note 2**.

2. Place strip tubes in a Tetrad or Dyad thermal cycler, place caps firmly on tubes, and begin thermal cycling.
3. The PCR conditions are as follows: an initial denaturation of 96°C for 5 min is followed by 35 cycles of 96°C for 20 s; 65°C for 45 s; 72°C for 1 min, finishing with 72°C for 5 min.
4. Analyze 5 µL of each PCR by 2% (w/v) agarose gel electrophoresis and dilute to approximately 5–25 ng/µL compared to the 500-bp or 1000-bp band in a 100-bp DNA ladder (Bioline).

3.2. HLA Class II Locus-Specific Amplification

1. Set up class II PCRs in a rack on ice (*see* **Notes 1** and **2**). Each PCR contains the following:
   a. 1 µL 10 µ*M* each primer (*see* **Table 3** for *DPB1*, **Table 4** for *DQB1*, and **Table 5** for *DRB1*, *3*, *4*, *5*) ***(7–9)***.
   b. 5 µL of 10X AmpliTaq Gold reaction buffer.
   c. 5 µL of betaine (*see* **Note 3**).
   d. 3 µL of $MgCl_2$.
   e. 0.4 µL of dNTP mix.
   f. 0.4 µL of AmpliTaq Gold DNA polymerase.
   g. 5 µL of genomic DNA (50 ng/µL).
   h. 29.2 µL of MilliQ H_2O.

Table 3
HLA-DPB1 Amplification and Sequencing Primers

Amplification

5DPB1: AggACCACAgAACTCggTACTAggA
3DPB1: TgAATCCCCAACCCAAAgTCCCC

Sequencing

DPB5RF (forward): gAgAgTggCgCCTCCgCTCA
DPB3RF (reverse): CCggCCCAAAgCCCTCACTC

Data from **ref. *7***.

Table 4
Amplification and Sequencing Primers for HLA-DQB1

Amplification primers

Sense primer name	Location	Sequence (5'→3')
5DQIn1–L1 (L1)	Intron 1, 1239–1259	gCggATTCCCgAAgCCCCCAg
5DQIn1–L2 (L2)	Intron 1, 1239–1259	gCAgATTCCAgAAgCCCgCAA
5DQIn1–L3 (L3)	Intron 1, 1232–1253	TAggAAggCggATTCCCgAAgA
Anti-sense primer name	**Location**	**Sequence (5'→3')**
3DQIn2–R1 (R1)	Intron 2, 105–125	CCTgTCCCCTggggTggAATA
3DQIn2–R2 (R2)	Intron 2, 92–112	gTggAATgAACKRggCTCAgA
3DQIn2–R3 (R3)	Intron 2, 105–125	CCTgTCCTCTggggTggAACA
3DQIn2–R4 (R4)	Intron 2, 105–127	CTCCTgTCMSCYggggTggAACg

Sequencing primers

Primer mix	Antigen/alleles	Sequencing primer	Sequence (5'→3')
L1R1	DQ5	3DQIn2, 20–39	ggCCAASggTgSgCCTCACg
L1R2	DQB1*06011/012	3DQIn2, 20–39	ggCCAASggTgSgCCTCACg
L2R2	DQB1*0602–0609	3DQIn2, 20–39	ggCCAASggTgSgCCTCACg
L3R3	DQ2	3DQIn2, 19–38	gCCAAgRRTgggCCTCgCAg
L3R4	DQ7	3DQIn2, 19–38	gCCAAgRRTgggCCTCgCAg
L1R4	DQ4, DQ8, DQ9	3DQIn2, 19–38	gCCAAgRRTgggCCTCgCAg

Data from **ref. *8***.

Table 5
HLA-DRB1 Amplification and Sequencing Primers

Primer Mixes

DR specificity	Forward primer	Reverse primer	Size	Sequencing primer
DR1	RB1	RB2	426 bp	RBSeq6
DR2	RB3	RB28	720 bp	RBSeq6
DR3	RB5	RB28	760 bp	RBSeq6
DR4	RB6	RB7	492 bp	RBSeq1
DR3,11,13,14	RB9	RB28	465 bp	RBSeq6
DR12	RB8	RB28	586 bp	RBSeq6
DR14	RB11	RB28	535 bp	RBSeq6
DR7	RB12	RB13	384 bp	RBSeq2
DR8	RB14	RB28	784 bp	RBSeq6
DR9	RB15	RB28	442 bp	RBSeq2
DR10	RB22	RB35	459 bp	RBSeq6
DR52	RB25	RB27	738 bp	RBSeq6
DR53	RB20	RB30	671 bp	RBSeq6
DR51	RB21	RB28	695 bp	RBSeq2

Sense primer sequences (5'→3'):

RB1	TCCCAgTgCCCgCTCCCT
RB3	ggTgggTgCTgTTgAAggT
RB5	AgCACTAAggAAgggTTCAg
RB6	CTTgggATCAgAggTAgATTTT
RB8	AACAggCTggAggTACggAC
RB9	TggTgggCgTTggggCg
RB11	gTTTTCCCgCCTggACCCT
RB12	CggCgTCgCTgTCAgTgTT
RB14	AgCgCAggCCAggCTCAAA
RB15	CAgTTAAggTTCCAgTgC
RB20	TTTCAggTgAAgACTCCCAgA
RB21	gATgAgAgAAggAgCACAgAT
RB22	ggCgTTgCgggTCggCg
RB25	CACAAggTCATCACTAAgAAAg

Antisense primer sequences (5'→3'):

RB2	ACACACTCAgATTCTCCgCTT
RB7	ACACACACACTCAgATTCTCC
RB13	TCAgATTCCCAgCTCggAgA
RB27	CAggCCCCgCCCCCCgA
RB28	ACACACACACTCAgATTCCCA
RB30	ACACACATACACAgATTCCCC
RB35	ACACACAgAgTCAgATTCCCA

Table 5 ***(Continued)***
HLA-DRB1 Amplification and Sequencing Primers

Sequencing Primers (5'→3'):	
RBSeq1	ATAgTgTCTTCCCCggAg
RBSeq2	ggAggCCgCCCCTgTgA
RBSeq6	CTgTCAgTgTCTTCTCAggAg

Data from **ref. *9***.

2. PCR conditions: An initial "hot-start" (*see* **Note 4**) of 12 min at 96°C is followed by 20 cycles of 96°C for 20 s, 65°C for 45 s, 72°C for 1 min; followed by 15 cycles of 96°C for 20 s, 60°C for 45 s, 72°C for 50 s; followed by 10 cycles of 96°C for 20 s, 55°C for 45 s, 72°C for 50 s; finishing with 72°C for 5 min.
3. The PCRs are analyzed as described in **Subheading 3.1.**, **step 4**.

