PREVENTION OF THALASSAEMIAS AND OTHER HAEMOGLOBIN DISORDERS

VOLUME 2: LABORATORY PROTOCOLS

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PREFACE

Volume 2 of the Prevention Book presents the major technical procedures that are useful for the laboratory diagnosis of the thalassaemias and abnormal haemoglobin disorders. This book was written for use in combination with Volume 1, in which the various types of disorders requiring prenatal diagnosis and the strategies used for carrier screening are described.

Most of the protocols described here are in current use in the authors' and contributors' laboratories. However it was not possible to include details of every known practical method for screening and mutation detection, and similarly it is not possible for a laboratory to develop expertise in the application of every method. Thus to guide laboratories in deciding which techniques to use or develop, the first chapter of this book concentrates on the best practice in carrier screening and molecular analysis. This is followed by five chapters that describe detailed protocols and technical tips not only for the classic procedures used for the prevention of thalassaemia, but also for some of the more advanced and specialized techniques that a diagnostic centre might consider to introduce into service. The final chapter highlights some of the emerging technologies that are being developed, such as the use of gene-chips or microarrays and next generation sequencing.

Many innovations have occurred since the publication of the First Edition of this book in 2005, and therefore every chapter in this Second Edition has been revised and updated. We are confident that this book, together with the revised Volume 1, will continue to provide a valuable tool and resource for diagnostic laboratories engaged in the prevention of the thalassaemias and other haemoglobin disorders.

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FOREWARD

Dear readers,

Welcome to the updated and completely revised new edition of theThalassaemia International Federation's book 'Prevention of Thalassaemias and Other Haemoglobin Disorders Vol.2'. In the first volume of the series, the authors, who are all international experts in the field, present an overview of the aspects and necessary components that need to be considered when setting up a national prevention strategy.

The Thalassaemia International Federation (TIF) has adopted prevention, the limitation of the number of new births, as a basic policy and a necessary element in the control of the thalassaemias and haemoglobinopathies. The other element in control, is the provision of the best possible treatment of those who suffer from these hereditary disorders. In this respect, TIF advised the authors to go a step forward and make recommendations in this second volume regarding the details of the laboratory techniques, methodologies, algorithms that they themselves use in their own centers. Therefore the objective of the second volume in the series is to guide other laboratory specialists and public health policy officers who are currently setting up large scale prevention programmes, and to provide the specific details that they will need to put theory into practice.

The team of authors includes specialists from the UK, Italy, Greece and Cyprus. In most of these countries, population-level prevention was adopted more than 30 years ago and most of the authors have been involved in their own country's programme from the very beginning. Hence, their contributions to the first edition of this book are based on years of experience and an extensive knowledge of all the technologies that have been used over the years, coupled with the latest, state-of-the-art methods.

TIF believes that this second edition of the book which is upgraded and revised to include all new advances in the field that have taken place since 2004 (when the first edition was published) will continue to provide valuable support to all laboratory professionals who are setting up a service for thalassaemia and haemoglobinopathy prevention. The first edition of this book has been considered by experts in the field as an accurate reference book, and I am confident that this updated version will continue to assist laboratory specialists in choosing and applying the most suitable methods for their population, its genetic characteristics and the financial resources available to them.

TIF is very grateful to each and every one of the authors (editors) for their hard work and commitment, and welcomes the contribution of additional eminent scientists from Netherlands to this group. We also deeply appreciate the support from all others who have contributed to the completion of what I am confident, is going to be a most useful companion of every Prevention Laboratory Service.

TIF, faithful to its mission, will proceed as it does with all its publications to translate this volume's context into as many languages as possible, thus extending its access by laboratory specialists/policy makers.

We are confident that this book, together with Vol 1 which is also in the process of revision and updating, will constitute a rich source of pooled knowledge and experience for the diagnostic laboratories providing services in the field of haemoglobin disorders. The completion of these updated versions, Vol. 1 & Vol. 2, are indeed very timely considering the tremendous focus that has been given in recent years on the prevention and diagnosis of Rare Diseases in the context of EU recommendations, and the NCD/genetic diseases in the context of WHO resolutions.

Your comments and/or suggestions on the context and format of the book are most welcome.

Dr Androulla Eleftheriou
Executive Director
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BSc, MSc, PhD, Dip Mgt

ABOUT TIF

TIF: The Thalassaemia International Federation (TIF) is a non-profit, non-governmental organisation founded in 1987 by a small group of patients and parents representing mainly National Thalassaemia Associations in Cyprus, Greece, UK, USA and Italy – countries where thalassaemia was first recognised as an important public health issue and where the first programmes for its control, including prevention and clinical management have started to be promoted and implemented.

MISSION: The development of National Control Programmes, including both components of prevention and management and the promotion of their establishment across 'affected' countries.

VISION: Establishment of equal access to quality health care for every patient with thalassaemia wherever he or she may live.

OBJECTIVES: The objectives of the Federation in addressing effectively the needs of the world thalassaemia family have since its establishment remained the same and include:

- The establishment of new and promotion of existing National Thalassaemia Patient/Parents Associations
- Encouraging, motivating and supporting studies and research for further improving prevention strategies, clinical care and for achieving the long-awaited final cure and
- Extending the knowledge and experiences gained from countries with successful control programmes to those in need

TO DATE: TIF has developed into an umbrella federation with 102 member associations, from 60 countries of the world, safeguarding the rights of patients for quality health care.

TIF since 1990 has organised 60 national/local, 6 regional workshops and 14 international conferences. TIF has an extensive range of educational material including 15 books published, translated some in more than 25 languages and distributed worldwide as free of charge service. Target audience: patients/parents, medical professionals and the community at large.

JOIN US, become a member of our world thalassaemia family

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''Knowledge is our power''
''Unity is our strength''

OTHER TIF PUBLICATIONS

All found on TIF's website, www.thalassaemia.org.cy

1. All About Thalassaemia (2010), Eleftheriou A. (Cartoon Booklet)
2. Sickle Cell Disease (2008), Inati-Khoriaty A
3. Guidelines for the Clinical Management of Thalassaemia 2nd Edition Revised (2008), Cappellini M-D, Cohen A, Eleftheriou A, Piga A, Porter J, Taher A
4. About Thalassaemia (2007), Eleftheriou A.
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8. Patients' Rights (2007), Eleftheriou A.
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11. Prevention of Thalassaemias and Other Haemoglobin Disorders Vol.1 (2003), Galanello R, Eleftheriou A, Trager-Synodinos J, Old J, Petrou M, Angastiniotis M (eds.)
12. Compliance to Iron Chelation Therapy with Desferrioxamine,
13. DVD with all publications

CHAPTER 01

BEST PRACTICE RECOMMENDATIONS

This chapter is an updated overview based on the Best Practice Guidelines published by the European Molecular Genetics Quality Network (EMQN) (web site: http//www.emqn.org/emqn.php) following a best practice meeting organised in 2002. The guidelines document has now been retired from their web site and awaits revision following a second EQMN best practice meeting planned for 2012, although copies are still available upon request at the time of writing. These recommendations will focus on best practice in laboratory methods and interpretation of results.

Haemoglobinopathies are possibly unique amongst all genetic diseases in that identification of carriers is possible (and preferable) by haematological (biochemical) tests rather than DNA analysis. Any at-risk couples can then be offered reproductive choice and avoid the birth of an affected child by undergoing prenatal diagnosis, which involves mutation characterization in the parents and subsequent fetal DNA analysis. Thus genetic services for haemoglobinopathies require close collaboration between several specialities, most notably haematology and molecular genetics (1-3).

"Screening" is distinct from "definitive" diagnosis in that the purpose of screening is to test for a defined set of conditions using simple biochemical tests. Screening programmes are designed using a protocol of first and second line methods in order to obtain a reliable diagnosis, which is essentially a presumptive diagnosis. If an unequivocal, definitive diagnosis is required, characterisation methods based on either protein or DNA analysis must be utilized.

With the thalassaemias, screening will detect most cases of ß-thalassaemia trait but there is no specific screening test for α-thalassaemia trait that often remains a diagnosis made by exclusion. If an abnormal haemoglobin is found, the results obtained constitute a presumptive identification of the haemoglobin. It is important to remember that with phenotypic screening it is possible that some rare conditions will not be detected and this has to be taken into account in the interpretation and reporting of data. For all samples, screening using haematological methods is the first step in genetic diagnosis (4).

Good laboratory practice also includes the minimization of clerical errors, particularly crucial in haematology laboratories undertaking large numbers of samples for carrier screening, sometimes numbering more than 1000 blood counts each day. Careful sample identification is essential (including: Full Name, Date of Birth, Sample date, if transfused in last 4 months). Bar coding is recommended. Laboratory error rates for methods utilized (if known) should be available to patients.

1.1 HAEMATOLOGY METHODS

1.1.1. COMPLETE BLOOD COUNT

Method: The recommended method is by electronic measurement.

Interpretation of results: All of the red cell indices (and other parameters) are important in evaluation, including Hb, RBC, MCH, MCV and RDW. Important cut-off values indicating possible heterozygosity for thalassaemia include MCV <78 fl and MCH <27 pg.

Note: Evaluation of blood count in samples >24 hours old should be made with caution, as the red cells increase in size, leading to falsely raised MCV (although different analysers have variable sensitivity to this problem).

1.1.2 HAEMOGLOBIN (Hb) PATTERN ANALYSIS

Methods include: For a presumptive identification of an abnormal haemoglobin, the methods include:

1. **Haemoglobin electrophoresis at pH 8.6 using cellulose acetate membrane.** This method will reliably detect the common haemoglobin variants, ie Hb's S, C, D-Punjab, E, O-Arab and the Lepores. HbH and Hb Bart's may also be detected if suitable run times are used. Many other variants are also detectable, eg J's, N's, Q's, Hasharon.
2. **Haemoglobin electrophoresis at pH 6.0 using acid agarose or citrate agar gel.** This method is useful for distinguishing Hb's C, E, and O-Arab from each other, also HbS and Hb D-Punjab from each other. Note that the migration patterns are different for acid agarose gels and citrate agar gel.
3. **Isoelectric focusing (IEF).** IEF is a sensitive method, giving good separation of haemoglobin variants but requires considerably more expertise for interpretation than electrophoresis since adducted fractions also separate.
4. **High Performance Liquid Chromatography (HPLC).** This method is recommended for simultaneous detection and quantitation of haemoglobin fractions. Since the systems are automated, operation of the analysers is simple, but interpretation of the chromatograms requires expertise. As a good laboratory practice, with all methods, QC should be used to monitor %A2 and %F imprecision. Like all automated methods, its cost per test is relatively high, the application is useful for large scale screening programmes. An advantage of this approach is that HPLC chromatograms for more than 300 rare alpha and beta chain variants have now been published to aid a presumed diagnosis (in a book by Barbara Bain et al [42] and in the Bio-Rad Hb Variant CD library, available from Bio-Rad).
5. **Capillary Electrophoresis (CE).** Automated capillary zone electrophoresis is a new and complementary screening technique to HPLC for the routine detection and measurement of haemoglobins and variants (5). CE patterns for abnormal haemoglobins are simple and easy to read (as long as Hb A is present in the sample), as the method does not separate haemoglobin derivatives such as glycated fractions. CE is able to detect and quantify HbH and Hb Bart's. Should be noted that two out

of three Bio-Rad HPLC methods are also able to detect and quantify HbH and Hb Bart's; if needed a software function can allow quantification with the third method.

Recommendations

1. In the presence of an abnormal haemoglobin, the use of a single test to establish presumptive identification is inappropriate and second or even third line testing procedures should be in place.
2. On most HPLC systems, derivatives of HbS may co-elute with HbA and HbA_2; thus whenever HbS is present, it is essential to run alkaline or acid electrophoresis to determine if HbA is present.
3. To quantify HbA_2 in the presence of HbS, electrophoresis and elution, or microcolumn chromatography with appropriate reagents for HbS are recommended methods, rather than HPLC (although the presence of at least 50% HbA should exclude co-existing ß-thalassaemia).
4. Quantification of HbA2 in the presence of HbC or HbE: A study has shown that HPLC will accurately quantify Hb A2 in the presence of HbC, but not in the presence of HbE or glycated Hb S. CE will accurately quantify Hb A2 in the presence of HbE or glycated HbS, but not in the presence of HbC [43]. As is the case for HbS, the presence of 50% HbA or greater should exclude a co-inherited ß-thalassaemia mutation with HbC and HbE in almost all cases.
5. Always analyse fresh blood samples if HbH disease is suspected, as HbH is unstable.

1.1.3 QUANTITATION OF HbA_2

Methods include:

1. **Hb electrophoresis with automatic densitometry** - not recommended.
2. **Electrophoresis and elution** - accurate but time-consuming.
3. **Microchromatography** - accurate but time-consuming.
4. **HPLC** - accurate in the absence of variants (see above) and high-throughput.
5. **Capillary Electrophoresis** - accurate in the absence of variants (see above) and high-throughput. Has an advantage over HPLC in that it separates HbE from HbA_2, thus providing a clean measurement of HbA_2 in patients with HbE.

Interpretation of results: The important cut-off value indicating heterozygosity for ß-thalassaemia is HbA_2 >3.5%. Borderline levels of 3.2%-3.8% (depending upon laboratory) indicate further investigation required (see Table 1.1).

Note: WHO International Reference Reagents are available for HbA_2 quantitation by electrophoresis & elution, microcolumn chromatography, and by HPLC. Every lab should establish its own normal HbA_2 ranges, in the absence or in the presence of variants, based on the results for its own population.

1.1.4 QUANTITATION OF Hb F

Methods include:

1. **Alkali denaturation** - The modification by Pembrey et al (6) has excellent reproducibility, in most ranges of HbF, giving worthwhile results in virtually all clinical situations (if used carefully).
2. **HPLC** - on some systems may be inaccurate for HbF values <1%, although for the Biorad HPLC

system, accurate quantitation of HbF can be achieved using the lytic solution for HbA_{1C}.
Capillary electrophoresis - the Sebia system can be used as an alternative to HPLC for HbF measurement, although it has been shown that it is less precise than HPLC on detecting HbF in the lower ranges [43].

Interpretation of results: Important cut-off value indicating heterozygosity for δß-thalassaemia are HbF>5% in the presence of low red cell indices and a normal HbA_2 level. However, HbF may increase up to 3% in pregnancy, making values in the range of 3-5% difficult to interpret. Values above 5% may indicate the presence of heterocellular HPFH. Values over 20% may indicate deletional HPFH. Follow-up at 6 months postnatal would clarify the individual's usual level.

Note: WHO International Reference Reagents are available for HbF quantitation using the 2-minute alkali denaturation method by Pembrey et al (6).

1.1.5 VARIATIONS OF HAEMATOLOGICAL FINDINGS NOT CONSISTENT WITH TYPICAL ß-THALASSAEMIA TRAIT

Haematology not consistent with typical ß-thalassaemia trait can be interpreted according to the following guidelines:

1. **Reduced red cell indices & normal Hb electrophoresis (including HbA_2)**
   The possible interpretation of these haematological parameters is
   - Iron deficiency
   - heterozygous α-thalassaemia
   - heterozygosity for mild ß-thalassaemia mutations (sometimes HbA_2 is borderline raised) - see Table 1.1 below.
   - co-inheritance of heterozygous δ- with ß-thalassaemia
   - heterozygous γδß-thalassaemia

2. **Normal/borderline reduced red cell indices with raised HbA_2**
   The possible interpretation of these haematological parameters is
   - Interaction of α- with ß-thalassaemia
   - HIV drug therapy
   - Hyperthyroidism
   - KLF1 gene mutations

3. **Normal or reduced red cell indices with raised HbF (and normal HbA_2).**
   The possible interpretation of these haematological parameters is
   - Heterozygous δß-thalassaemia or HPFH.