*3.3. Whole-Gene Amplification (Class I) (***see *Note 5)***

1. PCRs for whole locus amplification (class I) are set up on ice as above (*see* **Notes 1** and **2**) and each contains:
   a. 1 µL of 10 µ*M* each primer (*see* **Table 6**) ***(10,11)***.
   b. 5 µL of 10X Expand PCR buffer.
   c. 5 µL of DMSO (*see* **Note 6**).
   d. 3.5 µL of $MgCl_2$.
   e. 0.75 µL of dNTP.
   f. 0.75 µL of Expand DNA polymerase (*see* **Note 7**).
   g. 5 µL of genomic DNA (50 ng/µL).
   h. 28 µL of MilliQ H_2O.
2. The cycling parameters are as follows: 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 45 s at 65°C, and 3 min at 68°C finishing with 7 min at 68°C.
3. Analyze the PCRs using 0.8% (w/v) agarose gel electrophoresis.
4. Dilute to 5–25 ng/µL by comparison with a suitable ladder prior to sequencing.

*3.4. Splitting Class I Alleles Using Allele-Specific Primers (***see *Note 8)***

Prepare two 50-µL PCRs to amplify specifically one allele:

1. For the first PCR:
   a. 1 µL of 10 µ*M* primer that anneals in 5' untranslated region (*see* **Table 6**) ***(10,11)***.
   b. 1 µL of antisense allele-specific primer ***(12)***.
   c. 5 µL of 10X Expand PCR buffer.
   d. 5 µL of DMSO (*see* **Note 6**).

Table 6
Amplification Primers for Whole Locus Amplification of HLA Class I Genes

Locus	Primer	Sequence 5'→3'	Location
A	HLA5AUT	TgggCCAAgACTCAgggAgACAT	–330 to ATG start
A	3A18	TTggggAgggAgCACAggTCAgCgTgggAAg	+175 after term
B	HLA5BUT	ggCAgACAgTgTgACAAAgAggC	–320 to ATG start
B	3B38	CTggggAggAAACACAggTCAgCATgggAAC	+175 after term
C	HLA5CUT	TCAggCACACAgTgTgACAAAgAT	–320 to ATG start
C	3C22	TCggggAgggAACACAggTCAgTgTggggAC	+175 after term

Data from **refs. *10,11***.

e. 3.5 µL of $MgCl_2$.
f. 0.75 µL of dNTP.
g. 0.75 µL of Expand DNA polymerase (*see* **Note 7**).
h. 5 µL of genomic DNA (50 ng/µL).
i. 28 µL of MilliQ H_2O.

2. For the second PCR:
   a. 1 µL of 10 µ*M* primer which anneals in 3' untranslated region (*see* **Table 6**) ***(10,11)***.
   b. 1 µL of sense allele-specific primer ***(12)***.
   c. 5 µL of 10X Expand PCR buffer.
   d. 5 µL of DMSO (*see* **Note 6**).
   e. 3.5 µL of $MgCl_2$.
   f. 0.75 µL of dNTP.
   g. 0.75 µL of Expand DNA polymerase (*see* **Note 7**).
   h. 5 µL of genomic DNA (50 ng/µL).
   i. 28 µL of MilliQ H_2O.
3. The cycling parameters are as follows: 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 45 s at 60°C, and 3 min at 68°C finishing with 7 mins at 68°C.
4. Analyze the PCRs using 0.8% (w/v) agarose gel electrophoresis.
5. Dilute to 5–25 ng/µL by comparison with a suitable ladder prior to sequencing.

3.5. Cycle Sequencing and Separation of Sequenced Products (see *Note 10*)

3.5.1. Cycle Sequencing

1. Thaw and dilute BigDye™ Terminator Cycle Sequencing kit threefold with dilution buffer.
2. Set up cycle sequencing reactions in strip tubes and on ice, as if preparing PCRs (*see* **Notes 1** and **2**).
3. Each reaction contains:
   a. 8 µL of diluted sequencing mix.
   b. 1 µL of sequencing primer (3.2 pmol/µL) (*see* **Tables 3**, **5–7**).
   c. 11 µL of PCR (50–100 ng DNA).

Table 7
HLA Class I Sequencing Primers for Exons 2 and 3

Locus	Primer	Exon	Direction	Location	Sequence (5'→3')
HLA-A	5AIn1-99	Exon 2	Forward	Intron 1, 99–119	AgCCgCgCCKggASgAgggTC
HLA-A	3AIn2-37	Exon 2	Reverse	Intron 2, 37–57	ggCCCgTCCgTgggggATgAg
HLA-A	5AIn2-150	Exon 3	Forward	Intron 2, 150–171	gTTTCATTTTgRgTTKAggCCA
HLA-A	3AIn3-41	Exon 3	Reverse	Intron 3, 41–66	gAAACSgCCTCTgYggggAgAAgCAA
HLA-B	5BIn1-77	Exon 2	Forward	Intron 1, 77–99	ggAgCCgCgCCgggAggAgggTC
HLA-B	3B44	Exon 2	Reverse	Intron 2, 25–44	ggATggggAgTCgTgACCT
HLA-B	5BInt2	Exon 3	Forward	Intron 2, 226–243	ACKgKgCTgACCgCgggg
HLA-B	3BCIn3-12	Exon 3	Reverse	Intron3, 12–35	ggAgATggggAAggCTCCCCACT
HLA-C	5CIn1-77	Exon 2	Forward	Intron 1, 77–99	ggAgCCgCgCAgggAggWgggTC
HLA-C	3C26	Exon 2	Reverse	Intron 2, 25–44	ggAggggTCgTgACCTgCgC
HLA-C	5CInt2	Exon 3	Forward	Intron 2, 229–247	CTTgACCRCgggggCgggg
HLA-C	3BCIn3-12	Exon 3	Reverse	Intron 3, 12–35	ggAgATggggAAggCTCCCCACT

4. Centrifuge briefly to collect liquid.
5. Place tubes in a thermal cycler.
6. Cycle sequencing conditions: 98°C for 10 s followed by 30 cycles of 96°C for 10 s and 65°C for 1 min.

3.5.2. Removal of Unincorporated Dyes (see Note 9)

Unincorporated fluorescent dyes are removed by gel filtration either using AGTC™ cartridges or 96-well trays as follows:

1. Centrifuge cartridges in a microcentrifuge at 750*g* for 2.5 min to remove preservative.
2. Place the cartridges in fresh tubes.
3. Pipet each sequencing reaction directly onto the surface of the gel.
4. Centrifuge cartridges as above and discard.
5. Dry down sequencing reactions by vacuum centrifugation.
6. Centrifuge 96-well trays in a centrifuge at 850*g* for 2 min to remove preservative.
7. Discard bottom tray and replace with a new one.
8. Pipet each sequencing reaction directly onto the surface of the gel.
9. Spin tray 850*g* for 5 min.
10. Discard tray containing gel.
11. Dry down sequencing reactions by vacuum centrifugation.

3.5.3. Separation of Sequenced Products

1. Resusupend sequence products in either 5 µL of formamide–EDTA–blue dextran (ABI 373) or 10 µL of Hi-Di formamide (ABI 3100).
2. Electrophorese using a "Rapid Run" protocol of 1 h (ABI 3100) or electrophorese for minimum 8 h (ABI 373).

3.6. HLA Sequencing Analysis (see Note 11)

1. Set custom analysis to 9000 (ABI 3100) data points and start Sequencing Analysis™
2. Process analyzed sequences through MatchTools™.
3. Align sense and anti-sense sequences for each exon and compare to a locus-specific consensus sequence using MTNavigator™.
4. Deduce a consensus for that sample and save this as a text file.
5. Save MTNavigator™ layout.
6. Compare this consensus sequence against the appropriate libarary of HLA alleles using MatchTools™.
7. Print the batch report.