Note 1: Multiple a-gblobin gene alleles (triplicated, or rarely, quadruple), or mild ß-thalassaemia mutation maybe associated normal haematological findings (normal red cell indices with a normal Hb A_2).
Note 2: Some Hb variants are not detected by electrophoretic or chromatographic procedures, but may be suspected due to the presence of abnormal haematological parameters and/or clinical

symptoms. In such cases it is recommended that samples are analysed by mass spectrometry or DNA methods. Occasionally hyperunstable variants are present and these may only be found by DNA analysis as the protein produced is so unstable.

Table 1.1 Genotypes associated with borderline HbA_2 levels - a guideline of related haematological and biosynthetic characteristics.

GENOTYPE	MCV (fl)	MCH (pg)	HbA_2 (%)	α/ß ratio
ß -101 (C→T)	88.5 ± 7.8	30.1 ± 1.0	3.1 ± 1.0	1.3 ± 0.04
ß -92 (C→T)	83.0 ± 6.0	28.3 ± 2.0	3.5 ± 0.4	1.3 ± 0.08
ß +33 (C→G)	82.0 ± 9.2	27.1 ± 3.4	2.5 ± 1.4	1.3 ± 0.06
ß CAP+1 (A→C)	77.5 ± 2.5	24.5 ± 1.5	3.5 ± 0.3	-
ß IVSI-6 (T→C)	71.0 ± 4.0	23.1 ± 2.2	3.4 ± 0.2	1.9 ± 0.05
ß IVSII-844 (C→G)	96.0 ± 4.0	30.3 ± 1.8	3.2 ± 0.2	1.0 ± 0.06
ß +1480 (C→G)	88.3 ± 9.5	27.9 ± 6.0	2.7 ± 0.8	1.6 ± 0.04
ααα/αα	85.5 ± 7.8	30.4 ± 5.0	2.8 ± 0.6	1.2 ± 0.04
δ- & ß-thalassaemia	67.6 ± 7.6	21.8 ± 3.6	3.3 ± 0.4	1.7 ± 0.06
KLF1 mutations*	84.0 ± 6.6	28.2 ± 2.4	3.6 ± 0.3	0.9 ± 0.1

Values (mean ±2 SD) are a guideline and represent those reported in various studies on carriers of these mutations (prepared by R. Galanello). *Perseu et al, Blood 118, 4454-8. 2011

Other ß-thalassaemia mutations reported to have a borderline HbA_2 /silent phenotype are: -102 (C→A), -101 (C→G), CAP+8 C→T), CAP+10 (-T), CAP+33 (C→G), CAP+45 (C→G), IVSII-844 (C→A), Poly A (AATAAA→CATAAA), 5'UTR +10 (-T), 3'UTR +6 (C→G).

Note: Subjects with borderline HbA_2 levels, including spouses of a typical ß-thalassaemia carrier, should be extensively investigated (α and ß-gene analysis, globin biosynthesis). Although most usually have normal ß and α-globin genes, some will be ß-thalassaemia carriers. Borderline HbA_2 levels in many normal individuals may be explained as the extreme distribution of the normal range of the HbA_2 or possibly associated with artefacts of the analysis technologies.

1.2 SUPPLEMENTARY HAEMATOLOGICAL METHODS

1.2.1 IRON (Fe) STATUS DETERMINATION

Methods include:

1. **Zinc protoporphyrin (ZnPP)** - sample can be analysed from same tube as blood count, and sample is stable for long time period. Analysis is fast, simple and cheap, although it requires specific instrument. ZnPP is elevated in iron deficiency, but may be falsely high in lead intoxication or if the

bilirubin levels are raised.
2. **Ferritin** - most popular test for indicating iron deficiency, but it is expensive and may be falsely high during infection, liver disease or neoplasia.
3. **Transferrin saturation (Iron/Total Iron Binding Capacity)** - more accurate than ferritin but there is no internationally recognized standard protocol.

Interpretation of results: Measurement of iron status in samples with hypochromic, microcytic indices and normal HbA_2 and HbF values is useful to distinguish between cases of uncomplicated iron deficiency and those with possible α-thalassaemia trait or silent ß-thalassaemia trait in whom the iron status is normal. This is a useful approach not only to prevent unnecessary further investigation but in some cases inappropriate iron therapy. However, it is important to note that iron deficiency can co-exist with the thalassaemias, and such cases could be misinterpreted. It is sometimes necessary to recommend repeating the haematology screen after correction of iron deficiency (assuming that there is no time limit with an on-going pregnancy).

1.2.2 FUNCTIONAL TESTS FOR Hb VARIANTS

Methods include:
1. **Sickle tests** - if there is an abnormal fraction that runs in the position of HbS, then the sickle solubility test should be undertaken. Note: Some other (rare) haemoglobins also have reduced solubility and thus have a positive solubility test but do not migrate to the same position as HbS.
2. **Heinz body formation** - not very specific, but useful for detecting presence of unstable variants.
3. **Oxygen dissociation curve** - maybe useful for implicating presence of Hb variants with altered oxygen affinity.

1.3 GLOBIN CHAIN SYNTHESIS

This technique may provide useful information for diagnosing atypical cases.

Methods include:
1. **Carboxymethyl cellulose (CMC) chromatography method** - The original method for evaluating the relative rate of globin chains synthesised in reticulocytes, which can be very accurate, but is time consuming and has a low throughput. The method for carboxymethy cellulose chromatography is detailed in Chapter 4.
2. **Reverse phase high performance liquid chromatography (HPLC) and isoelectric focusing (IEF)**. These methods require specialist equipment and are now not widely used (7).

1.4 MOLECULAR DIAGNOSIS

Almost all methods for DNA analysis of haemoglobinopathies currently in use are based on the polymerase chain reaction. There are now many different PCR-based techniques that can be used

to detect the globin gene mutations, including dot blot analysis, reverse dot blot analysis, the amplification refractory mutation system (ARMS), restriction endonuclease analysis, gap-PCR and multiplex ligation-dependent probe amplification (MLPA). All are recommended for use as best practice, each method having its own advantages and disadvantages (Chapter 5). The particular methods chosen by a laboratory for the diagnosis of the globin gene point mutations or deletions 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 (population groups) being tested. It is best practice for any DNA diagnostic laboratory to have at least two alternative methods for detecting each mutation.

It is also best practice to use the same method on two independent DNA samples to confirm the presence of a causative mutation, preferably at different DNA concentrations in order to control the potential problem of allele drop out. Similarly, DNA sequencing should be done on both the plus and minus strands to confirm the presence of a mutation. Finally it is best practice to have an additional 'check' on the molecular diagnostic results by looking at the haematology, so that any additional interacting genetic factors are considered.

The haemoglobinopathies are mostly regionally specific, with each population having a unique combination of abnormal haemoglobins and thalassaemia disorders. The spectrum of mutations and their gene frequencies have been published for most populations, usually consisting of a limited number of common mutations and a slightly larger number of rare mutations (8). Therefore knowledge of the ethnic origin of a patient simplifies the diagnostic strategy, enabling a quick identification of the underlying defects in most cases. A list of mutations and their relative gene frequencies are presented in Annexe 2 of Volume 1.

1.4.1 α-THALASSAEMIA

Molecular diagnosis of nearly all known inherited α-thalassaemia mutations can be carried out by a combination of three different techniques: gap-PCR for the diagnosis of the common deletion mutations, multiplex ligation-dependent probe amplification (MLPA) for rare and novel α^0-thalassaemia deletions, and DNA sequencing for non-deletion α^+-thalassaemia mutations.

Gap-PCR (amplification across the breakpoints of a deletion) provides a quick diagnostic test for α^+-thalassaemia and α^0-thalassaemia deletion mutations but requires careful application for prenatal diagnosis, since the method may be susceptible to false negative results caused by allele drop out (9,10). The five commonest α^0-thalassaemia deletions can be diagnosed by gap-PCR: 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 commonest α^+-thalassaemia deletions are also diagnosed by gap-PCR: the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ alleles. The former is found in African, Mediterranean, Asian and Southeast Asian populations, while the latter is found in Southeast Asia and the Pacific populations.

The technique of MLPA analysis has now replaced the classical method of Southern blot analysis for the detection of α-thalassaemia deletion mutations [11]. MLPA detects all the common, rare

and novel forms of deletional α-thalassaemia (in contrast to gap-PCR) and provides a reliable alternative screening method for the prenatal diagnosis of α^0-thalassaemia [12]. The technique will also identify gene rearrangements which lead to the duplication of the alpha globin genes in the form of triple and quadruple α–gene alleles [13]. However, unlike gap-PCR, MLPA does not provide a definitive diagnosis of a particular deletion mutation, only a result that is consistent with a known deletion.

Non-deletion α^+-thalassaemia mutations are screened for by the technique of selective amplification of each α-globin gene followed by DNA sequence analysis (14). For the detection of known mutations, pyrosequencing provides a quick and cheap alternative to Sanger sequencing (15). Other methods developed for the diagnosis of selected non-deletion mutations include reverse dot blotting and the amplification refractory mutation system (ARMS) (16,17,18). Several non deletion mutations alter a restriction enzyme site and may be diagnosed by selective amplification and restriction endonuclease analysis, eg the mutation for Hb Constant Spring in Asians, or the ATG ACG α2-gene mutation and the IVSI donor site (–5bp) deletion in Mediterraneans (19).

1.4.2 ß-THALASSAEMIA

A limited number of ß-thalassaemia mutations are prevalent in most of the populations at risk for severe thalassaemia and in practice this permits the most appropriate probes or primers to be selected according to the carrier's ethnic origin. The most commonly used screening procedures for known mutations are the reverse dot blot analysis with allele specific oligonucleotide probes (20), and primer specific amplification (ARMS) (21). Restriction enzyme analysis of amplified ß-gene product is useful for a limited number of mutations (22). Pyrosequencing has also been developed for the diagnosis of known mutations. However the cost of direct DNA sequencing has now reduced sufficiently for many laboratories to switch to Sanger sequencing as the primary method of screening for both known and unknown ß-thalassaemia point mutations.

When a ß-thalassaemia mutation cannot be defined by one of the direct detection methods, characterization of the mutation may be done by using denaturing gradient gel electrophoresis (DGGE) (23) or single-¬strand conformation polymorphism (SSCP) analysis (24) to localize possible mutations within the ß-globin gene, followed by direct sequencing on amplified single strand DNA either manually or automatically (25). DGGE is also useful for directing mutation identification when using mutation specific assays such as ARMS or RE-PCR, since most mutations have characteristic heteroduplex patterns with DGGE analysis. However the gold standard method for the identification of unknown mutations is DNA sequencing and is thus the primary method of most laboratories, although it must be emphasised that there are pitfalls with this method (26).

Small nucleotide deletions resulting in $ß^0$-thalassaemia and some of the larger deletions that remove the whole of the ß-globin gene may be identified by gap-PCR (including Hb Lepore, some δß-thalassaemia deletions and the HPFH1/2/3 deletion mutations) (27). However all of the larger ß-globin gene deletions can be detected by MLPA (11) and this approach has quickly become the most useful screening technique for the larger ß- and δß-thalassaemia deletion mutations found within a population (28).

1.4.3 COMMON Hb VARIANTS

The clinically important variants, namely HbS, HbC, HbE, Hb D-Punjab and Hb O-Arab, can be diagnosed by the methods of dot blot hybridisation, the ARMS technique or direct sequencing. All except HbC can also be diagnosed by restriction endonuclease digestion of amplified ß-gene product (RE-PCR). For the many other haemoglobin variants, positive identification at the DNA level is achieved by selective globin gene amplification and DNA sequence analysis (Chapter 5) or by mass spectrometry (29).

1.5 FETAL DNA ANALYSIS

It is best practice for all couples undergoing prenatal diagnosis to be counselled by a qualified health professional well versed in the molecular diversity of the haemoglobinopathies. No woman should undergo prenatal diagnosis unless she has been counselled by a qualified health professional. Problems related to PCR-based prenatal diagnosis include the high sensitivity to maternal DNA contamination and the complex battery of probes and primers necessary to detect a wide range of thalassaemia mutations. The following procedures are intended to minimise the diagnostic error rate.

1.5.1 PARENTAL BLOOD SAMPLES

Copies of haematology results should be sent to molecular diagnostic laboratory. Blood samples should be obtained from both parents to confirm phenotype of parents by full blood count and haemoglobinopathy screen such as electrophoresis and as source of control DNA for the molecular analysis. This should be repeated with every prenatal diagnosis that any couple undergoes.

1.5.2 PARTNER NOT AVAILABLE FOR TESTING

There are cases where a carrier woman requests prenatal diagnosis although her partner is unavailable for testing. In such situations it is important to evaluate risk of a major haemoglobinopathy in the fetus:

a. For a sickle cell trait mother and untested partner. If an AS genotype is diagnosed in fetus then test for common beta thalassaemia mutations and any other haemoglobinopathy genes (especially $ß^C$ or $ß^D$) known to exist in the partner's ethnic group. Alternatively, the method more commonly used, is fetal DNA sequencing, to detect any rare mutations present.

b. For a ß-thalassaemia trait mother and untested partner. If the mother's ß-thalassaemia mutation is diagnosed in fetus, the possibility of the fetus being homozygous or compound heterozygous for ß-thalassaemia should be excluded, either by testing for the ß-thalassaemia mutations and any other ß-gene haemoglobinopahies found in the fathers ethnic group. Alternatively, the method more commonly used, is fetal DNA sequencing to detect any rare mutations present.

1.5.3 FETAL SAMPLING

There are three possible procedures, chorionic villus sampling, amniocentesis and fetal blood sampling. Prenatal diagnosis of haemoglobinopathies should preferably be carried out by a chorionic villus sample in the first trimester of pregnancy (10-12 weeks).

Methods:

1. **Chorionic Villus Sampling.** Provides good source of DNA. Risk of maternal contamination is low with careful microscopic dissection to remove contaminating maternal decidua. There is a risk of maternal contamination if sample is cultured, although this should not be necessary if sample is of adequate size. Risk of miscarriage is low if sample taken in experienced centre. Result is available early in pregnancy.

2. **Amniocentesis.** Amniocytes can be used for molecular analysis directly spun down from the amniocentesis sample. This usually yields sufficient DNA for analysis with PCR-based methods. For greater amounts of fetal DNA, samples have to be cultured for 10-14 days. Culture of the cells reduces risk of maternal contamination, but result is delayed. Risk of miscarriage following amniocentesis is low if sample taken in experienced centre. Result is available later in pregnancy as amniocentesis cannot be taken earlier than about the 16th week.

   Note: Direct analysis should be carried out with caution as the fetal cells are invariably contaminated with maternal cells.

3. **Fetal Blood Sampling.** The analysis of fetal blood by globin chain biosynthesis was widely used before the development of PCR-based molecular diagnostic techniques. 1-2 ml of fetal blood is usually obtained, which can be used for molecular analysis or, very rarely today, globin chain biosynthesis studies. The latter was used in the past when parental mutations were not known, when a couple presented late for prenatal diagnosis, or when partner was unavailable for testing. The diagnosis is based on the relative synthesis of ß-globin (representing HbA) and γ-globin (representing HbF). A ß/γ chain synthesis ratio above 0.02-0.03 (slightly variable between laboratories) indicates an unaffected fetus.

   Note: When using this technique results should be interpreted with care as mild $ß^+$ mutations can produce higher levels of ß-globin, leading to risk of misdiagnosis (30).

Recommendations

- Prenatal diagnosis using globin chain biosynthesis in fetal blood is no longer recommended by most centres. The approach is associated with a higher rate of miscarriage and is carried out late in pregnancy (after 18-20 weeks).
- Chorionic villi sampling is the recommended approach for fetal DNA diagnosis.
- However fetal blood sampling has been used in some centres when alpha thalassaemia hydrops fetalis is suspected by ultrasound examination in the fetus. The advantage is that the sample may be tested by HPLC or similar quick method for the presence of HbF, with a result available within 30 min.