4. Notes

1. All PCRs should be set up in a designated "pre-PCR" laboratory that is distinct from the ("post-PCR") laboratory used for analysis and sequencing of amplicons. The amplification methods described here work very well with genomic DNA

prepared by a number of commercial kits as well as "in-house" methods such as salting out. Genomic DNA isolated using magnetic beads amplifies well as long as the DNA remains bound to the beads—elution of DNA from the beads invariably results in poor amplification. The AmpliTaq Gold method, described for HLA class II genes in **Subheading 3.2.**, can also be used for class I. The class I amplicons are approx 900 bp.

2. It is convenient to prepare a mix with all components other than DNA; aliquot 45 µL of this into the tubes, briefly centrifuge the rack in a centrifuge (i.e., IEC Centra 8) to collect liquid, and then add DNA into appropriate tubes.
3. Betaine (0.5 *M* final concentration) is used as an additive to enhance the amplification of GC-rich templates and help prevent preferential allele amplification ***(13–15)***.
4. A number of thermally stable DNA polymerases are available in which the enzmye's active site has been mutated or chemically modified such that it requires a heat activation step ("hot start") before the enzyme can start to work copying the template. This is useful in preventing the formation of dimers between primers.
5. Whole class I gene amplification is used when we know that the alleles present have polymorphisms outside of exons 2 and 3. **Table 8** lists alleles that are indistinguishable from their exon 2 and 3 sequences, where they differ, and which sequencing primer should be used to resolve these alleles. **Table 9** lists these primers.
6. Whole gene amplification has an absolute requirement for DMSO, which probably helps to remove structures produced during this long (3.4 kb) amplification.
7. The Expand DNA polymerase system works optimally at 68°C and is a mixture of *Taq* DNA polymerase and a DNA polymerase from *Pseudomonas woelii* which has proofreading activity. This reduces the rate of errors that are incorporated by *Taq* DNA polymerase into DNA during thermocycling and amplification.
8. When the PCR-SBT results indicate the presence of a variant allele, this allele has to be separated from the other allele of the same locus. There are two ways to do this: (a) cloning using *E. coli* and (b) allele-specific PCR. In our experience cloning generates a significant number of sequence artefacts that have probably occurred in the PCR (even if a so-called high-fidelity enzyme system is used). Therefore, we always use method (b) to isolate a suspected new allele. In method (b) two PCRs are carried out. In the first a sense allele-specific primer ***(12)*** can be used in conjunction with a locus-specific primer that anneals in the 3' UTR and in the second an anti-sense allele-specific primer ***(12)*** can be used in conjunction with a locus-specific primer that anneals in the 5' UTR. The annealing temperature to use these allele-specific primers is reduced to 60°C (**Subheading 3.3.**).
9. Unincorporated dyes must be removed before separation of the sequenced products. These dyes will leave a large fluorescent "flare" obscuring the first 50–100 nucleotides of sequence if they are not removed.
10. Enzymes used for cycle sequencing have been optimized by manufacturers (by site-directed mutagenesis or chemical modification) to maximize read lengths

Table 8
HLA Class I Polymorphisms Outside of Exons 2 and 3 (*see* Table 9)

Alleles	Exon difference	Sequencing primer
A*68012, A*6811N	1	3AIn2–37
A*7401, A*7402	1	3AIn2–37
A*01011, A*0104N	4	5A24
A*02011, A*0209, A*0243N	4	5A24
A*2402101, A*2409N, A*2411N	4	5A24
B*27052, B*2713	1	3B44
B*44021, B*4419N	1	3B44
B*51011, B*5111N	4	5B44
B*0705, B*0706	5	5ABC48
Cw*1701, Cw*1702, Cw*1703	1	3C26
Cw*0501, Cw*0503	4	5C17
Cw*0701, Cw*0706	5	5Cex5
Cw*1801, Cw*1802	5	5Cex5
Cw*04011, Cw*0409N	7	5ABC47

Table 9
Sequencing Primers for Other HLA Class I Exons (*see* Table 8)

Locus	Primer	Location	Sequence (5'→3')
A	5A24	Intron 3, 483–503	TTCTgTgCTCYCTTCCCCAT
B	5B44	Intron 3, 502–522	CTggTCACATgggTggTCCTA
A, B, C	5ABC47	Exon 7, 1055–1073	ACAgTgCCCAgggCTCTgAT
A, B, C	5ABC48	Exon 4, 808-828	gCTgTggTggTgCCTTCTggA
C	3C26	Intron 2, 25–44	ggAggggTCgTgACCTgCgC
C	5C17	Intron 3, 553–576	CAAAgTgTCTgAATTTTCTgACTC
C	5Cex5	Exon 5, 901–921	TCTTCCCAgCCCACCATCCCC

while maintaining even peak height. For most applications in HLA SBT it is not necessary to sequence more than 300 bp, as all polymorphic exons are approx 270 bp or less. Even peak height is, however, critical because it is very important to identify heterozygous positions. Four different sequencing primers are used to read the nucleotide sequence of both strands of class I exons 2 and 3. HLA-DQB1 or HLA-DRB amplification usually produces a homozygous amplicon, so only one sequencing primer is required. Occasionally, an ambiguous class I SBT result is obtained because the heterozygous nucleotide sequence of one pair of alleles is identical to that of one (or more) other pair of alleles. For example, exon

2 and 3 sequences of B*07021, B*1402 are the same as B*0726, B*1403. These can be resolved by using B*07 (or B*14) allele-specific primers ***(12)*** as sequencing primers, thus determining which B*07 allele is present. This is not really a problem for *DQB1* or *DRB* SBT (it could be resolved in the same way as class I) but it is a problem with *DPB1* SBT because of the nature of the polymorphism in exon 2. We use a commercial high resolution SSP kit (Dynal Biotech; Wirral, Cheshire, UK) to resolve DPB ambiguities.

11. Software developed by Applied Biosystems "trims" sequences to precise exons, reversing and complementing if necessary (Sequencing Analysis™, MatchTools™). Heterozygous positions are identified and assigned IUB codes. A consensus sequence for each sample (exon 2 or exons 2 + 3 or exons 2 + 3 + 4) is compiled using MTNavigator™ and compared against a library of the appropriate locus-specific alleles. A batch report is produced with a list of the closest match(es) and a sequence of the polymorphic positions in the sample compared with the closest match(es).

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Analysis of Chimerism After Stem Cell Transplantation

Peter Bader and Hermann Kreyenberg

1. Introduction

In recent years, considerable progress has been made in the analysis of hematopoietic chimerism after allogeneic stem cell transplantation (SCT). The introduction of polymerase chain reaction (PCR)-based methods for the characterization of "variable number of tandem repeats" (VNTR) and "short tandem repeats" (STR) has made it possible to analyze donor/recipient chimerism in a sensitive and quantitative fashion in all patients except those with syngeneic donors. In turn, this has allowed the serial monitoring of engraftment and study of the kinetics of graft failure and graft rejection after allo-SCT ***(1–4)***. These methods also allow the identification of patients with the highest risk of relapse after allo-SCT ***(5–7)***. In consequence, analysis of chimerism has become a routine diagnostic instrument at many centers performing allo-SCT ***(8–11)***. This chapter concentrates on PCR-based techniques for the amplification of STRs, using either (a) conventional PCR and polyacrylamide gel electrophoresis (PAGE) of reaction products or (b) PCR using fluorescent labeled primers with size resolution by capillary electrophoresis. Both methods are described in detail.

2. Materials

2.1. DNA Isolation

1. QIAmp Blood Mini Kit (Qiagen).
2. 99.7% Isopropanol.
3. Double-distilled water (ddH_2O).
4. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

From: *Methods in Molecular Medicine, Vol. 91: Pediatric Hematology: Methods and Protocols*
Edited by: N. J. Goulden and C. G. Steward © Humana Press Inc., Totowa, NJ

2.2. PCR Reaction Using PAGE

1. PCR reaction buffer (Qiagen).
2. 5 U/µL of *Taq* DNA polymerase, (Qiagen).
3. TE buffer.
4. Tris–borate–EDTA buffer (TBE): 0.089 *M* Tris, 0.089 *M* orthoboric acid, 0.002 *M* EDTA.
5. Deoxynucleotides: dATP, dCTP, dGTP, dTTP.
6. Oligonucleotides (*see* **Table 1**).
7. TEMED: *N,N,N',N'*-tetramethlylethylenediamine (Roth).
8. Ammonium persulfate 10% (Sigma-Aldrich).
9. 10X TBE buffer: 0.89 *M* Tris, 0.89 *M* orthoboric acid, and 0.02 *M* EDTA.
10. Loading buffer for PAGE: 30% glycerol, 25% bromphenol blue Na salt (Sigma-Aldrich).
11. DNA-Ladder VIII (No. 13360459) (Roche).
12. Ethidium bromide: 10 µg/mL in 1X TBE. **Caution**: Extremely toxic (Roth).

2.3. Fluorescent PCR

1. PCR reaction buffer (Qiagen).
2. 5 U/µL of *Taq* DNA polymerase (Qiagen).
3. TE buffer.
4. Deoxynucleotides: dATP, dCTP, dGTP, dTTP.
5. Fluorescent-labeled oligonucleotides (*see* **Table 1**).
6. Formamide solution (Applied Biosystems).
7. Standard solution Gene Scan 500 Rox (Applied Biosystems).
8. HPLC-grade H_2O (Merck).

3. Methods

3.1. DNA Isolation

DNA isolation is performed using DNA isolation kits from Qiagen according to the manufacturer's instructions and quantitated spectrophotometrically as described below (*see* **Notes 1** and **2**).

3.1.1. DNA Spectrophotometric Quantitation

1. Add 95 µL of ddH_2O to a clean glass 100-µL cuvette.
2. Add 5 µL DNA solution.
3. Place cuvette in Genequant® spectrophotometer (Amersham Pharmacia Biotech).
4. Optical density (OD) readings are taken at 260 nm and 280 nm. An OD_{260} of 1 corresponds to 0.05 µg/µL for double-stranded DNA. Pure preparations of DNA have OD_{260}/OD_{280} values of 1.8.

3.2. Chimerism Analysis After PAGE Resolution Using Primers for VNTR and STR

Pay strict attention to possible sources of contamination (*see* **Note 3**). All reagents should be kept on ice. It is advisable to keep primers in small aliquots to minimize freeze–thawing which might lead to degradation. Try also to minimize the time that the *Taq* DNA polymerase is out of the freezer.

To obtain an informative primer pair, patient pretransplant and donor samples are first amplified with four of the primer pairs listed in **Table 1**. The most informative primer is then used for follow-up investigations (*see* **Note 4**).

3.2.1. Sample Preparation and Preparation of Master Mix

All samples should be amplified using 50-µL reaction volumes (*see* **Note 5**).

1. Take an appropriate number of clearly labeled PCR tubes.
2. Add 250 ng of template DNA to each PCR tube.
3. Add H_2O to make a final volume of 20 µL.
4. Prepare a master mix. This is a bulk mix containing the following multiplied by the number of individual PCR reactions being performed:
   a. 7 µL of 10X PCR Qiagen PCR buffer (containing 15 m*M* $MgCl_2$) (*see* **Note 6**).
   b. 4 µL of dNTP (prepared by mixing equal volumes of 10 m*M* dATP, dCTP, dGTP, dTTP.
   c. 1 µL of primer solution (containing 1 m*M* of both forward and reverse primers each).
   d. 0.5 µL of *Taq* DNA polymerase (Qiagen).
   e. 17.5 µL of H_2O.
5. Add 30 µL of this mastermix to each PCR tube.
6. Gently shake the tubes and transfer to a PCR cycler.

3.2.2. PCR Cycling Parameters

These depend on the particular primer pair being used, as follows:

1. Primer pairs no. 2 or 3:
   a. Initial denaturation at 94°C for 3 min.
   b. Then 35 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 90 s.
2. Primer pairs no. 1, 4, 5, 6, 7, 8, and 9:
   a. Initial denaturation at 94°C for 3 min.
   b. Then 35 cycles of 94°C for 1 min, 62°C for 45 s, and 72°C for 90 s.

3.2.3. Analysis of PCR Products by 8% PAGE

1. Prepare 8% polyacrylamide gel with minimum thickness of 1.2 mm. Use 1X TBE as the buffer system (*see* **Note 7**).
2. Mix 50 µL of each samples with 14 µL of loading buffer.
3. Load 50 µL of sample onto the gel.

Table 1
Primer Characteristics

No.	Name	Chromosome location	Genome database Acc. ID[a]	Type	Heterozygosity	Alleles	Product size	Primer sequences 5'→3'
1	D11S533	11q 13	182143	TetNR	0.90	10	400–1100	Forward: GCCTAGTCCCTGGGTGTGGTC
								Reverse: GGGGGTCTGGGAACATGTCCCC
2	F13A1	6p 24–25	180362	TetNR	0.78	8	180–203	Forward: GAGGTTGCACTCCAGCCTTT
								Reverse: ATGCCATGCAGATTAGAAA
3	MYCL	1p 32	750055	TetNR	0.87	8	140–209	Forward: TGGCGAGACTCCATCAAAG
								Reverse: CCTTTTAAGCTGCAACAATTTC
4	HUM RENA-4	1q32	185507	TetNR	0.77	6	251–271	Forward: AGAGTACCTTCCCTCCTCTACTCA
								Reverse: CTCTATGGAGCTGGTAGAACCTGA

5	HUM FABP2	4q28-31	185505	TetNR	0.80	9	199–220	Forward: GTAGTATCAGTTTCATAGGGTCACC
								Reverse: CAGTTCGTTTCCATTGTCTGTCCG
6	HUM THO1	11p15.5	212652	TetNR	0.87	7	179–203	Forward: GTGGGCTGAAAAGCTCCCGATTAT
								Reverse: ATTCAAAGGGTATCTGGGCTCTGG
7	HUM CD4	12p	119767	PenNR	0.82	5	88–128	Forward: GCCTGAGTGACAGAGTGAGAACC
								Reverse: TTGGAGTCGCAAGCTGAACTAGC
8	P-450CYP 19	15q21.1	119830	TetNR	0.91	5	154–178	Forward: GCAGGTACTTAGTTAGCTAC
								Reverse: TTACAGTGAGCCAAGGTCGT
9	vWF II	12p12pter	177640	TetNR	0.79	7	139–167	Forward: GCAGGTACTTAGTTAGCTAC
								Reverse: TTACAGTGAGCCAAGGTCGT