1.5.4 GENOTYPE ANALYSIS

Recommendations

• The laboratory carrying out the molecular analysis should choose the technique(s) that best suits their laboratory, expertise and population.
• Always analyse parental and the appropriate control DNA's simultaneously with the fetal DNA and use a blank control sample.
• Perform duplicate tests to minimize human errors.
• To monitor potential laboratory errors such as partial digestion or allele drop use two independent diagnostic methods on each sample for each mutation being investigated.
• Use a limited number of amplification cycles to minimise co-amplification of any maternal DNA.

1.5.5 MATERNAL DNA CONTAMINATION

Recommendations

• Use polymorphism analysis to exclude maternal contamination (and may also identify non-paternity).
• Check for maternal DNA contamination in every case, especially when the fetal genotype is the same as the mother's genotype. The choice of polymorphic markers available is wide, including Short Tandem Repeat (STR) markers such as D21S11, D21S1414, D18S535 (31). Commercial kits are also now available (eg. ABI AmpF lSTR Identifiler Kit) that tests for up to 16 STR's. Alternatevely use Variable Number Tandem Repeat (VNTR's) markers such as ApoB, IgJH and Has-ras (32). Note that STR kits require sizing on an automatic DNA sequencer, whereas VNTRs can be sized by agarose gel electrophoresis.
• When the fetal genotype is the same as the mother's, and the VNTR/STR markers do not exclude maternal contamination, the fetal diagnosis report should state these findings and indicate greater risk of error in the fetal result. It is strongly recommended that another fresh fetal sample or a cultured sample should be requested for analysis in these cases.

1.5.6 PATIENT CONSENT AND REPORTS

There should be a consent form signed by patient and counsellor accompanying the fetal sample. The fetal DNA report should detail types of DNA analysis performed and clearly state the risk of misdiagnosis based on reported technical errors of the protocols utilized. Laboratory error rates should be documented and explained to patients for all methods.

1.5.7 PRENATAL DIAGNOSIS FOLLOW-UP

Ideally, a fetal DNA diagnosis should be confirmed at birth through a request for a cord blood sample that can be sent out with fetal diagnosis report. Haematological, haemoglobin and DNA analysis also requested by some centres. Ideally, fetal material should be requested when affected pregnancies are terminated to confirm prenatal diagnosis result.

1.6 AUDIT

National registers should exist to audit services for prenatal diagnosis. In the UK the three diagnostic laboratories enter data for each diagnosis onto a shared register and aggregated data can be used for national audit of antenatal carrier screening and utilisation of prenatal diagnosis by risk, ethnic group and region (33). It can also be used to report on the accuracy of prenatal diagnosis (34).

1.7 BEST PRACTICE RECOMMENDATIONS FOR PGD

PGD is now an established reproductive alternative for couples with a high-risk of transmitting inherited disorders such as thalassaemia or sickle-cell syndromes. The selection of embryos for transfer supports initiation of pregnancies unaffected for the disease, thus precluding the need of pregnancy termination following an affected diagnosis with conventional prenatal diagnosis (see Volume 1, Chapter 8,). However, PGD is still relatively unregulated and lacks standardization compared to other forms of diagnostic testing, although, this is changing and an EQA scheme for PGD is now running (35). PCR-based protocols for PGD are subject to the limitations of single-cell PCR, including total PCR failure, failure to detect both alleles (allelic drop-out, or ADO), and sample contamination, the latter potentially occurring during any stage of the PGD procedure (assisted reproductive treatment, embryology or genetic analysis). PCR failure, although undesirable, will not lead to an unacceptable misdiagnosis. On the other hand, ADO and contamination may lead to serious misdiagnosis. Thus, compared to more routine genetic testing, there is a comparative difficulty in achieving the highest levels of accuracy and reliability when analysing single cells as part of PGD. In this context quality assurance is of fundamental importance when offering PGD, and to support this it is advisable to follow best practice recommendations.

In 2005, the European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium published a set of Guidelines for Best Practice in PGD to give information, support, and guidance to potential, existing and fledgling PGD programs (36). The rapidly changing nature of PGD, specifically the technologies associated with its use, along with increasing patient access and demand, has necessitated review and revision of the original ESHRE PGD Consortium guidelines (36). As a result, the ESHRE PGD Consortium has prepared several guidelines, two of which are relevant to the application of PGD for haemoglobinopathies. The first relates to the requirements when organizing a PGD Centre and the other relates to PGD protocols based on DNA amplification, which should be followed in conjunction.

The guidelines directing the organisation of PGD Centres (37) contain information on personnel, the inclusion and exclusion criteria of couples requesting PGD, all aspects of genetic counselling and informed consent relevant to assisted reproductive treatment (ART) and the genetic diagnosis of the PGD itself, how an IVF centre should be set up for PGD, the prerequisites for transport PGD (when the ART and subsequent PGS diagnosis is performed different locations and by independent teams of specialists, requiring "transport" of embryo-biopsy samples), quality assurance and quality control (QA/QC) and finally the process of accreditation for PGD centres (35).

In the best practice guidelines for PGD protocols based on DNA amplification (38), the topics covered include the general uses of amplification-based PGD testing, and all laboratory aspects relating to amplification-based testing, pre-examination validation, examination process, and post examination process. The PGD International Society (PGDIS) has also drafted guidelines which were recently updated and are more in-depth than the ASRM (39). Of course, owing to variations in local or national regulations and specific laboratory practices, there will remain differences in the ways in which PGD is practiced (from initial referral through IVF treatment, single cell analysis to follow-up of pregnancies, births and children). In addition all PGD treatment must also take into consideration the unique needs of individual patients according to professional judgement.

Although PGD is an established alternative to conventional prenatal diagnosis, specific methodologies to PGD will not be described in this volume because there are too many different methodologies in use due to the wide variation of genotypes to be addressed (40,41).

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CHAPTER 02

HAEMATOLOGICAL METHODS

Haematological methods contribute the basis of identification of carrier of the thalassaemias. Red cell indices and morphology, HbA_2 quantification and Hb fraction separation are all used. No one technique by itself is sufficient to prove the carrier status. This requires a combination of tests that must be evaluated together. A flow chart for thalassaemia is described in Volume1, chapter 4. In this chapter haematological techniques are described which assist in the diagnosis prior to the use of more definitive procedures such as the haemoglobin pattern analyses and the molecular methods. These are methods often used as economical screening methods or as elements in the description of the carrier status.

2.1. RED BLOOD CELL INDICES

Principle: Red blood cell (RBC) indices determination is the most common laboratory test and is usually carried out by automated electronic cell counters. These counters produce many parameters of which only a few, such as the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and haemoglobin (Hb) concentration, are strictly relevant and useful for haemoglobinopathies screening. The principles on which the different cell counters are based are beyond the aims of this book and are available from the manufacturers. As a general rule the preference should be for counters that directly measure the mean corpuscular volume. The counter needs to be calibrated daily with appropriate material to obtain accurate results.

Interpretation of the results: MCV and MCH are variably reduced in thalassaemia carriers. MCH is more reliable than MCV, since the MCV does not remain stable due to a tendency for the red cells to increase in size over time. The MCH is derived from the calculation: (MCH = Hb/RBC number).

The value at which the normal range for a parameter changes to an abnormal range is called the cut-off value. The most widely used cut-off values of MCV and MCH for indicating thalassaemia are 79 fl and 27 pg, respectively. Values below these may indicate α- or ß-thalassaemia or iron deficiency and other methods are needed to make a precise diagnosis. For ß-thalassaemia, the degree of microcytosis and hypochromia as reflected by the lower than normal MCV and MCH is related to the severity of the underlying mutation which determines the production of ß–globin chains. In general mild ß-thalassaemia mutations cause less microcytosis and hypochromia than ß° and

severe ß+ mutations, although there is some overlap (1,2). Carriers of silent ß-thalassaemia mutations have normal or only slightly reduced MCV and MCH values. However carriers for both ß and α-thalassaemia may have normal MCV and MCH values because of less globin chain imbalance (3). α-Thalassaemia carriers have lower than normal MCV and MCH values. α+-Thalassaemia (-α/αα) carriers may have normal or reduced MCV and MCH values, while in -α/-α and --/αα carriers the MCV and MCH are always reduced (4). δß-Thalassaemia carriers have slightly reduced MCV and MCH values. Note that an individual's iron status should always be taken into account when evaluating MCH and MCV values for thalassaemia screening.

2.2. RED BLOOD CELL MORPHOLOGY

Principle: Morphological changes of red cells can be detected in most thalassaemia carriers. An examination of a stained peripheral blood smear may be helpful in the evaluation of cases.

Preparation of smears: EDTA anticoagulated venous whole blood or capillary blood is used to prepare a blood film. After drying at room temperature the blood film can be stained with May-Grumwald (or Wright stain).

Reagents:

a. May-Grunwald's stain powder (0.3 g)
b. Methyl alcohol (100 ml)
   The staining solution is made up by swirling to mix, then warming in a 56ºC water bath for 15 min. Allow to cool to room temperature with occasional shaking, then filter after standing for 24 hrs.
c. Giemsa's stain powder (1 g)
d. Glycerol (66 ml)
e. Methyl alcohol (66 ml)
   Staining solution is made up by adding the stain to the glycerol, then warming in a 56ºC water bath for 90-120 min. Allow to cool to room temperature with occasional shaking, then filter after standing for 24 hrs.
f. Phosphate buffer 0.066 M pH 6.8 (Sorensen's):
   KH_2PO_4: 9.1 g
   Na_2HPO_4: 9.5 g (or $Na_2HPO_4.2H_2O$: 14.2 g)
   Dissolve in 1 litre distilled H_2O to make.066 M buffer of pH 6.8 .
   Add 50 ml of phosphate buffer 0.066 M pH 6.8 to each litre of water used to dilute stains and wash films.

Note: Ready staining solutions are commercially available.

Method:

1. Smear small drop of blood on slide using a spreader slide at an angle of 45º.
2. After drying in air, fix film in a jar of methanol for 15 min.
3. Immerse in a jar of May-Grunwald's stain freshly diluted with an equal part of buffered distilled water for 5 min.

4. Transfer to a jar of Giemsa's stain freshly diluted with nine parts of buffered distilled water for 15 min.
5. Transfer to a jar of buffered distilled water, wash with 3 to 4 changes of buffered water and let stand for 5 –12 min to differentiate.
6. Stand slide upright to dry.
7. After drying examine by microscope under immersion oil.

Interpretation: Red cell morphology is illustrated in Figure 2.1.1. Microcytosis, hypochromia and anisopoikilocytosis (variation in the size and shape of the red cells) are the most typical changes in thalassaemia. Other less common findings are basophilic stippling and the presence of some target cells. A high percentage of target cells are found in HbC syndromes. Nucleated red blood cells are indicative of bone marrow hyperactivity and can be found in homozygous ß-thalassaemia. Polychromasia (blue-grey and slightly bigger red cells) is associated with the presence of reticulocytosis. Howell-Jolly bodies (fragments of nuclear DNA) can be found after splenectomy or in the functional asplenic condition in sickle-cell syndromes, where sickle shaped cells are sometimes seen on the stained film as well.

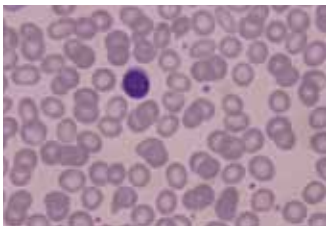

FIG. 2.1.1
Normal Red cell morphology

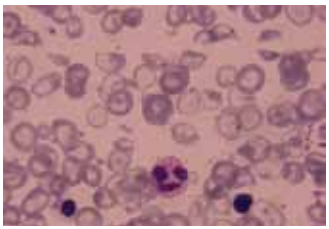

FIG. 2.1.2
Red cell morphology in homozygous ß-thalassaemia

2.3. RETICULOCYTE AND HbH INCLUSION BODY DETECTION

Principle: In thalassaemia carrier screening reticulocyte count does not have a diagnostic value. However in the detection of α-thalassaemia, especially HbH disease, the brilliant cresyl blue stain will detect the characteristic HbH inclusion bodies. Supravital stains (brilliant cresyl blue or new-methylene blue) are able to stain residual mRNA in immature red blood cells. There are now several automated electronic cell counters able to perform a reticulocyte count using specific RNA staining.

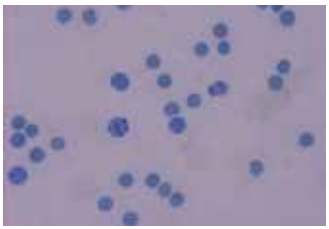

FIG. 2.2
Staining for reticulocytes

Sample: Capillary or EDTA anticoagulated blood.

Reagents

a. Brilliant cresyl blue (BCB)
b. 3% trisodium citrate (3 g in 100 ml of distilled water)
c. 0.9 % NaCl

d. Staining solution is made up using: 1 g BCB, 20 ml 3%Tri-sodium citrate and 80 ml 0.9% NaCl. Dissolve by stirring for 20 minutes, then filter.

Method:

1. Add in a tube containing 2-3 drops of staining solution, 2 or 3 drops of blood.
2. Mix and leave at 37°C for 15-20 min.
3. Make smears, and after drying examine by microscope under immersion oil (Figure 2.2).
4. Reticulocytes (red cells containing purple inclusions or filaments) as a percentage of a total of 500-1000 red cells should be counted.
   The absolute number is derived as follows:

$$\frac{\%\ \text{reticulocytes} \times \text{RBC number/l}}{100}$$

Normal values:
% Reticulocytes = 0.0-2.0%
Absolute number = 20-100 x 109/l

Interpretation of the results: Reticulocyte number is useful in the evaluation of erythropoiesis and is significantly increased when haemolysis (or bone marrow regeneration) is present.

2.4 HEINZ INCLUSION BODIES

Principle: Under appropriate staining conditions intra-erythrocytic inclusions can be visualized. These inclusion bodies (Heinz bodies) are intracellular haemoglobin precipitates detected by supravital stains. These may be found in some forms of thalassaemia (mainly α-thalassaemia) and in unstable haemoglobin disorders.

Reagents and method:

1. BCB staining as described for reticulocytes.
2. Methylviolet stain (0.5 g in 100 ml of 0.9% saline) can be also used.
3. Dry blood films are made after 30 min, 1h and 3h, and examined by microscope under immersion oil.

Interpretation: Heinz bodies appear as simple or more commonly multiple inclusions (Figure 2.3). They are found in variable percentages (5-50%) in HbH disease, in unstable haemoglobins and sometimes in very low number (1:1000-10.000 RBC) in -α/-α and - -/αα carriers. In these carrier states they are rare and so in most cases it is time consuming to attempt confirmation of the presence of α^0-thalassaemia through inclusion body detection. Absence of inclusions does not exclude α-thalassaemia.

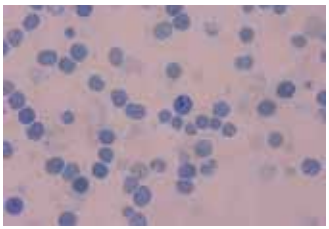

FIG. 2.3
HbH blood smear showing Heinz bodies

2.5 OSMOTIC FRAGILITY TEST

Osmotic fragility test (OFT) was the first method used for screening of thalassaemia and was introduced as a simple approach to detect thalassaemia carriers by Silvestroni and Bianco in the 1940s. This fast and simple method has been applied as a screening test in large populations. The availability of electronic counters for the measurement of MCV and MCH has decreased the use of OFT. It is still used in low resource countries to screen large rural or tribal populations. Several variations of the basic method have been proposed. The most used test at present is NESTROFT, the acronym for Naked Eye Single Tube Redcell Osmotic Fragility Test (5-7).

Principle: Microcytic red blood cells are resistant to lysis when exposed to hypotonic solutions.

Sample: Blood in EDTA.

Reagents:

a. 0.36 % buffered saline: Dilute a 10% stock solution of sodium chloride (90 g), disodium hydrogen phosphate (13.65 g) and sodium dihydrogen phosphate (2.43 g) in 1000 ml of distilled water (pH 7.4). The original method (kindly given by Prof. Ida Bianco) uses the Tyrode's solution, diluted 4:10 with distilled water.

b. Tyrode's solution (1 Litre):

NaCl	8.2 g
KCl	0.2 g
$MgCl_2.6H_2O$	0.2 g
$CaCl_2.2H_2O$	0.2 g
$NaH_2PO_4.H_2O$	0.1 g
$NaHCO_3$	0.05 g

Tyrode's solution should be stored at 4°C and the work solution prepared few hours before use.

Method:

1. 20 µl of whole blood collected in EDTA is pipetted into a glass test tube (100 x 10 mm) containing 4 ml of 0.36% buffered saline solution.
2. Shake the tube and leave at room temperature for 30 minutes.
3. Shake again the tube and immediately hold the tube in front of a piece of paper with text.

Interpretation: If the words on the paper are clearly visible through the tube, the test is negative; whereas if the words are not clearly visible the test is positive (thalassaemia trait) due to the turbidity of the solution.

Comments: The method is easy to perform, fast, cheap and does not require sophisticated equipment. However, it needs careful standardization. It is particularly useful in places where the electronic cell counters are not available. The test is positive both in ß- and in α-thalassaemia carriers, in sickle cell trait and iron deficiency anaemia. False positive results are obtained in patients with

iron deficiency and therefore subjects positive with NESTROFT need further investigation to define the diagnosis. False negative results have also been reported (8).

2.6 HB STABILITY TEST

Disruption of the normal structure of the haemoglobin molecule can result in reduced stability, which leads to precipitates in the erythrocyte causing its destruction. The clinical consequence is often a haemolytic anaemia of variable severity.

Amino acid substitutions in the globin chains, particularly those involving the non-polar amino acids that constitute the haem pocket, may result in an unstable haemoglobin. In general the amino acid substitution also produces a modification of electrophoretic mobility. However, if the substituted amino acid is internal or the total charge of the molecule is unchanged, the haemoglobin variant will not be detected by conventional electrophoresis. Therefore, if an unstable haemoglobin is suspected clinically, a specific test for unstable haemoglobin should be performed even if the electrophoretic pattern is normal. Milder instability may also be associated with perturbations of bonding to the haem group (Hbs) or between subunits (eg. HbH or variants with altered oxygen affinity). There are two stability tests: isopropanol test and heat test.

2.6.1 ISOPROPANOL TEST

Principle: The presence of isopropanol makes the buffer less polar, weakening the haemoglobin hydrophobic bindings that facilitate its denaturation and precipitation (9).

Sample

a. Wash 1 ml of fresh blood (in any anticoagulant) twice in 0.9% NaCl. The packed cells are lysed by the addition of one or two volumes of distilled water. Mix gently and wait few minutes. Centrifugation to remove the erythrocyte ghosts is not necessary. For some Hb variants early precipitation and binding to the red cell membrane may take place.
b. Therefore the use of organic solvents, such as toluene or carbon tetrachloride, which dissolve the red cell membrane should be avoided.
c. Prepare a fresh normal sample in the same conditions as a control.
It is advisable to test the reagents by using a positive control (eg a HbH blood sample).

Reagents

a. Tris-HCl 0.1 M, pH 7.4:
(i) 1.21 g tris-hydroxymetyl-amino-methan
(ii) 100 ml distilled H_2O
Bring to pH 7.4 with 4 M HCl
b. Tris-isopropanol buffer:
(i) Tris-HCl 0.1 M, pH 7.4 solution: 83 ml
(ii) Isopropanol: 17 ml
Keep stoppered at room temperature

Method

1. Two small stoppered tubes (one for the sample the other for the control) each containing 2 ml of tris-isopropanol buffer and 0.2 ml of haemolysate are mixed by inversion and placed at 37°C in water bath.
2. Check for the presence of flocculent precipitate at 5, 20 and 30 minutes.

Interpretation: Flocculent precipitate indicates the presence of an unstable haemoglobin. The control solution should remain clear.

Comments: The presence of HbF levels higher than 3% may give false positive results for its mild instability. Storage of the blood sample for several days may results in methaemoglobin formation and possible false positive results. The hemolysate should be fresh prepared. False negative results may be obtained adding KCN to the lysate.

2.6.2 HEAT TEST

Principle: Normal haemoglobin precipitates only in a very minimal amount at 50°C for 30 minutes, while unstable haemoglobins in these conditions are completely denatured.

Sample:

a. Fresh blood sample in any anticoagulant.
b. A control blood sample must always be tested at the same time.

Reagent and solutions:

a. Phosphate buffer 0.01 M pH 7.4:
   (i) Solution A: $NaH_2PO_4.2H_2O$: 15.6 g in 1 litre distilled H_2O.
   (ii) Solution B: Na_2HPO_4: 14.2 g in 1 litre distilled H_2O.
b. Add 19.2 ml of solution A to 80.8 ml of solution B.
c. Mix and leave for 10 minutes at room temperature. Check pH.

Method:

1. Lyse as previously described (see isopropanol test).
2. Transfer 5 ml of the lysate and 5 ml of phosphate buffer pH 7.4 to test tube.
3. Mix and centrifuge at 3000 rpm for 10 minutes.
4. Transfer 2ml of supernatant to a glass test tube, which should then be heated at 50°C for 30 to 60 minutes.

Interpretation: A precipitate easily visible to the naked eye will be formed if a heat-unstable haemoglobin is present. The control sample should remain clear.

Comments: Slight precipitation is of doubtful significance and, if present, the test should be repeated. The presence of HbF levels higher than 3% may give false-positive or doubtful results.

2.7 HbF QUANTITATION BY ALKALI DENATURATION

Fetal haemoglobin is the prevalent haemoglobin type in the newborn (about 60-80%) then progressively decreases to very low levels, of less then 1%, by the second year of life. The decline is slower in ß-thalassaemia carriers.

HbF levels may be increased in adults in a number of inherited (δß-thalassaemia, deletional and non-deletional HPFH, some ß-thalassaemia mutations) and acquired conditions, including pregnancy, recovery after bone marrow transplantation and aplastic anaemia, myelodisplastic syndromes, juvenile chronic myeloid leukaemia. The HbF is restricted to a sub-population of erythrocytes termed "F-cells".

Detection and quantification of increased levels of HbF are important for genetic counselling and prognosis in ß-thalassaemia and sickle cell anaemia. An increased level of HbF may be associated with a milder phenotype.

Since the range of HbF may be from 1% to 95%, no single method is accurate for its quantitation over the whole range. High performance liquid chromatography (HPLC), gives the best estimation of HbF over the whole range (10,11). However other methods are available for HbF determination, such as alkali denaturation, immunological techniques (by immunodifusion, ELISA and immunoradiometric), IEF, or column chromatography.

Principle: HbF is relatively resistant to denaturation by alkalis as compared to HbA. The method described (12) gives reliable results in the range of 0.8 to 25%, which are the levels most commonly found in carriers of δß-thalassaemia and of most of the forms of HPFH.

Sample:

a. Blood in any anticoagulant may be used.
b. Wash the red cells 3 times in 0.9% saline and then lyse by adding to the packed cells 2 volumes of water and 1 volume of toluene or carbon tetrachloride (CCl_4). *(Toxic reagents, use with care).*
c. After shaking in a mechanical agitator, centrifuge at 3000 rpm (1200 g) for 30 minutes and pipette of the clear haemoglobin solution.

Reagent and solutions:

a. Drabkin's solution:
   $K_3Fe(CN)_6$ 200 mg
   KCN 200 mg
   Distilled water 1 Litre
   Note: Ready to use reagents are commercially available.
b. NaOH 1.2 M :
   4.8 g in 100 ml distilled H_2O (fresh prepared)
c. $(NH_4)_2SO_4$ saturated solution:
   706 g in 1 Litre distilled H_2O
   Heat to dissolve, then slowly at room temperature.

Procedure:

1. Add 0.6 ml of haemolysate (Hb concentration 8-10 g/dl) to 10 ml Drabkin's solution (Hb is converted to cyanmet form). 2.8 ml of the Hb solution is added to 0.2 ml of alkali solution and the mixture is well agitated.
2. Exactly after 2 minutes add 2 ml of $(NH_4)_2SO_4$ solution.
3. After vigorous mixing the denaturated material is allowed to precipitate for 5-10 minutes.
4. Remove the precipitate by filtration through a double layer of filter paper (Whatman no. 6 or no. 42) and read the optical density of the filtrate at 415 mm.
5. A control solution is prepared by mixing 1.4 ml of Cyammet-Hb, 1.6 ml of distilled H_2O and 2 ml of saturated ammonium sulphate. Dilute this solution 1:10 with distilled H_2O to obtain a suitable optical density.

Calculation:

$$\%HbF = \frac{OD^{415} \text{ test sample} \times 100}{OD^{415} \text{ control} \times 20}$$

Interpretation: The HbF determination is one of the most difficult of the routine procedures for haemoglobinopathies study. The difficulty is not in the methodology but in obtaining accurate and reproducible results, which can be achieved only when the method is in frequent use and performed by an experienced technician.

Percentage HbF for normal adult is 0.2-1.0%. Values of HbF higher than 20% are slightly underestimated with this method. Higher levels can be determined with Jonxis and Visser method or with HPLC. Some variability has been found with different batches of filter paper. The concentration of the haemolysate is also important: more diluted samples may give higher results.

2.8 HbF INTRACELLULAR DISTRIBUTION

Principle: HbF is not uniformly distributed among red cells, except in the condition of deletional HPFH. Cells with detectable amounts of HbF are called F-cells and they can be detected on blood smears by two techniques: the acid elution test of Kleihauer (13,14), and the immunofluorescence test using specific anti-HbF monoclonal antibodies (15,16). Methods of staining red cells in suspension have been developed making possible the quantitation of erythrocytes by flow cytometry (17). Quantitative evaluation of F-cells may be useful to screen for HPFH, to monitor F cells in patients with sickle cell anaemia treated with hydroxyurea and to detect fetal cells in adult blood such as in case of fetal-maternal haemorrhage. The performance of different methods of flow cytometry for F cell counting has been recently reported (18).

2.8.1 F-CELLS BY ACID ELUTION TECHNIQUE

Principle: The acid elution technique is based on the differential elution of fetal and adult haemoglobins from ethanol fixed red blood cells at acid pH (14).

Reagents:

a. Citric-acid phosphate buffer pH 3.3:
   (i) Solution A: (citric acid 0.1 M): 21.01g of citric acid in 1 litre of distilled water.
   (ii) Solution B: (NaH_2PO_4 0.2 M): 35.6 g $NaH_2PO_4.2H_2O$ in 1 litre of distilled water.
   Mix 73.4 ml of solution A with 26.6 ml of solution B; check pH and adjust if necessary to pH 3.3.
b. Ethanol: 80 % vol.
c. Stains:
   (i) 0.1% of erythrosine in water.
   (ii) Erlich's acid haematoxyline: dissolve 4.0 g of crystalline haematoxyline in 200 ml of ethanol 95% vol and add 8 ml of 10% sodium iodate. Add 200 ml of water and boil the mixture. Cool and add 200 ml of glycerin 6.0 g of aluminium ammonium sulphate and 200 ml of glacial acetic acid. Allow to stand the solution for at least 14 days.
   Note: Complete kits of reagents are commercially available.

Method:

1. Blood in any anticoagulant. Make thin smears and air dry for 10 to 60 minutes.
2. Fix in ethanol 80 vol% for 5 minutes at 20-22°C.
3. Rinse the smears with tap water and air dry.
4. Stain with (i) for 3 minutes, then rinse with water and air dry.
5. Counter-stain with (ii) for 3 minutes.
6. Rinse with tap water and air dry.
7. Examine under light microscopy without oil immersion.

Interpretation: F cells are densely stained with erythrosine. Cells containing HbA appear as ghost cells. Normal values for adults are below 0.01%.

Comments: The method is not quite sensitive and gives too low values. HbF at low concentration is eluted from red cells together with HbA. Pink stained cells are of difficult interpretation.

2.8.2 F-CELLS BY IMMUNOFLUORESCENCE

Materials:

a. Cleaned microscope slides: use pre-washed and pre-cleaned slides from BDH Superfrost (Cat.no. 406/0169/02).
   (i) Clean slides with acetone followed by ethanol, and allow to air dry for at least 1 hour.
   (ii) Prepare 4-5 very thin smears, one cell thick, per individual using 1 µl of fresh blood on the cleaned slides. Allow to dry for at least two days, for best results dry over one week.
b. Fixative: acetone/ethanol/methanol (6:2:2 v/v).
c. Phosphate buffered saline (PBS) Sigma P-4417.
d. Trypsin solution: trypsin 0.1% in calcium chloride ($CaCl_2$) 0.1% pH 7.8.
   (i) Prepare 10 ml solution [10 mg of trypsin (ICN Cat.No.150213) +10 mg $CaCl_2$ +10 ml distilled water] and store at –20°C in 1 ml aliquots.
   (ii) Alternatively, use trypsin tablets (Sigma; Cat.no. T-7168). Dissolve 1 tablet in 1 ml deionised

water, store at –20°C in 10 µl aliquots. Before use, make up to 500 µl (i.e. add 490 µl) with deionised water. Pre-warm to 37°C before use.
Note: The pH of $CaC1_2$ is very important.

e. Anti-γ monoclonal antibody (Sigma T-6653): undiluted supernatant.
f. Tetramethylrodamine Isothiocyanate (TRITC) conjugate anti-mouse IgG (Sigma T-6653).
   (i) Store frozen in 10 µl aliquots.
   (ii) Working solution: dilute 1:32 in PBS.
g. Humidity chamber: moist tissue in plastic chamber.
h. Glycerol: PBS (1:1 v/v) or anti-fade (Vectashield, Mounting Medium for Fluorescence; Vector Cat. No. H-1000).

Method:

1. Mark on the smear a small area of approximately 5 mm diameter using a diamond cutter.
2. Fix smears in acetone: ethanol: methanol fixative for 20 minutes. Air dry for 2 minutes (do not overdry).
3. Re-hydrate in PBS for 5 minutes (use plenty of PBS in a large glass container) and then rinse very briefly in distilled water. Air dry.
4. Cover the circled area with 8 µl of pre-warmed (37°C) trypsin solution and incubate at 37°C for 15 minutes in a humidity chamber.
   Note: time of trypsinization varies with age of slides but generally 15 minutes is sufficient.
5. Wash in PBS for 5 minutes with gentle agitation and rinse in distilled water. Air dry.
6. Cover trypsinized area with 5-10 µl anti-γ antibody, incubate at 37°C in humidity chamber for 30-40 minutes.
7. Wash as before and air dry.
8. Cover circled area with 5-10 µl fluorescent anti-mouse IgG and incubate at 37°C for 20-30 minutes. In the humidity chamber.
9. Wash as before with PBS, rinse in distilled water, air dry.
10. Mount in Glycerol: PBS or anti-fade.

Interpretation and comments: We suggest - 1,000 red cells are counted, which corresponds to 4-5 high power fields. Adult normal values are 0.3 to 4.4% F-cells. Females have a higher number of F-cells than males. The number of fields, of course, depends on the density of cells. Hence, it is very important to master the art of making thin blood smears. Whole blood can be stored up to a week prior to making smears, but it is preferable to use fresh blood. The air-dried smears can be stored wrapped in tissue paper at room temperature for years.

2.9 HbA_2 DETERMINATION

This technique is discussed in chapter 3.

2.10 HbS DETECTION

When Hb electrophoresis, HPLC or CE shows a fraction that runs in the position of HbS, a functional test for Hb S should be undertaken. Some common haemoglobins migrate to the same position as Hb S but do not sickle. These are listed in Table 2.1a. It should also be noted that 11 other (rare) haemoglobins have reduced solubility, and have therefore a positive solubility test, but most of which do not migrate to the same position as Hb S. These very rare sickling variants are listed in Table 2.1b. Examples are HbS-Antilles and Hb S-Oman, both of which have been observed in patients with severe sickle cell disease (see chapter 7, Volume 1).