[a]The Genome Database: http://www.gdb.org/gdb/

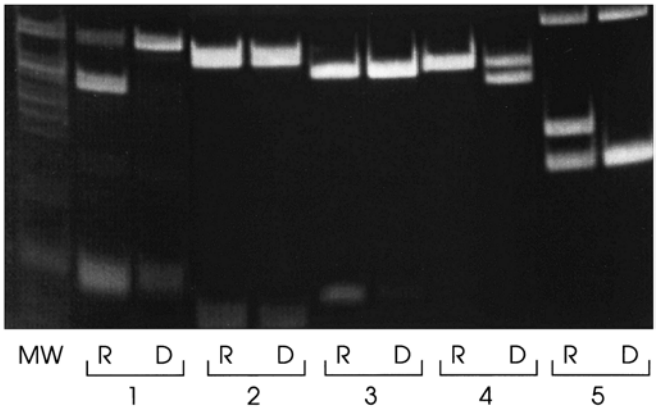


Fig. 1. A primer screening test is shown using primers 1–5 from the list given in **Table 1**. Resolution was performed using PAGE. MW, molecular weight marker; R, recipient; D, donor.

4. Resolve at 10 V/cm for 3 h at room temperature.
5. Stain with ethidium bromide for 7 min. Rinse in water prior to visualization.
6. An image of a typical PAGE gel showing amplification products of a screening PCR is shown in **Fig. 1**.

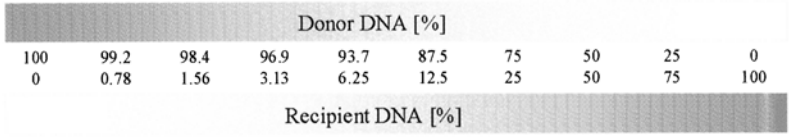
3.2.4. Generation of Patient Specific Standard Curves for Semiquantitative Analysis

For semiquantitative analysis of mixed chimeric samples it is necessary to generate patient specific standard curves to achieve reliable values (*see* **Note 8**). The first step involves making serial dilutions of recipient DNA into donor DNA (*see* **Fig. 2**).

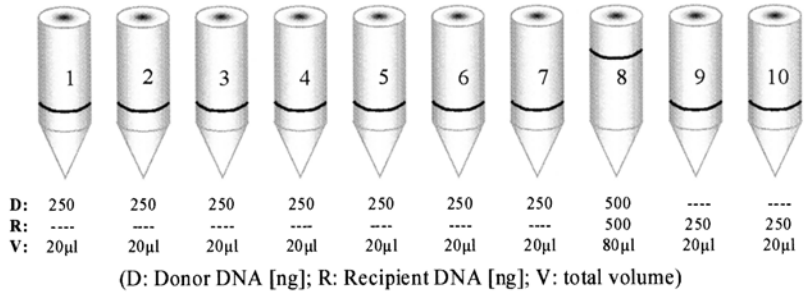
3.2.4.1. Generation of DNA Dilution Series

1. Number Eppendorf tubes 1–10.
2. Add 250 ng of donor template DNA in 20 µL of H_2O to tubes 1–7.
3. Add 500 ng of donor template DNA in 40 µL of H_2O and 500 ng of recipient template DNA in 40 µL of H_2O to tube 8.
4. Add 250 ng of recipient template DNA in 20 µL of H_2O to tubes 9 and 10.
5. Mix tube 8 carefully.
6. Transfer 20 µL of DNA mixture from tube 8 to tube 7. Mix tube 7 carefully.
7. Transfer 20 µL of DNA mixture from tube 7 to tube 6. Mix tube 6 carefully.
8. Transfer 20 µL of DNA mixture from tube 6 to tube 5. Mix tube 5 carefully.
9. Transfer 20 µL of DNA mixture from tube 5 to tube 4. Mix tube 4 carefully.
10. Transfer 20 µL of DNA mixture from tube 4 to tube 3. Mix tube 3 carefully.
11. Transfer 20 µL of DNA mixture from tube 3 to tube 2. Mix tube 2 carefully.

I. desired mixing ratio



II. prepare dilution series by filling vials with recipient and donor DNA



(D: Donor DNA [ng]; R: Recipient DNA [ng]; V: total volume)

II. starting from vial 8: transfer 20µl aliquots to the neighbouring vials as described in the text (3.2.4.1.)

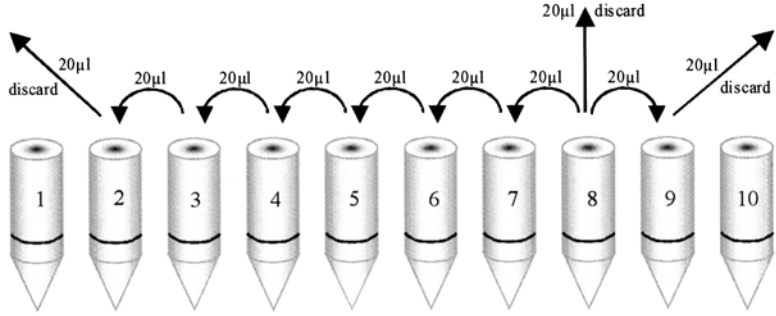


Fig. 2. Flow chart for preparing the dilution experiments. For more information *see* **Subheading 3.2.4.1.).**

12. Remove and discard 20 µL of DNA mixture from tube 2.
13. Transfer 20 µL of DNA mixture from tube 8 to tube 9. Mix tube 9 carefully.
14. Remove and discard 20 µL of DNA mixture from tube 8.
15. The final concentration of donor and recipient DNA in the different tubes is shown in **Table 2**.

Table 2
Dilution Series DNA Concentrations

Final concentration of:	Recipient DNA (%)	Donor DNA (%)
Tube 1	0	100
Tube 2	0.78	99.2
Tube 3	1.56	98.4
Tube 4	3.13	96.9
Tube 5	6.25	93.7
Tube 6	12.5	87.5
Tube 7	25	75
Tube 8	50	50
Tube 9	75	25
Tube 10	100	0

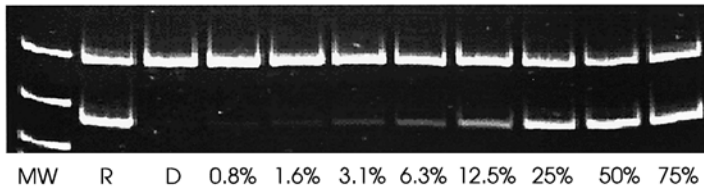


Fig. 3. PCR amplification of a dilution experiment is shown. MW, molecular weight marker; R, recipient; D, donor. Numbers indicate the relative amount of recipient DNA given to the respective sample.

16. Add to each single tube 30 µL of mastermix reaction containing the informative primer pairs as described in **Subheading 3.2.1.**
17. Gently shake the PCR tubes and transfer them to the PCR cycler using the appropriate cycling program.
18. Load samples onto 8% PAGE as described in **Subheading 3.2.3.**
19. **Figure 3** shows a photograph of a typical PAGE gel on which the amplification reactions used to generate a standard curve have been resolved.