Table 2.1a: Non-sickling haemoglobin variants with S-like mobility (√) after electrophoresis at alkaline pH. Their IEF and HPLC (BioRad Variant II) mobilities are also compared to HbS: (X) denotes a different mobility to HbS; (?) denotes mobility unknown.

Haemoglobin	Amino acid substitution	% in carriers	Alkaline electrophoresis	IEF	HPLC	Population
G-Philadelphia	α68 Asn → Lys	20%	√	x	x	African Mediterranean
Hasharon	α47 Asp → His	15-20%	√	x	x	Ahskenazi Jews Italian
D-Punjab	ß121 Glu→Gln	30-40%	√	x	x	Indian Mediterranean
G-Galveston	ß43 Glu → Ala	30-40%	√	?	?	African
G-San Jose	ß7 Glu→ Gly	30-40%	√	x	x	Italian Mexican
P-Galveston	ß117 His → Arg	45-50%	√	?	?	African
Lepore	δß fusion	7-15%	√	x	x	Mediterranean
Osu Christiansborg	ß52 Asp → Asn	45-50%	√	√	x	African
Stanleyville II	α78 Asn → Lys	15-25%	√	√	x	African
Memphis	α23 Glu → Gln	15-20%	√	x	√	African
Russ	α51 Gly→Arg	10-15%	√	x	√	Caucasian
G-Pest	α74 Asp→Asn	20-25%	√	x	√	Hungarian
G-Waimanalo	α64 Asp →Asn	20-25%	√	√	√	Fillipino Indian
Summer Hill	ß52 Asp → His	30-45%	√	√	√	Turkish
Machida	ß6 Glu → Gln	35-45%	√	√	√	Japanese
G-Makassar	ß6 Glu → Lys	25-45%	√	√	√	African

Table 2.1b: The sickling haemoglobin variants, plus their mobilities on alkaline electrophoresis, IEF and HPLC where known.

Haemoglobin	Amino acid substitution	Alkaline electrophoresis	IEF	HPLC	Population
Hb S	beta 6(A3) Glu→Val	Separate band	8.5 mm from HbA	RT 4.5 (±1) min	African, Indian, and many other
Hb S-South End	beta 6(A3) Glu→Val & beta 132(H10) Lys→Asn	?	?	Runs with HbA	
Hb S-Antilles	beta 6(A3) Glu→Val & beta 23(B5) Val→Ile	?	Separate band	Separate peak	Martinique
Hb C-Ziguinchor	beta 6(A3) Glu→Val & beta 58(E2) Pro→Arg	Runs in HbC position	Runs in HbC position	unknown	Senegal
Hb C-Harlem	beta 6(A3) Glu→Val & beta 73(E17) Asp→Asn	Runs in HbC position	?	Runs with HbA_2	African
Hb S-Providence	beta 6(A3) Glu→Val & beta 82(EF6) Lys→Asn	Runs with HbA	?	Separate peak	African
Hb S-Travis	beta 6(A3) Glu→ Val & beta 142(H20) Ala→Val	Separate band	?	Separate peak	African
Hb C-Ndjamena	beta 6(A3) Glu→Val & beta 37(C3) Trp→ Gly	?	Runs with HbC	Separate peak	Chadian
Hb S-Oman	beta 6(A3) Glu→Val & beta 121(GH4) Glu→Lys	?	Separate band	Separate peak	Oman
Hb S-Clichy	beta6(A3)Glu→Val & beta8(A5)Lys→Thr]	?	Separate band	Separate peak	French
Hb S-Cameroon	beta 6(A3) Glu→Val & beta 90(F6) Glu→ Lys	?	Separate band	Separate peak	Cameroonian
Hb Jamaica Plain	beta 6(A3) Glu→Val & beta 68(E12) Leu→Phe	?	Runs with HbS	Runs with HbS	Puerto Rican

2.10.1 SICKLING TEST

Carrying out the sickling test is part of the diagnostic work up in patients suspected of having a sickle cell syndrome. The test should be also carried out if there is an abnormal electrophoretic or chromatographic haemoglobin fraction in the position of HbS (Table 2.1a). Many tests have been described and several commercial kits are available. We will describe a simple method that has proved very reliable and simple to carry out.

Principle: Sodium metabisulphite reduces the oxygen tension inducing the typical sickle-shape of red blood cells.

Sample: Fresh blood in any anticoagulant.

Reagents: 0.2 g of sodium metabisulphite in 10 ml of distilled water.
Stir until dissolved. Prepare fresh each time.

Method:

1. Mix 1 drop of blood with 1 drop of 2% sodium metabisulphite solution on a microscope slide.
2. Cover with a cover slip and seal the edge with wax/vaseline mixture or with nail varnish. Allow to stand at room temperature for 1 to 4 hours.
3. Examine under a microscope with the dry objective.

Interpretation: In positive samples the typical sickle-shaped red blood cells will appear (Figure 2.4). Occasionally the preparation may need to stand for up to 24 hours. In this case put the slides in a moist Petri dish. False negative results may be obtained if the metabilsulphite has deteriorated or if the cover slip is not sealed properly.

A positive test does not distinguish the sickle cell trait from sickle cell disease. It is important to examine the preparation carefully and in particular near the edge of cover slip.

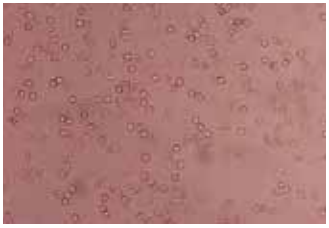

FIG. 2.4
Sickling test showing typical sickle-shaped red blood cells

2.10.2 SOLUBILITY TEST

HbS is quite insoluble when in the reduced state in high phosphate buffer solution. It forms tactoids (water crystals) which refract and deflect light rays and produce a turbid solution, permitting a solubility test (19).

Reagents:

a. Stock 2.58 M phosphate buffer:
Dissolve 239.66 g K_2HPO_4 and 164 g K_2HPO_4 in distilled water; then make up to 1 litre with distilled water. The pH should be 6.5.
b. High molarity Buffer (2.24 M):
Dilute 434 ml of stock buffer above to 500 ml distilled water. Mix and stopper.
c. Low molarity Buffer (1.1 M):
Dilute 213 ml of stock buffer above to 500 ml distilled water. Mix and stopper

Buffers may be kept at room temperature and used as long as they are clear and uncontaminated.
Note: Kits for this test are commercially available (eg. Sickledex, Sickle prep).

Method:

1. Label two small (12 x 75 mm) test tubes for each patient. Mark one low molarity and one high molarity.
2. Pipette 1 ml of high molarity buffer (2.24 M) into its properly labelled tube and 1 ml of low molarity buffer into other labelled tube.
3. Add two drops CCl_4 haemolysate into each tube with a disposable, labelled Pasteur pipette and mix.
4. Add a pinch (about 10-20 mg) of sodium dithionite powder to all tubes. Mix and read immediately.
5. Run positive and negative control bloods by following the same steps given above.

Interpretation: Haemoglobin S is present if a precipitate forms in the tube labelled "high molarity." "Low molarity" tubes must all be negative for precipitate. When cellulose acetate electrophoresis shows a band in the HbS position, interpret the solubility test as follows:

Low Molarity	High Molarity	Results
no precipitate	precipitate	HbS
no precipitate	no precipitate	HbD or Hb G
precipitate	precipitate	inconclusive: poor reagents or specimen

False positive results may be due to polycythaemic blood and a variety of abnormal haemoglobins including Hb's I, Bart's, C-Georgetown, Alexandra and C-Harlem. Positive test should be confirmed by haemoglobin fractionation. High concentration of HbF may inhibit the reaction. The solubility test is unlikely to be reliably positive result until after 6 months of age.

2.11 HbE DETECTION BY DCIP TEST

Principle: The HbE mutation occurs from the mutation on the beta globin gene at codon 26, GAG→AAG, resulting in the amino acid change from Glu → Lys. This results in a mildly unstable haemoglobin and exposure of the -SH group, which can be oxidized by certain chemical agents including the dye DCIP (dichlorophenolindophenol) at the neutral pH (7.5). HbE and other unstable haemoglobin molecules such as Hb H will be precipitated when exposed to this dye at 37°C (20).

Reagents:

DCIP Reagent:

(i)	Tris base	4.36 g
(ii)	EDTA $Na_2.2H_2O$	2.68 g
(iii)	DCIP (Sigma)	0.0276 g
(iv)	Saponin	0.05 g

Dissolve in distilled water and adjust pH to 7.5 by 6 M HCl and adjust the volume to 500 ml. This working solution should be kept in 4°C.

Method:

1. Add 30 µl of whole blood or 20 µl packed red cell into 5 ml DCIP solution.
2. Gently mix and incubate at 37°C for 1 hour.

Interpretation: Precipitated hemoglobin can be visualized by the naked eye at the bottom of the tube.

Results and comments: In homozygous HbE, a heavy sediment will form at the bottom of the test tube. In HbE trait, and HbE/ß-thalassaemia, the precipitation of HbE produces a cloudy or an evenly distributed particulate appearance. The test is positive also in HbH disease and other unstable haemoglobins. To overcome these false positive results a modified DCIP test has been described (21) and more recently the CMU-E (Chiang Mai University-E) has been found to have 100% sensitivity and 99.1% specificity (22).

2.12 IRON STATUS DETERMINATION

Analysis and determination of iron status is quite often necessary in screening for thalassaemia carriers. Iron status can be measured using many haematological and biochemical indices. Each parameter reflects changes in different body iron compartments (storage, transport, end product, receptors) and is affected at different levels of iron deficiency. However, the presence of iron deficiency should be evaluated with simple tests such as Zinc protoporphyrin (ZnPP), serum iron and transferrin and in some cases serum ferritin. The red blood cell ZnPP determination is the fastest and easiest method for screening of iron deficiency. Iron depleted red blood cells show increased levels of ZnPP. A diagnosis of iron deficiency has to be confirmed by serum iron and transferrin determination, to calculate transferrin saturation. Some laboratories prefer to determine the iron status using serum ferritin, but it should be pointed out that with this parameter there are several limitations (i.e. false positive and negative results).

2.12.1 ZINC-PROTOPORPHYRIN (ZnPP)

Principle: ZnPP is a metabolite normally produced in trace amounts during heme biosynthesis. In the presence of iron deficiency or impaired iron utilisation, zinc becomes the alternative to iron for ferrochelatase-mediated chelation by protoporphyrin, leading to increased ZnPP formation (23). The substitution of zinc for iron is one of the first biochemical responses to iron depletion, causing increased ZnPP to appear in circulating erythrocytes.

Method: ZnPP is a fluorescent compound and haematofluorometry is the fastest and easiest method of determining ZnPP in blood specimens (in any anticoagulant). Dedicated haematofluorometers can measure the ratio of ZnPP fluorescence to haem (haemoglobin absorption) directly in whole blood or in washed erythrocytes. ZnPP is determined in oxygenated blood by illuminating the surface of a glass slide containing a layer of blood with light (wavelength 415 nm) and by measuring

the emitted fluorescence at 596 nm.
Currently, two haematofluorometers are commercially available (Aviv Associates, Inc., Lakewood NJ, and the ProtoFluor Z, by Helena Laboratories, Beaumont, Tx). The methodology for both instruments is cheap, fast and requires only one drop of whole blood.

1. 50 µl of blood is placed and spread on the 25 x 25 mm2 cover glass.
2. The instrument should be adequately calibrated with 3 control blood of different known protoporphyrins values.

Results: In normal non iron deficient individuals, the ZnPP values are lower than 30 µg/dl whole blood. In non iron deficient ß-thalassaemia carriers, the ZnPP values are lower than 40 µg/dl.

Comments: One advantage of determining ZnPP is that it can be performed from the same sample used for the complete blood count. The ZnPP concentration in a blood sample is stable for several days (unless haemolysis is present). ZnPP is increased also in conditions of relative iron deficiency, when iron is delivered to the marrow at a rate inadequate to meet the requests of increased erythropoiesis. Examples are conditions associated with ineffective erythropoiesis. Conditions of impaired iron utilization, including sideroblastic anaemia and anaemia of chronic disease, lead to increased ZnPP as well. ZnPP is also increased in lead intoxication because of the inhibition of ferrochelatase. Haemolysis and hyperbilirubinaemia may give false increased values.

Despite this low specificity ZnPP determination is a very good screening tool for iron deficiency and is considered a suitable screening test also for lead intoxication. Transferrin saturation should always be determined to confirm the presence of iron deficiency.

2.12.2 SERUM IRON

Serum iron levels are reduced after the complete depletion of iron stores but before the haemoglobin level drops. Several manual and automated methods are available and their description is beyond the scope of this book.

Limitations associated with serum iron determination include a wide diurnal variation of serum iron concentrations, (lower in the morning as compared to the afternoon), and the diet ingested during the day before (eg. a high intake of meat may increase the serum iron levels). Serum iron has a low specificity as low levels may be found in pregnancy, during chronic infections and inflammations, pyrexia, malignancy. Serum iron should be used in combination with serum transferrin to calculate the percentage of saturation.

2.12.3 SERUM TRANSFERRIN

Transferrin is the iron-transporting protein which can be determined using normal or automated techniques as total iron binding capacity (TIBC), ie. the amount of added iron specifically bound by plasma. Alternatively transferrin can be measured as protein using immunological methods. Se-

rum transferrin increases in iron deficiency, and is falsely reduced in acute inflammation, chronic infections, renal diseases, and malignancy. Several manual and automated methods are available and their description is beyond the scope of this book.

Transferrin saturation: Transferrin saturation is the ratio of serum iron to iron-binding capacity and is the most accurate indication of iron supply to the bone marrow. Normal values are higher than 16% in adults and higher than 10% in children.

2.12.4 SERUM FERRITIN

Serum ferritin is usually measured using a immunoradiometric assay (IRMA), radioimmunoassay (RIA) or by enzyme-linked immunosorbent assay (ELISA). Several manual and automated methods are available and their description is beyond the scope of this book. The WHO reference standard is recommended. Normal values are 15 to 300 µg/l in males and 15 to 200 µg/l in females. Serum ferritin levels are increased in acute and chronic infections and inflammations, in liver disease, and in malignancy. Falsely reduced levels can be found in association with ascorbate deficiency.

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CHAPTER 03

HAEMOGLOBIN PATTERN ANALYSIS

3.1 CHROMATOGRAPHIC METHODS (FOR HbA_2 DETERMINATION)

Quantitative HbA_2 determination is the most valuable test for ß-thalassaemia carrier identification. Several methods have been set up, but only a few are now recommended for their accuracy. It should be pointed out that the precision and accuracy of HbA_2 determination using densitometry scanning after cellulose acetate electrophoresis is unsatisfactory and its use has to be avoided (1). Isoelectric focusing (IEF) has an excellent resolution allowing for an accurate quantification, but it is cumbersome and time-consuming. Capillary electrophoresis (CE) is being used more and more in a clinical diagnostic setting for diagnosis of haemoglobinopathies. Several studies showed an excellent correlation between capillary electrophoresis and high performance cation-exchange chromatography (HPLC) for the qualitative and quantitative haemoglobin analysis (2-4).

The most used methods for HbA_2 measurement are CE, HPLC and less frequently the microchromatography on DE-52 (5-8). These methods are very accurate, fast and simple. In addition CE and HPLC identify and measure many variant haemoglobins, including the commonly encountered variants HbS, HbC, HbE and Hb D-Punjab. Figure 3.1 shows examples of HPLC chromatograms from 4 different analysers (Variant II, HA 8160, G7 and Ultra 2) in comparison to CE.

3.1.1 HPLC FOR HAEMOGLOBINOPATHIES SCREENING

Cation: exchange high performance liquid chromatography (HPLC) has emerged as the method of choice for quantification of HbA_2, HbF and for detection and quantitation of the Hb variants, particularly those which may interact with ß-thalassaemia such as HbS, E, C, O-Arab, D and Lepore.

Principle: In this method phosphate buffers at different concentrations (mobile phase), pass under pressure through an ionic exchange column (stationary phase). The stationary phase consists of a temperature controlled analytical cartridge containing a resin of anionic or cationic particles (3-5 µm). The chromatographic station delivers a programmed buffer gradient of increasing ionic strength and pH to the cartridge by two dual-piston pumps, and the haemoglobins are separated according to their ionic interaction with the stationary phase.

FIG. 3.1
Examples of HPLC chromatograms in comparison to CE (adapted from van Delft et al. 2009 ref 4). From left to right: Capillarys CE, Variant II, HA 8160, G7 and Ultra 2. a = normal; b = HbA/S; c = HbA/C; d = HbA/E; e = HbA/D; f = High HbA_2 Heterozygous ß-thalassaemia.

FIG. 3.1.a

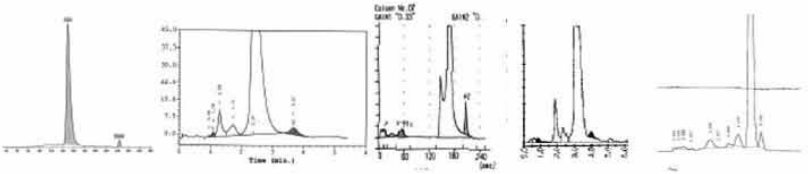


FIG. 3.1.b

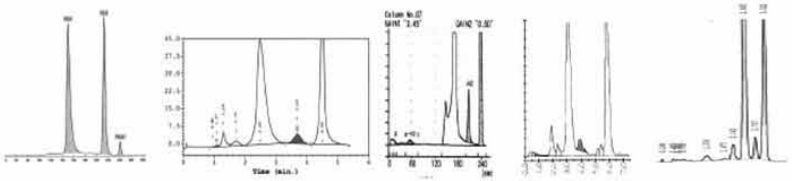


FIG. 3.1.c

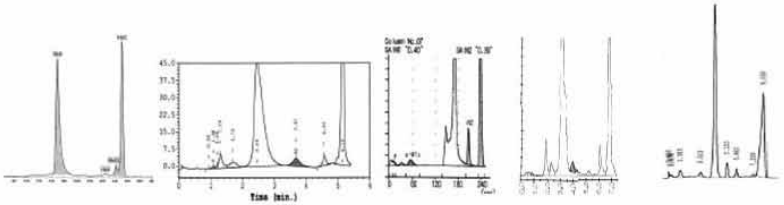


FIG. 3.1.d

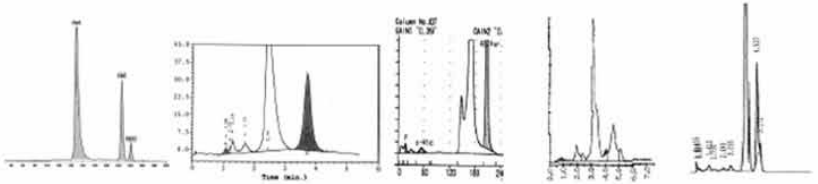


FIG. 3.1.e

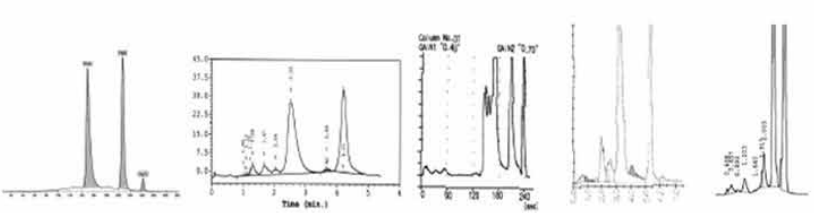
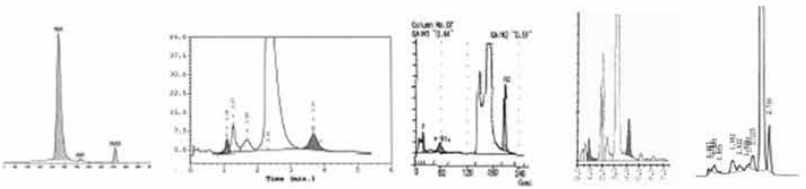


FIG. 3.1.f



The separated haemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance (415 nm) are measured; background variations are corrected by an additional filter at 690 nm. Each haemoglobin is characterised by a specific retention time, which is the elapsed time from the sample injection to the apex of a haemoglobin peak.

The calibration factors for HbA_2, F, A_{1C} are automatically calculated by processing a calibration sample at the beginning of each run. Specific software turns the raw data collected from each analysis into a report showing the chromatogram, with all the haemoglobin fractions eluted, the retention times, the areas of the peaks and the values (%) of the different haemoglobin components. The report presents the percentages of haemoglobins F, A_{1C}, A and A_2 and provides qualitative and quantitative determination of abnormal haemoglobins.

Sample: Venous blood in any anticoagulant.

Method: A commonly used apparatus is the Variant (Bio-Rad Laboratories). However, reliable results have been recently obtained also with other instruments, such as HA816 (Menarini Arkay) and G7 (Tosoh- Bioscience) (4).

Results and interpretation: The expected normal range for HbA_2 is between 1.7% and 3.2% in normal subjects, while in ß-thalassaemia carriers when it is between 4.0% and 7%. HbA_2 values are considered borderline when between 3.2% and 3.8%. Samples with these levels need further investigation for possible normal HbA_2 thalassaemia (see Table 1.1). The normal range for HbF is usually less than 1.5% of total haemoglobin.

HPLC machines have analyte identification windows that help in the interpretation of normal and abnormal haemoglobins detected in the blood sample (9). The windows are defined retention time intervals in which the common haemoglobin variants are eluted (eg HbS, C and D). However, it should be pointed out that since other Hb variants may have a similar retention time to the common variants, (Tables 3.1a and 3.1b), the Hb variant identification is only presumptive (Figure 3.1) and DNA or globin amino acid analysis is necessary for definitive Hb variant identification (10). HPLC is also being used for neonatal haemoglobinopathy screening programmes (11).

Limitations of the procedure: Since Hb Lepore and HbE are co-eluted with HbA_2, their presence in the sample gives a falsely high percentage (>10%) of HbA_2. This amount of HbA_2 is almost never present in ß-thalassaemia carriers (6). Therefore samples found to have a level of HbA_2 greater than 10% should be further tested for the possible presence of a haemoglobin variant running with the HbA_2 peak. The increase of HbA_2 levels above 3.5% in HbS carriers is due to co-elution of minor components with HbA_2 (possible post-translational modifications of HbS). This may also occur with haemoglobin variants eluting after HbA_2. HbH and Hb Bart's can be detected in the chromatogram but not quantified because they are eluted prior to the start of integration.

Table 3.1a Elution times of some haemoglobin variants observed with the BioRad Variant-II HPLC system.

Hb Window	F	A_{1C}	A_0	A_2	D	S		C
Retention Time	1.05-1.10	1.60-1.70	2.3-2.7	3.6-3.7	4.1-4.2	4.4-4.5	4.7-4.8	5.1-5.2
	J-Sardinia J-Iran Dagestan S. Florida	Le Lamentin N-Baltimore J-Baltimore J-Broussais J-Paris 1 J-Norfolk J-Pontoise Luton Andrew Mi Harbin Dublin Interlaken I-High Wycombe Bexiers J-Wenchang-Wuming	Athens-GA Tyne Creve Couer New York Hounslow Ty Gard Koln	E Bari Lepore G-Copenhagen D-Ibadan Belfast Matera Zurigo Fort Worth Kenya D-Iran Deer Lodge G-Tapei Gainsville-GA	D-Punjab G-Norfolk G-Phil. G-St Jose Stanleyville 2 Dallas Kempsey Tak Tarrant Matsue-Oki San Antonio Radcliffe Woodville West One	S Winnipeg Memphis Manioba 2 Yakima Savaria G-Waimanalo G-Pest Russ Richmond St Lukes Summer Hill	Hasharon O-Padova O-Arab Shelby Q-Iran Q-India Setif M-Milwaukee-2 Ta-Li M-Iwate Titusville	C C-Rothschild G-Siriraj S-Oman

Note: Different elution patterns are obtained with different instruments.

Table 3.1 b (part 1) Elution zones of some haemoglobin variants observed with the Sebia Capillarys Capillary Electrophoresis system: Zones 1-7.

Zone	1	2	3	4	5	6	7
Hb Window			HbA_2				HbF
Potential Hb variants	Santa Ana F-Hull T-Cambodia A_2' HbA_2 variants of: "Hasharon" "Winnipeg" "Q-Thailand" "Q-India" "G-Norfolk" "G-Pest" "Inkster" "Memphis" "Chapel Hill" "Arya" "Fort de France"	C Constant Spring F-Texas C-Harlem HbA_2 variants of: "Setif" "Bassett" "Swan River" "Manitoba I" "Manitoba II"	O-Arab Chad E-Saskatoon	E Köln Agenogi G-Siriraj Santa Ana A2-Babinga M-Saskatoon Denat. C HbA_2 variants of: "M-Iwate"	S Dhofar Arya Hasharon Handsworth Ottawa S-Antilles Fort de France Hamadan Montgomery Denat. O-Arab HbA_2 variants of: "Lombard" "Cemenelum" "Jackson"	Stanleyville II Osu Christansborg Leiden Muravera Matsue-Oki Muskegen Summer Hill D-Ibadan D-Bushman D-Punjab D-Ouled Rabah D-Iran Lepore Korle-Bu Köln Fort Worth G-Norfolk G-Philadelphia G-Coushatta G-Taipei	Q-Thailand Alabama Chapel Hill Bassett Barcelone Geldrop Sant Anna Swan River Presbyterian Burke Manitoba II Richmond G-San-José Porto Alegre denatured S HbA_2 variants of: "J-Paris-I"

						G-Siriraj G-Pest Inkster Memphis P-Nilotic Q-India Q-Iran Willamette Winnipeg Setif denatured E HbA_2 variants of: "J-Toronto" "J-Rajappen" "J-Anatolia" "J-Oxford" "J-Broussais" "Mexico" "J-Habana" "J-Rovigo"	

Table 3.1 b (part 2) Elution zones of some haemoglobin variants observed with the Sebia Capillarys Capillary Electrophoresis system: Zones 8-15.

Zone	8	9	10	11	12	13	14	15
Hb Window	Hb F acetylated	HbA		Denatured HbA zone				
Potential Hb variants	Atlanta Alberta Hinsdale Kempsey Athens-GA	Toulon Okayama Fontainebleau Camperdown Gorwihl Phnom Penh Silver Springs La Coruna Bougardirey-Mali Austin Buenos Aires Chicago Raleigh Hekinan Mosella Dallas Aztec Little Rock Frankfurt Bethesda M-Boston Brisbane Mizuho Grange Blanche San Diego M-Saskatoon Malmö Minneapolis Laos Syracuse	Hope M-Iwate Camden	Kaohsiung Providence K-Woolwich Lombard Fannin Lubbock Andrew Minneapolis Jackson Himeji HbA_2 variants of: "I-Texas"	Hb Bart's Cemenelum Providence J-Toronto J-Mexico J-Baltimore J-Calabria J-Rajappen Grady J-Anatolia J-Broussais J-Chicago J-Oxford J-Meinung Ube-2 Mexico J-Habana J-Paris-I	J-Rogivo N-Baltimore J-Norfolk J-Kaohsiung	N-Seattle	Hb I-Texas

3.2 ELECTROPHORETIC METHODS

Electrophoresis is a separation technique based on the mobility of ions in an electric field. It is the classical method of identifying and quantifying the haemoglobin proteins.

The haemoglobin molecules (HbA, HbA_2, HbF and variants) in solution are electrically charged at any given pH. They can have a positive charge or a negative one according to the ionisable groups (acidic or basic side chain) that they have. Total haemoglobin, which is a mixture of these molecules, has a net negative charge. When an electrical potential difference is applied, particles will migrate either to the cathode or the anode depending on their net charge, and molecules with different overall charges will begin to separate.

3.2.1 CLASSIFICATION OF ELECTROPHORETIC METHODS

There are several electrophoretic methods, mostly classified according to the supportive media. The broad classification is into "free electrophoresis", in which the molecules are made to migrate in liquid and "zone electrophoresis", in which the molecules, dissolved in buffer, are made to migrate on a more solid medium.

The supportive media are described as:

a. **Liquid** - the only "free" method still used to separate haemoglobin molecules is capillary electrophoresis.
b. **Solid** - this includes paper, which is no longer used and cellulose acetate, which is one of the most commonly used media.
c. **Gel** - such as starch (which is also no longer in use), agar, agarose, and polyacrylamide.

Some of these media effect separation of molecules by utilizing differences in size (mass) of the molecules, shape of the molecule and the frictional effects of the medium. Therefore the choice of medium will affect the quality of separation. Likewise the rate of migration and thus the resolution depends on:

a. The strength of the electric field
b. The molecular mass
c. Whether the molecule is hydrophobic
d. The ionic strength of the buffer
e. The temperature of the buffer.

For example high voltage means faster separation but excessive current causes heat which will distort the bands by causing evaporation, siphoning of electrolytes and denaturation of molecules. The choice of buffer is also important since it determines the pH, which influences the rate and direction of movement of the protein, and the ionic strength, which influences the rate of separation. The composition of the buffer may interact with a protein causing a change in charge density.

In practice the choice of the electrophoretic method will be influenced by all of the considerations mentioned above and at least two different methods are recommended to positively identify the haemoglobins because some haemoglobins have identical migration rates on one medium but separate on the other. For example HbS, HbD and Hb G-Philadelphia migrate together on alkaline electrophoresis, as do HbE and C, while their mobility differs on citrate agar. In a reference laboratory other methods, such as Isoelectric Focusing (IEF), Capillary Electrophoresis (CE) and /or High Pressure Liquid Chromatography (HPLC), should also be available to resolve diagnostic problems.

In this chapter, the most commonly used electrophoretic methods in thalassaemia population screening programmes or for diagnosis of homozygote states and identification of variants will be described. These are Capillary Electrophoresis, cellulose acetate electrophoresis, citrate agar electrophoresis and isoelectric focusing.

Samples:

a. 2-3 ml of whole blood in EDTA is the usual sample taken for electrophoresis. Other anticoagulants may also be used.
b. The red cells are lysed in distilled water and carbon tetrachloride and then centrifuged to give a clear haemolysate that can be used for electrophoresis. The haemolysate is better utilised fresh although if stored at –80ºC it can be used later. In automated systems, like for example the Capillarys of Sebia, haemolysing buffer is delivered by the manufacturer.

3.2.2 CAPILLARY ELECTROPHORESIS FOR HAEMOGLOBINOPATHIES SCREENING

Capillary Electrophoresis is an emerging diagnostic tool in many clinical chemistry labs to separate Hb fractions and calculate the percentage of each fraction. A commercially available apparatus, used in several European labs, is for example the Capillarys from Sebia (Lisses, France).

Principle: Capillary Electrophoresis (CE) is the technique of performing electrophoresis in buffer-filled narrow capillaries, 25-100 µm in diameter. The separation relies on differences in the speed of migration (migration Velocity) of ions or solutions, but the vitally important feature of CE is the bulk flow of liquid through the capillary, which is called Electric Osmotic Flow (EOF).

The inside surface of the capillary has ionisable silanol groups, which readily dissociate giving a negative charge to the capillary wall. The negative charge attracts the positive charged ions from the buffer, creating an electrical double layer and therewith a potential difference close to the capillary wall. When a voltage is applied across the capillary, cations in the diffuse layer are free to migrate towards the cathode, carrying the bulk solution with hem. The result is an Electro Osmotic Flow and separation of the differently charged Hb fractions. These fractions are detected directly at an absorbance wavelength of 415 nm, which is optimal to haemoglobins, in the following order from cathode to anode: HbC, A_2, E, S, D, F, A, Bart's, J and H.