3.2.4.2. Densitometric Analysis and Construction of Standard Curve

Patient/donor specific standard curves are generated by analyzing the intensity of donor and recipient specific bands densitometrically from digitally stored gel pictures (e.g., **Fig. 3**) using appropriate software (e.g., WinCam 2.1, Cybertech, Berlin). The relative recipient/donor quotient (RQ) can be calculated using the equation below.

$$\text{RQ (relative quotient recipient/donor DNA)} = \frac{\text{area recipient}}{\text{area recipient + area donor}}$$

As an illustration, the results of densitometric analysis of **Fig. 3** are given in **Table 3**. The experiment was carried out in duplicate and mean values calculated for generation of a standard curve. The respective standard curve is shown in **Fig. 4**.

3.2.4.3. Semiquantitative Estimation of a Posttransplant Sample

1. For semiquantitative PCR analyses, the following samples should be amplified:
   a. Recipient DNA.
   b. Donor DNA.
   c. The latest analyzed posttransplant sample (if one has already been tested).
   d. The sample to be assayed.
2. After electrophoresis the products are visualized by ethidium bromide staining and analyzed by densitometry. In samples with mixed chimerism the relative recipient/donor quotient is calculated and referred to the standard curve. This allows a semiquantitative estimation of the amount of recipient DNA in a given sample.

3.3. Fluorescent-Based PCR Analysis of Chimerism

The same general guidelines apply as detailed in **Subheading 3.2.**

To obtain an informative primer pair, patient pretransplant, and donor samples are first amplified with all listed primer pairs. The most informative primer is then used for follow-up investigations. Details of primers used are listed in **Table 1** (*see* **Note 9**). A representative electropherogram of a primer screening test is shown in **Fig. 5**.

3.3.1. Sample Preparation and Preparation of Mastermix

All samples are amplified using 10.5-µL reaction volumes (*see* **Note 10**).

1. Take an appropriate number of clearly labeled PCR tubes.
2. Add 0.5 µL of DNA template solution containing 100 ng DNA to each.
3. Prepare a mastermix. This is a bulk mix containing the following multiplied by the number of individual PCR reactions being performed:
   a. 1.4 µL of 10X PCR Qiagen PCR buffer (containing 15 m*M* $MgCl_2$).
   b. 0.8 µL of dNTP (prepared by mixing equal volumes of 10 m*M* dATP, dCTP, dGTP, dTTP.
   c. 0.2 µL of primer solution (containing 1 m*M* forward and reverse primer).
   d. 0.1 µL of *Taq* DNA polymerase, 5 U/µL (Qiagen).
   e. 7.0 µL of H_2O.
4. Add 10 µL of mastermix to each PCR tube.
5. Gently shake the PCR tubes and transfer to the PCR cycler.

3.3.2. Post-PCR Processing of the Samples

1. Add 40 µL of H_2O to each PCR tube.

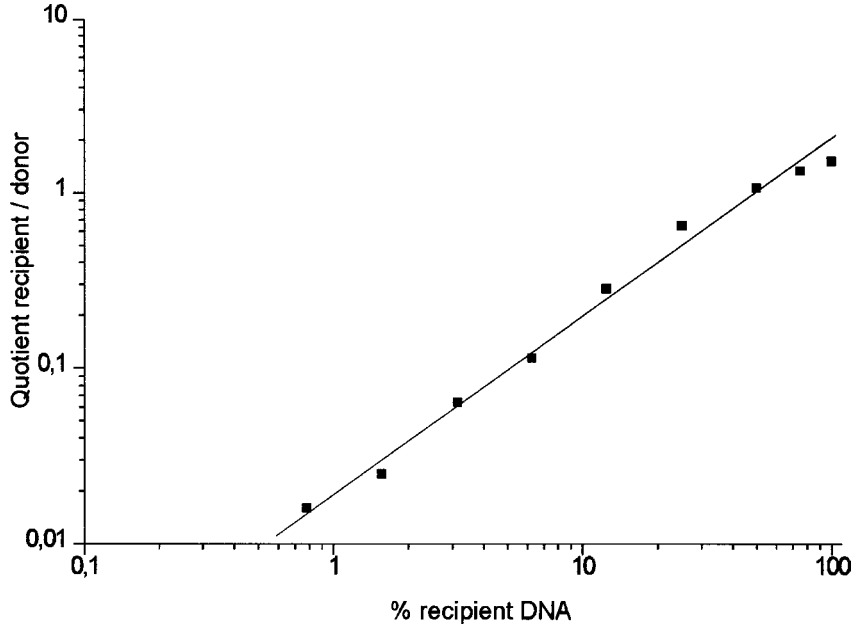


Fig. 4. The calculated standard curve of the gel shown in **Fig. 3**.

2. Prepare for capillary electrophoresis by adding 13.7 µL of formamide and 0.3 µL of standard (Gene Scan 500 ROX) to 1.0 µL of diluted PCR product (*see* **Subheading 3.3.1.**).
3. Denature by heating each sample at 90°C for 2 min and then place on ice immediately.

3.3.3. Analysis on ABI 310 Using Polymer POP 4

Parameters used are as follows:

1. Injection time: 5 s.
2. Injection energy: 15.0 kV.
3. Temperature: 60°C.
4. Time per tube: 18 min.

3.3.4. Quantification of Mixed Chimeric Samples

Quantitative analyses are performed using the Genotyper Software (Perkin-Elmer, Foster City, USA). It is best to place the recipient and donor pretransplant samples together with the last sample and the posttransplant sample being investigated on one page in the "align by size" mode so that the informative allele can be identified easily. The recipient and donor specific peaks are marked and the peak area under the curve is printed below the electrophero-

Table 3
Calculation of Relative Quotient (RQ) Values

	Standard dilution experiment I			Standard dilution experiment II			
Recipient DNA (%)	Peak 1	Peak 2	RQ	Peak 1	Peak 2	RQ	Mean value
0.00	3.355	0.000	0.000	3.132	0.000	0.000	0.000
0.78	3.250	0.067	0.021	3.831	0.046	0.012	0.016
1.56	3.529	0.102	0.029	3.472	0.072	0.021	0.025
3.13	3.270	0.234	0.072	3.190	0.177	0.056	0.064
6.25	2.942	0.355	0.121	2.805	0.302	0.108	0.114
12.50	2.887	0.943	0.327	2.585	0.616	0.238	0.282
25.00	2.391	1.562	0.653	2.318	1.481	0.639	0.646
50.00	1.831	2.000	1.092	1.692	1.740	1.029	1.060
75.00	0.847	1.091	1.289	2.150	2.935	1.365	1.327
100.00	2.157	3.455	1.602	2.188	3.079	1.407	1.504

Peak 1, Peak area donor; Peak 2, peak area recipient; RQ, relative quotient according to formula in **Subheading 3.2.4.2.**