In contrast with the pressure driven flow in HPLC, the flow profile of EOF is distributed uniformly along the capillary. No pressure drops are encountered and the flow velocity is uniform across the

capillary, which leads to higher separation efficiencies (12).

Equipment: The capillary electrophoresis device used is the Capillarys from Sebia (Lisses, France). Consumables are delivered by the manufacturer and include:

a. Dilution segments green and transparent.
b. Sample rack 0: For use of normal and pathogen control, with tube and barcode.
c. Sample rack 100: The system recognizes this rack as a maintenance rack and the option: "capi. cleaning "is available.
d. Sample rack Sebia: Used with the green dilution segments. The system recognizes automatically that dilution preparing is not necessary. For use of patient's samples and AFSC control (control containing mix of HbA, HbF, HbS and HbC).
e. Sample rack with a number: The system makes dilutions automatically from the tubes placed into the sample rack.

Sample preparation: The anticoagulant is EDTA, but the use of citrate or heparin is also acceptable.

Method:

1. Switch on the Capillarys with the button in the rear.
2. Turn on the computer and log on into the system.
3. Then double click the Phoresis icon on the desktop screen and log in to the system with appropriate ID and password.
4. Check and /or change the reagents by opening the lid on the right front side of the apparatus. Click OK in the window appearing when performing this procedure.
5. Enter the new Lot numbers and expiration dates and click "OK ".
6. Check the reagents levels visually and move the cursor buttons to adjust if necessary.
7. The status window will show the message "Busy" and changes to "ready": the apparatus is ready for use.

Migration control and control cycle:

1. The Control samples are available commercially and should be dissolved in distilled water according to the manufacturer's instructions. This control vial can be stored at –20ºC and used 15 times. The control should be placed in position 1 in the sample rack "0". Pour 4 ml of the haemolysing solution in a tube and place it in position 8. Place a new green dilution segment on the rack. (For manual dilution: mix 90 µl of the haemolysing solution with 18 µl of the control in a green segment and put it on the "0" rack with the appropriate tube. The controls made in this way can be frozen and thawed three times.)
2. When the status window changes from "busy" into "ready", the rack "0" containing the normal control, is inserted into the Capillarys. Place this rack in the apparatus and push the rack until you hear a beep. The green light of the feeder belt indicator will switch to red.
3. The "Select a control" window will appear: Select QC medium in the drop down menu, enter the lot number, select the right dilution: manual when a diluted green segment is used or automatic when the Capillarys has to make the new dilution and click "OK" (A second normal control run has to be done in the following circumstances:

a. After changing the analysis buffer
b. After capillary cleaning or capillary activation
c. After a software upgrade)
d. When the run is completed and the controls are in range, all eight capillaries are calibrated.

4. Insert rack "0" again with the pathological control and on the first position a tube with the appropriate barcode. A window appears in which the lot number should be entered, select manual or automatic dilution and click "OK ".
5. Collect the patient blood for making the dilution. Use the red blood cells after centrifugation. Mix the red cells carefully and prepare the dilutions by pipetting 90 µl haemolysing solution (Sebia) with 18 µl red cells, use the green dilution segments.
6. Insert the rack when the status window shows "Ready".
7. If migration is completed the curves are shown by clicking the video screen-icon (red arrow).

Results and interpretation: When the run has ended, prepare a Sebia worklist by clicking the worklist icon. The results of the controls appear automatically on the worklist (NORMAL CTL, PATHO CTL). Open the video screen-icon to analyze the curves:

To open the curves there are two options:

1. Click on a curve to open its review window
2. Click on the icon underneath the blue marker (the third icon from the left) and it shows the first curve of the run. Use the select buttons to bring up the next curve, the previous curve and the first or last curve. To return to the profiles of the current analysis: click the 'save and exit' button (with the red arrow).

The Capillarys recognizes automatically the A_0, F, C and A_2 peaks. Other peaks have a specific position (zones) for instance HbS, HbD, HbE, HbH and HbJ but they are all labelled as HbX. It is possible to add a comment with the most probable name of the abnormal Hb variant.

3.2.3 CELLULOSE ACETATE ELECTROPHORESIS

This is an acetate salt of cellulose produced by treating cotton with acetic acid using sulphuric acid as a catalyst. Migration takes place on the buffer film on the surface of the cellulose acetate plate or membrane. Separation of the proteins is primarily by charge. Cellulose acetate electrophoresis may be used for qualitative identification of variants, but also with elution for quantitation of the haemoglobins, A_2, A, S, D, Lepore, α-chain variants, HbH and Hb Bart's. The positions of various haemoglobin fractions on cellulose acetate electrophoresis are shown in Figure 3.2.

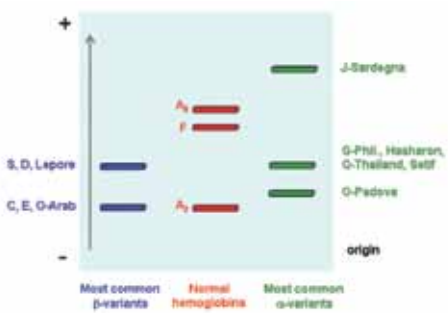


FIG. 3.2
Position of fractions on cellulose acetate electrophoresis.

Reagents and materials:

a. Tris-EDTA Boric Acid (TEB) buffer, pH 8.4:
   (i) Tris hydroxymethyl amino methane (TRIS): 10.2 g
   (ii) Ethylene diamine tetracetic acid (EDTA): 0.6 g
   (iii) Boric Acid: 3.2 g
   Make up to 1 litre with distilled water.
b. Whatman No. 3 chromatography paper.
c. Cellulose acetate membranes are supplied Scheicher and Schuell, 40 x 300 mm. Alternatives are made by Sartorius and by Shandon.
d. HbA_2 control, as supplied by the National Institute for Biological Standards and Control (NIBSC). The control has been produced by freeze drying a solution of haemoglobin prepared from human cells and made stable by the addition of sucrose (200 mM), potassium cyanide (6 mM) and chloramphenicol (1 mg/dl). This control was established by the World Health Organization in 1994 as the international Reference Reagent with an assigned value of 5.3% of total haemoglobins present.

Equipment required:

a. Power supply capable of delivering a constant current, 0-80 mA and up to 400 volts.
b. An horizontal electrophoresis tank with adjustable bridge gaps and a polarity indicator (eg. Shandon), as shown in Figure 3.2.1.
c. Roller mixer.
d. A single beam SP6-200 - Spectrophotometer Pye Unicam.

FIG. 3.2.1
Cellulose acetate electrophoresis equipment.

Method:

a. Haemolysate is prepared from whole blood (in K_2EDTA) as previously described.
b. The electrophoresis tank is prepared by filling the tank with 900ml approximately of TEB buffer wicks are cut from Grade No. 3 chromatography paper and were placed along the 22 cm long bridges in the tank.
c. The cellulose acetate membranes are cut in 40 x l00 mm each and soaked (shiny side down) in TEB buffer for 5 minutes. Five strips are plotted and placed on the electrophoresis tanks.
d. Voltage current is applied at 250 V for 5 minutes to the membranes to equilibrate the membranes with the buffer.
e. The current is turned off and 8-10 µl haemolysate (10 g/µl) is applied on each membrane at the cathodal end using a capillary tube.
f. Then the voltage is set at 250-300 V working at constant current of 2 mA for each strip.

g. The electrophoresis is run for approximately 45 minutes to one hour until there is a clear area between the bands.
h. The current is then turned off and the separated HbA_2 on the cellulose acetate membrane is cut and immersed in a tube containing 4 ml of distilled water and the HbA in a tube containing 16 ml distilled water. If a haemoglobin variant is present then this is cut separately into 4 or 16 ml distilled water depending on the quantity of the variant present. Note that a blank is prepared from the same run by cutting a piece of clear cellulose acetate strip that was immersed in 4 ml distilled water.
i. The tubes are then placed on a roller mixer for 30 minutes for the haemoglobin elution.
j. The strips are removed and the tubes are then centrifuged for 10 minutes at 3000 rpm.
k. The absorbance of each haemoglobin is read at 413 nm against the blank on a spectrophotometer.

Interpretation:
The results are calculated as follows:

$$\% \text{ of } HbA_2 = \frac{\text{Absorbance of } HbA_2 \times 100}{\text{Absorbance of Total (HbA} \times 4) + \text{absorbance of } HbA_2}$$

The haemoglobins migrate on the cellulose acetate membrane from cathode to anode in the following order: HbA_2, HbE, HbC, HbD, HbS, Hb Lepore, HbF, HbA and the fast moving haemoglobins Bart's and HbH (see Figure 3.2). The normal range for HbA_2 is 2.4% to 3.2%. A typical example of cellulose acetate electrophoresis is shown in Figure 3.2.2.

Factors affecting the result are: correct pH, correct concentration of the buffer and the temperature of the buffer, which may be influenced by the voltage or the environmental temperature. It is advisable, especially in hot climates to keep electrophoresis tanks with buffer refrigerated at 4°C. Ideally the method should be conducted at an environmental temperature below 23°C. It is necessary, therefore, in warm climates to have air conditioning in the laboratory. The quality of the carrier membrane must be good and poor quality should be recognised and discarded. The membrane should be kept moist.

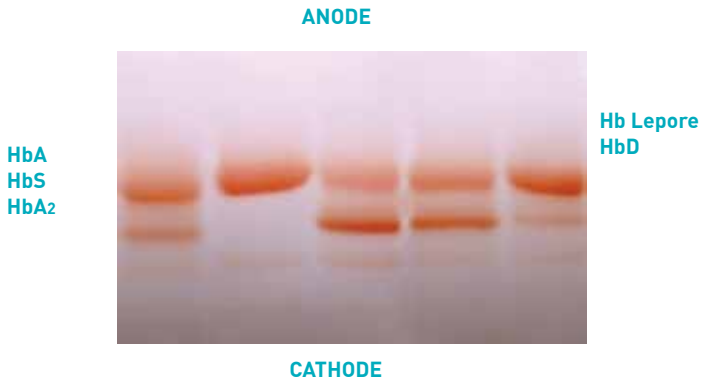


FIG. 3.2.2
Example of cellulose acetate electrophoresis.

3.2.4 AGAR GEL ELECTROPHORESIS

Agar is a gelatinous substance derived from the cell wall of red marine algae. It is composed of a matrix of cross-linked molecules with spaces between them. In an electric field the haemoglobin molecules will move through the matrix so that the migration rates depend on the size and shape of the molecules as well as the charge. This means that smaller, linear molecules with high electric charge will move through the gel at a faster rate.

Agar gel electrophoresis is not a satisfactory screening technique because it cannot distinguish many abnormal haemoglobins from HbA. However it can separate the C group into three fractions: HbC, O-Arab, and HbE plus HbA_2. The method can also distinguish HbS from HbD, HbF from HbA, Hbs Little Rock, Rainier and Bethesda from HbA, and HbH from HbI.

Reagents:
a) Stock buffer - Citrate buffer, pH 5.9:
Dissolve 73.5 g tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in approximately 700 ml distilled water. Adjust the pH to 5.9 using 0.5 M citric acid (10.5 g per 100 ml). Approximately 34 ml will be required. Make the solution up to 1 litre with distilled water, and store at 4°C.
b) Working buffer. Dilute stock buffer 1 in 5 with distilled water. The pH will be 6.
c) Gel: Pre-prepared made Titan IV Citrate Agar plates, provided by Helena Laboratories U.K.
d) Bromophenol blue - for staining of the gel.
Dissolve 20 mg bromophenol blue in 200 ml distilled water containing 2ml glacial acetic buffer.

Equipment:
a) Power supply capable of delivering 30-40 mA is necessary (Vokan SAE 2761, Vokan 400).
b) A southern horizontal electrophoresis tank.
c) Gel slot, a device for making sample application wells.

Method:
1. Using the gel slot, 5 slots are made on the gel, mid-way between anode and cathode.
2. Using a capillary tube enough haemolysate is delivered to fit each slot on the gel.
3. 700 ml of the working buffer is poured into the electrophoresis tank.
4. Wicks made from Whatman No.3 chromatographic paper, are placed along the bridges of the electrophoresis tank. The gel is placed along the wicks and is held in position using extra wicks made of Whatman No.3 chromatographic paper in order to ensure contact with the buffer.
5. A current 30 mA approximately (40 V) is applied across the gel.
6. Electrophoresis is run at 4°C.
7. After approximately 3 hours, separation is achieved.
8. The agar plate is stained for 20 minutes with the bromophenol blue stain and then rinsed with distilled water.

Interpretation: Typical results of agar gel electrophoresis are shown in Figure 3.3.

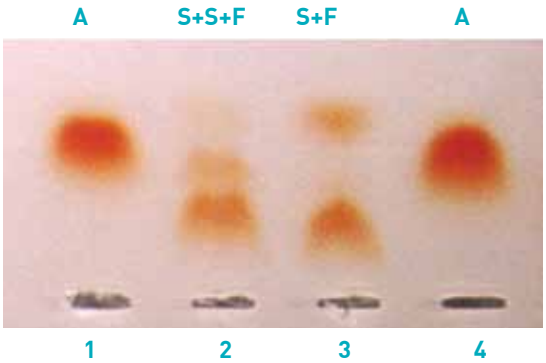


FIG. 3.3
Example of agar gel electrophoresis
1. Lanes 1,4: normal adult
2. HbS/β^+-thalassaemia
3. HbS/β^0-thalassaemia

3.2.5 ISOELECTRIC FOCUSING

Isoelectric focusing is the electrophoretic method that separates proteins according to the isoelectric points. The net charge of a protein is the sum of all negative and positive charges of its amino-acid chains and their ionisable groups (amino and carboxyl termini). The isoelectric point (pI) is the specific pH at which the net charge on the molecule is zero (proteins are positively charged at pH values below their pI and negatively changed at pH values above their pI) (13, 14).

A pH gradient is necessary so that under the influence of an electrical field, a protein will move to the position where its net charge is zero. A protein with a positive net charge will migrate towards the cathode becoming progressively less positively charged as it moves through the pH gradient until it reaches a point that corresponds to its pI value.

Isoelectric focusing requires solid support such as agarose gel and polyacrylamide gel. Polyacrylamide is a polymer with small interstices - approximately the size of proteins so that apart from surface charge, separation depends on the size of the molecules. Isoelectric focusing gels contain synthetic buffers called ampholytes that smooth the pH gradients.

Isoelectric focusing needs high voltage (1000 V or more). It gives good separation with a high resolution compared to any other method. Resolution depends on:

i. The pH gradient,
ii. The thickness of the gel,
iii. Time of electrophoresis,
iv. The applied voltage,
v. Diffusion of the protein into the gel.

Method 1: Pharmacia PhastSystemTM
The preferred isoelectric focusing electrophoresis method in the Cyprus laboratory is the PhastSystemTM with dry polyacrylamide gels soaked prior to use in a narrow pH gradient (6.7-7.7). The

pH gradient is made by Pharmalyte® which generates stable, linear pH gradients in the gels during the run. The haemoglobins migrate under an electric field to a point in the pH gradient that corresponds to their pI (isoelectric point). The separated Hbs on the stained gel are evaluated by visual inspection. Protein patterns from known haemoglobin variants are used as references to identify the protein bands from unknown Hb samples.