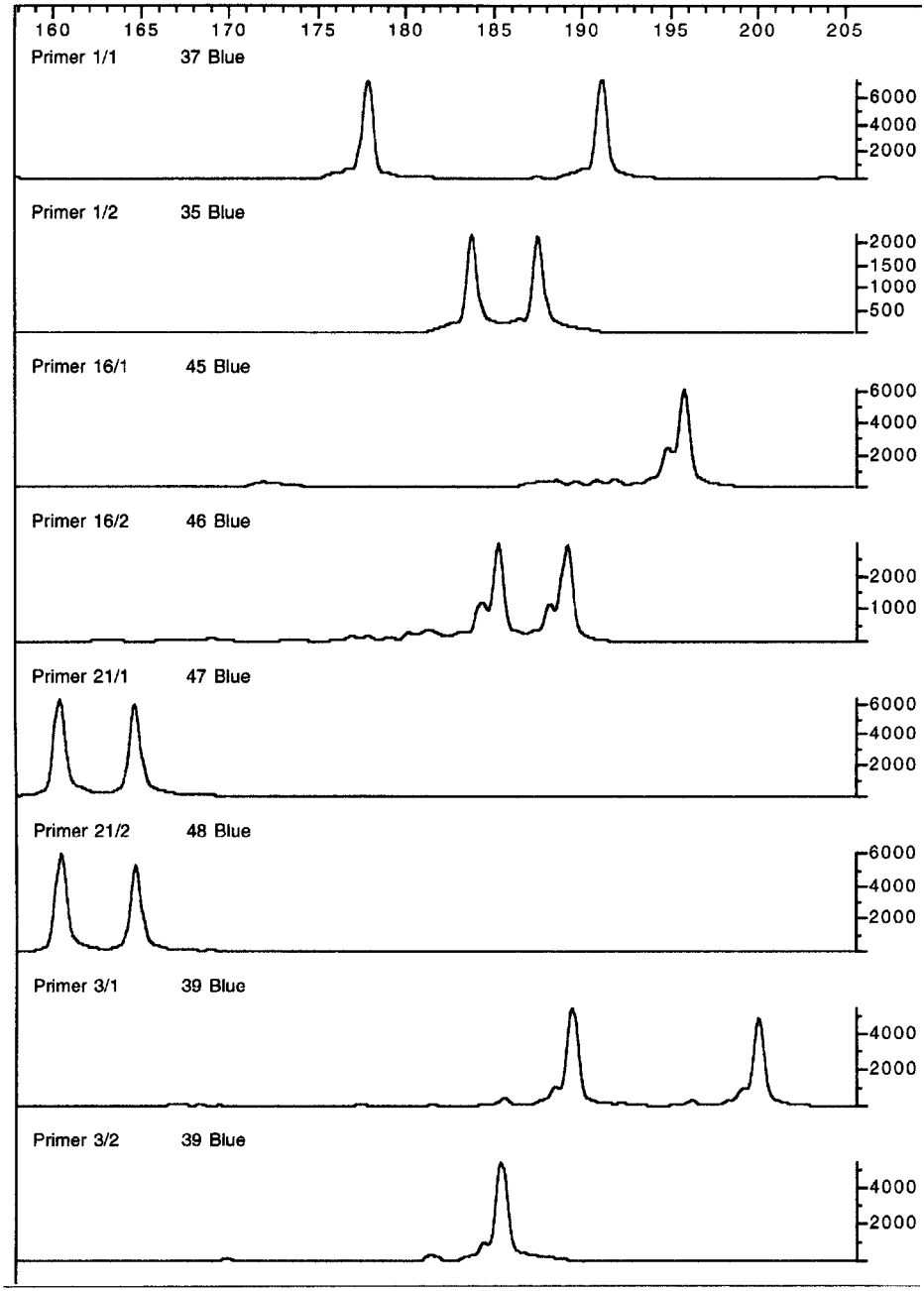
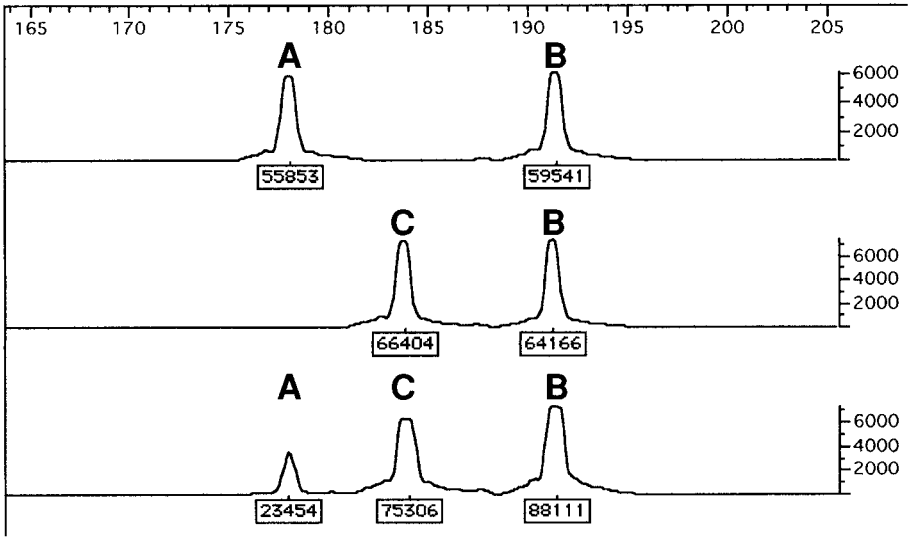


Fig. 5. A primer screening test using fluorescent labeled primers analyzed by capillary electrophoresis. Four different primers were used. In the first line recipient and in the second line donor samples are shown.



$$\% \text{ recipient DNA} = 100 \times \frac{\text{area A}}{\text{area A} + \text{area C}}$$

Fig. 6. A mixed chimeric sample is shown with one shared and one unique donor allele. In this case the quantitative calculation should be performed according to the equation below. (For further details *see* **Subheading 3.3.5.1.**) In the first line recipient DNA, in the second line donor DNA and in the third line a mixture is used for PCR amplification.

gram (*see* **Notes 11** and **12**). The calculation of the amount of recipient DNA in a given posttransplant sample is done using the general equation:

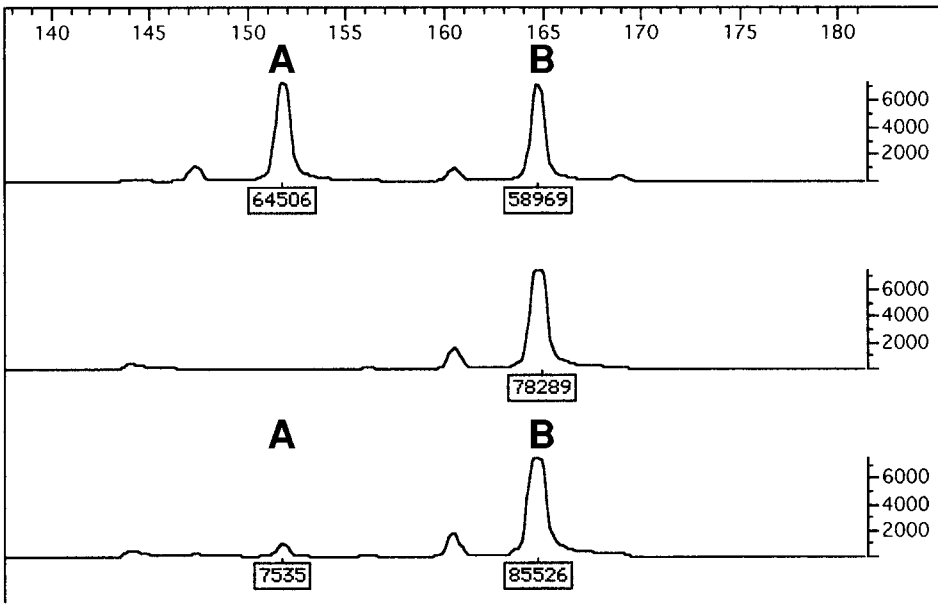
$$\% \text{ recipient DNA} = \frac{\text{area recipient}}{\text{area donor} + \text{area recipient}}$$

If the recipient and donor share one allele, the common allele can be disregarded for quantification. This is illustrated in the graph shown in **Fig. 6**.

If (a) either the recipient or donor is heterozygous and the other is homozygous and (b) both share one allele the calculation is slightly different, as illustrated in **Fig. 7**.

4. Notes

1. DNA can be isolated from peripheral blood and bone marrow samples after Ficoll purification and phenol–chloroform extraction. However, recently a variety of affinity-capture methods have been developed and made available commercially.



$$\% \text{ recipient DNA} = 100 \times \frac{2 \text{ area A}}{\text{area A} + \text{area B}}$$

Fig. 7. A mixed chimeric sample is shown where the recipient is heterozygous and the donor homozygous, with an allele shared with the recipient. In this case the quantitative calculation should be performed according to equation shown below. (For further details *see* **Subheading 3.3.5.**) In the first line recipient DNA, in the second line donor DNA and in the third line a mixture is used for PCR amplification.

In our laboratory a variety of these commercially available DNA isolation kits have been tested. The Qiagen DNA isolation kits gave reproducible DNA isolation to high purity. This allows direct DNA extraction from whole peripheral blood or bone marrow without Ficoll purification of mononuclear cells, and 500 µL of blood or bone marrow provides sufficient good quality DNA for many PCR amplifications.

2. Exact DNA quantification is a prerequisite for semiquantitative analysis of posttransplant chimerism. Therefore spectrophotometric DNA quantification is strongly recommended. This procedure also helps to check for DNA integrity and cross-contamination with protein.
3. The spillage of small volumes of PCR productions in the laboratory environment may cause serious problems with false-positive results. Effective prevention of "contamination" is normally possible following a few simple recommendations:

a. DNA extraction and PCR should be performed in an area separate from that used to process PCR products.
b. Separate pipets, glassware, tubes, and chemicals should be used. Aerosol-resistant tips might be useful to avoid contamination of pipets.

4. Screening recipient and donor DNA to obtain an informative primer pair can be done with four primers out of those listed in **Table 1** (*see* **Subheading 3.2.**). Shorter alleles may be amplified more effectively than long alleles. To achieve the highest possible sensitivity of detection of recipient cells in a posttransplant sample it is therefore advisable to use a primer combination in which the informative allele of the recipient is shorter than that of the donor. This is shown in **Fig. 1**, where primer pair 1 amplifies a shorter recipient allele. The relative effect of the differing patterns of bands is not yet clear, for example, whether having a shared band between donor and recipient impacts on sensitivity. This is being investigated by a European Study Group on Chimerism, which commenced work in September 2002. It is hoped that this group will (a) define the best microsatellite loci for chimerism analysis, (b) identify the best allele patterns for sensitive and reproducible detection, and (c) develop specific kits for chimeric analysis in conjunction with commercial companies. The findings of this group will be published at intervals in the journal *Leukemia*.
5. PCR amplification for gel resolution is performed in 50-µL assays. This offers the possibility to load at least 50 µL of PCR reaction onto the gel. Using lower amounts will result in fainter bands after visualization with ethidium bromide. This will also reduce the sensitivity for detection of recipient DNA in posttransplant samples.
6. In the assay described, an ammonium-based PCR buffer from Qiagen is used, although other PCR buffers containing appropriate concentration of $MgCl_2$ may provide equivalent amplification. However, the approach presented was optimized for the Qiagen buffer and the respective *Taq* polymerase. The assay may need to be recalibrated for other buffer/*Taq* systems and equivalent results cannot be guaranteed.
7. Analysis of PCR productions is performed after electrophoresis using 8% polyacrylamide gels (*see* **Subheading 3.2.3.**), as agarose gels often do not provide adequate resolution. 12% polyacrylamide gels may improve resolution of closely spaced short products. However, the time requirement for electrophoresis procedure will be longer. The sensitivity will increase with the diameter of the gel layer. Therefore spacers > 1 mm thick are highly recommended.
8. Generation of standard curves. For a sample that is heterozygous for a polymorphism of variable length, it is possible (*see* **Subheading 3.2.4.**) that shorter alleles are amplified more efficiently than the longer allele. This preferential amplification may result in a nonlinear relationship between the allelic band intensity and the amount of DNA present in the mixture, and it is often impossible to use a simple visual estimation of the band intensity to determine accurately the degree of mixed chimerism. This effect is augmented when products are analyzed using gel electrophoresis and ethidium bromide staining. Therefore

Table 4
Guide to Problem Solving

Problem	Probable cause	Solution
1. PCR and PAGE resolution		
Faint PCR signals	DNA	Check DNA quality; increase amount of DNA to sample
Low sensitivity for detection of recipient DNA in mixed chimeric samples	Wrong primer constellation	Check that informative allele is shorter than donor alleles; take care that the difference in length of the alleles allows proper identification.
High background signals	$MgCl_2$; PCR conditions	Check PCR conditions; increase annealing temperature. If still problems, reduce concentration of $MgCl_2$
Blurred PCR gel signals	Electrophoresis	Reduce voltage; check gel quality; increase PAGE concentration
2. Fluorescence-based PCR		
High peak intensity	Sample	Increase dilution of PCR product; decrease injection time to electrophoresis apparatus; reduce template amount
Low peak intensity	Sample	Reduce dilution of PCR product; increase injection time; increase PCR template
Nonspecific peaks close to informative allele	Primer constellation	Make sure that informative recipient allele is outside the stutter peak area (four or five base pairs)

semiquantitative analysis is most reliable after generating patient- and primer-specific standard curves.

9. Fluorescence-based PCR analysis (*see* **Subheading 3.3.**) Identification for an informative primer pair should be done using four of the listed primers according to **Table 1** and **Note 4.** It is advisable not to use those primers where the informative recipient allele is within the stutter peak area of the donor allele. Stuttering can occur within a length of four base pairs.
10. Owing to the low amount of PCR product needed for postamplification processing with the capillary electrophoresis, mastermix reactions are set up with only 10 µL. This also helps to reduce costs. It is recommended to use 100 ng of DNA for each sample. Less than 5 ng of DNA per sample will not provide reproducible results.
11. Quantitative analysis of mixed chimeric sample with fluorescent labeled primers offers the possibility to calculate the amount of recipient DNA in a given sample without prior construction of a standard curve. Quantification should be done according to the formula given in **Subheading 3.3.4.**
12. Possible causes and solutions to problems that may occur are shown in **Table 4**.

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