Reagents:

a. Phast-Gel Dry IEF plates *
b. Pharmalyte pH 6.7-7.7*
c. Kerosene
d. 20% trichloroacetic acid (TCA) fixative
e. Staining solution stock: one tablet of Phast Gel Blue R tablet * (1 tablet + 80 ml distilled H_2O + 120 ml methanol). The solution was stirred for 2 minutes and filtered twice
f. Methanol
g. Destaining solution containing 300 ml methanol + 100 ml acetic acid + 600 ml distilled water (3:1:6). $CuSO_4$ ($5H_2O$)
h. Working solution for staining was made by mixing 30 ml stock staining solution with 270 ml of 3:1:6 destaining solution and 0.45 g $CuSO_4$ ($5H_2O$). The solution is filtered and is prepared fresh before use.
i. 0.1% Triton

Note: *These items are provided by the Amersham-Pharmacia company.

Equipment:

Pharmacia LKB - Phast System. The system consists of:

a. a separation unit
b. a developing unit for staining procedures.
c. Phast Gel sample - applicator 8/1
d. Phast Gel sample – well stamp
e. Scanning Densitometer LKB Pharmacia

All functions are controlled by a microprocessor.

Gel preparation: Rehydration of dry gels: dry gels are rehydrated using a 1:16 solution of Pharmalyte 6.7-7.7 (100 µl Pharmalyte + 1500 µl distilled H_2O). The dry plate is placed with the gel side down on the drop of the solution that has been pipetted on a clean plastic surface, for 1 hr checking at intervals that the plate did not stick to the surface.

After rehydration excess of fluid is removed from the surface of the gel by wiping it gently with the edge of a piece of filter paper.

Sample preparation: Samples are prepared by diluting 5µl of whole blood in 200 µl 0.1% Triton. The mix is allowed to stand for 5 minutes and then mixed on a vortex mixer. 5µl is used for the sample application.

Sample application: To load the sample applicator, depressions are formed on a strip of Parafilm (using the PhastGel sample well stamp). 5 µl of each sample is placed on the depressions. The PhastGel sample applicator 8/1 is dipped in the 5 µl droplets on each sample.

Method:

1. The plates are placed on the cooling bed of the Phast System, on which two drops of kerosene are placed on each plate.
2. After application of the electrodes on the gel the pre-focusing stage is performed.
3. After pre-focusing, the samples are applied anodally to the gels using the 1 µl applicator.
4. For each type of plate a different separation program on the Phast System is used through the microprocessor of the Phast unit.
5. For running Phast Gel Dry IEF 6.7-7.7 the following separation program is used:

Sep. 1.1	1000 V	2.0 mA	2.0 W	15 t	75 vh
Sep. 1.2	300 V	1.0 mA	1.0 W	15 t	25 vh
Sep. 1.3	1500 V	5.0 mA	3.5 W	15 t	550 vh
Sep. 1.4	0V	0 mA	0 W	0 t	0 vh

6. End of separation program:
   Sample applicator down at 1.2 vh
   Sample applicator up at 1.3 vh
7. The method called separation method 1 was programmed on the Phast System previously and contains three separating steps, Sep 1.1, Sep 1.2 and Sep 1.3. A final step, Sep 1.4, was added to finalise the program.
8. The sample applications will be lowered onto the gels at after 75 vh during Step 1. During this 75 vh period, the sample applicators are loaded. An alarm sounded at 73 vh as a warning that the sample application will occur in 2 vh. The sample applicators are automatically raised at 100 vh of Step 3 and separation proceeds as indicated in the above program.

Staining: After the focusing steps have been performed, the gels are placed in the staining chamber. The plates are fixed with 20% TCA for 5 minutes at 20ºC, and washed with distilled water for 2 minutes. Subsequently they are stained for 10 min with 0.2% Coomassie Blue (1:10 solution, of stock solution: destain solution), containing 0.15 w/v % $CuSO_4$ ($5H_2O$) at 50ºC.

The plates are destained in the destain solution for 10 min at 50ºC and allowed to dry. Plates are scanned on a laser scanning densitometer for determination of pI values. Samples containing known haemoglobins (HbF, HbS, HbA_2, HbA, Hb Lepore, HbD) as well as the sample containing the unknown haemoglobin are run on the same plate. The method does not distinguish between Hb G-Philadelphia, Hb Lepore, HbE and Hb O-Arab.

Interpretation: The identification of the unknown haemoglobin is achieved by measuring the pI value of the unknown haemoglobin in a laser densitometer. The pI values of known haemoglobins, separated on the same plate, are also measured and are used as controls. Despite excellent reso-

lution achieved by isoelectric focusing, accurate quantification is not possible.

A Phast Gel plate pH 6.7-7.7 showing the separation of various haemoglobins is illustrated in Figure 3.4

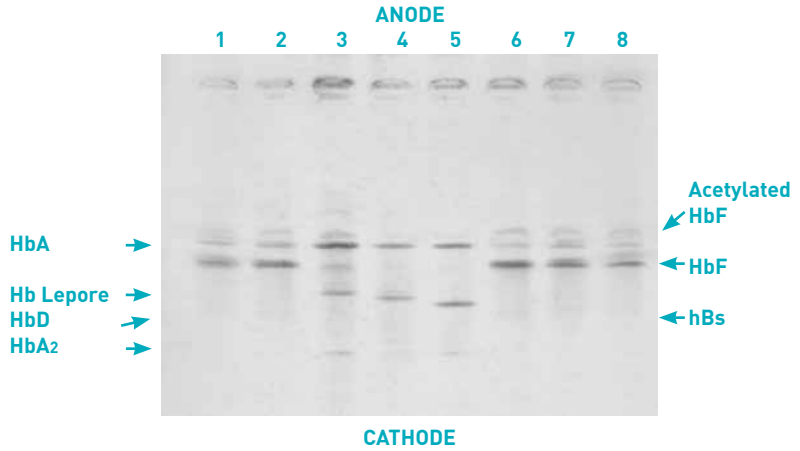


FIG. 3.4
A Phast Gel plate pH 6.7-7.7 showing the separation of various Hbs by IEF.
Lanes 1, 2, 6, 7, 8 are cord blood samples
lane 3 is Hb Lepore trait
Lane 4 is HbD trait
Lane 5 is HbS trait

Method 2: PerkinElmer RESOLVE® System
The preferred isoelectric focusing electrophoresis method in the Oxford laboratory is the RESOLVE® Systems K Haemoglobin Kit, used to detect haemoglobin variants in whole blood (EDTA) for antenatal screening or blood spot samples from Guthrie cards for neonatal screening.

Reagents:

a. RESOLVE® FR-9120 Haemoglobin Kit – contains 5 IEF gels, an anode and cathode solution, Hb elution solution, electrode wicks, blotting paper and strips and sample application templates.
b. JB-2 Staining System - 2x Gel Stain Concentrate, 2x Stain Buffer and 1x Stain Activator.
c. 10% Trichloroacetic acid -To make up to 500 ml 10% TCA, add 50 ml 6.1N TCA to 450 ml distilled water.
d. Vnbs Retention Time markers (FASE and FACD) are used as control samples. These are made up with 1ml de-ionised water, aliquotted into microvials, and frozen at -70ºC until required. Those in use are stored at 4ºC. Use FASE twice, one at the beginning and end of the run. Use FADC in the middle of the run (as well as FASE if space allows).

Equipment:
PerkinElmer RESOLVE® System consists of:

a. Multiphor II electrophoresis unit
b. A circulating water bath to provide constant temperature control to the electrophoresis unit
c. Programmable Power Supply

Sample preparation from whole blood:

1. Pipette 5 µl of whole blood sample into a labelled Eppendorf tube (try not to mix the blood and use

the packed cell portion).
2. Add 50 µl of Hb Elution solution provided with the IEF gel kit.
3. Vortex the tubes and leave to lyse for approximately 10 minutes.

Sample preparation from blood spots on Guthrie cards:

1. Allocate a well number for each specimen. Using a manual puncher, punch 3 mm holes from the Guthrie cards and place in the corresponding well of the microtitre plate. Use two spots for samples with HbA levels of less than 3% and abnormal variant levels of less than 2% on the Vnbs.
2. Add 80 µl of Hb Elution solution to each well.
3. Cover the microtitre plate and allow to stand for 30 min at room temperature. Mix the blood spots using a sonic bath for 5 min.

Electrophoresis:

1. The precast gels and solutions (Hb elution solution, cathode solution and anode solution) are kept 2-8ºC and should be taken out of the fridge approximately 30 minutes prior to use, to bring up to room temperature.
2. Start up the water bath by turning on the Power Switch and the Refrigeration Switch. The temperature at temperature controller (Walton SD) will start going down. After a few minutes, check the 'outlet' and 'inlet' tubing of the bath and the cooling plate in the electrophoresis unit to see if they are cooled down. If they are not cool, the bath needs to be switched off and switched on again observing for bubbles in the lines which may cause airlocks.
3. Pipette water onto the centre of the cooling plate. Take out the gel and using both hands hold the edges of the gel. Position the gel in the centre of the cooling plate between lanes 4 and 14 and bending gently, roll from side to side to ensure water spreads evenly under the gel. Be careful not to trap air bubbles between the gel and cooling plate.
4. Remove the topmost sheet on the gel and blot gently. Any excess water from the periphery of the gel should be removed with paper towel.
5. Evenly saturate one wick (rough side up) with the cathode solution on several paper towels and blot to remove excess water. Place the wick between positions 3-4 of the cooling plate. Remove gloves and repeat with another wick using the anode solution. Place this wick between positions 13-14. Change gloves again.
6. Place the sample template strip carefully onto the gel, next to the cathode wick. Run your finger gently along the sample template strip to remove air bubbles.
7. Load controls and specimens according to the worksheet (5 µl for control and neonatal samples, 3.5 µl for adult specimen).
8. Place the electrode holder over the gel with the cathode electrode on left (black) and centre the electrodes over the wicks. Connect the electrode leads into the connectors on the inside of the tank. Place the cover over the electrophoresis unit.
9. Switch on the power supply. Press "run" at each prompt until the voltage readout shows increasing voltage (In some cases the power does not reach the 1500 V but that usually does not cause a problem).
10. After 20 minutes, press 'standby' to pause the program. Remove the sample template strip and blot the gel. Press 'Run' to continue the program. Repeat blotting after 20 minutes (optional).

11. The program takes 1 hour and 30 minutes to run. However, if the bands are separated and clearly visible on the gel (after approximately 1 hour and 15 minutes), press 'stop' to terminate the run. Switch off both the cooler unit and the power supply. Remove the cover, disconnect the electrodes and remove the electrode holder.
12. Make sure that the cooling plate and electrodes are wiped down with deionised water and a paper towel after use. When cleaning the electrodes, do so gently so as to avoid bending and damaging the electrodes.

Fixing and Staining Gel:

1. Place the gel in a staining tray. Take it to the fume cupboard and add 100 ml of 10% TCA or sufficient to cover the gel, place the lid on the staining tray and mix it gently on the mixer for 10 minutes.
2. Pour off the TCA down the sink and run plenty of water afterwards. Add deionised water in the staining tray and mix it gently for a couple of minutes. Leave it to stand for 10 minutes whilst the gel stain is prepared. Always make the stain fresh.
3. Take the box with the stains into the fume cupboard. Add 15 ml of stain buffer and 27 ml of gel stain concentrate to the designated 500 ml measuring cylinder. Make up to 150 ml with deionised water. Add 3 ml of stain activator. Place parafilm over the cylinder and gently mix.
4. Pour off the water on the gel and add the stain. Place the lid on the tray and place it on the mixer. Leave it to agitate gently for 10-15 minutes or until the HbA band in the neonatal samples is clear.
5. Pour off the stain down the sink with copious amounts of water. Submerge the gel in deionised water and place it back on mixer for at least 1 hour, changing the water at least once.
6. Air-dry the gel on a paper towel overnight. Write the gel number on the paper towel.
7. Next day, number the lanes and write the gel number on the gel using a marker pen.

Calculation of Results:

1. Measure the gel distance, in mm, from HbA to HbS, HbA to HbC, HbA to HbD and HbA to HbE (use bands of the control samples: FASE and FADC).
2. Calculate the multiplication factor by dividing the reference distance by the gel distance. For example, if the gel distance from HbA to HbS is 8 mm, the multiplication factor for any band positioned between Hb A and Hb S will be 8.5 mm / 8.0 mm.
3. To determine the relative distance of an unknown band on the gel, measure the gel distance between HbA and the unknown band and multiply it with multiplication factor. The relative distance will then be obtained. Thus a band running a 6.5 mm below HbA is recalculated to a position of 6.9mm.
4. Some bands may appear above the HbA band - these are noted with a negative measurement.
5. The Hb variant may now be given a presumed identification by comparing its relative distance to those of known variants observed on previous gels, or against the positions marked on the published isoelectric focusing map of human haemoglobins (13, 14).
6. Additional genetic investigations should be done to confirm the presumptive diagnosis when it is needed.

Interpretation: An example of isoelectric focusing electrophoresis is shown in Figure 3.5. As each gel run is slightly different, the band positions for each run should be standardised using the refer-

ence band distances from the published isoelectric focusing map as described above. The reference distances are: HbA to HbF 3 mm; HbA to HbS: 8.5 mm; HbA to HbC: 16.0 mm. Refer to the isoelectric focusing maps of human haemoglobins (13, 14) for variants that are out of the AFSC range. Electrophoresis of old samples may yield "aging bands". This is due to the degradation or oxidisation of the haemoglobin molecule.

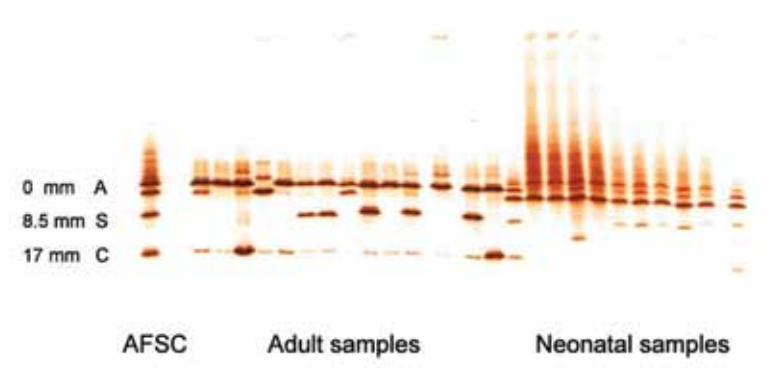


FIG. 3.5 Isoelectric focusing gel showing adult and neonatal samples using the RESOLVE system.

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CHAPTER 04

GLOBIN CHAIN SYNTHESIS

Globin chain synthesis analysis was introduced in the study of thalassaemia syndromes more than 30 years ago (1). It has greatly contributed to the understanding of the pathophysiological mechanisms of the different thalassaemia syndromes. Moreover, even in the DNA era it still remains a very sensitive diagnostic tool, very useful to define some complex or atypical forms of thalassaemia.

There are two established methods for globin chain synthesis analysis : one is the first classical method of Weatherall and Clegg, based on carboxymethyl cellulose chromatography for globin chain separation, which is described here and the other is based on reversed phase high performance liquid chromatography (HPLC) (2).

4.1 WEATHERALL AND CLEGG METHOD

The principle of this method is that the RNA in reticulocytes is used to synthesise haemoglobin *in vitro*. The reticulocyte rich cells are incubated with essential amino acids where one is radioactively labelled with tritium (^{3}H Leucine). The synthesised globin is precipitated with an acid/acetone mixture. The globin fractions are separated by column chromatography with a Urea buffer and a salt gradient. The radioactivity of each fraction is counted in a scintillation counter. The results are plotted on a graph and the globin fractions calculated.

This method has been used for prenatal diagnosis and the first prenatal diagnosis for haemoglobin disorders were based on this technique (3). Prenatal diagnosis is now routinely done using molecular techniques therefore globin chain biosynthesis is rarely used in prenatal diagnosis except in the rare situations where the parental mutations are not known. It may be used in some centres if a couple presents late for prenatal diagnosis, or if the partner is unavailable for testing. It is more commonly used to test individuals who are suspected to be carriers of ß-thalassaemia but have atypical haematological results, these can include individuals who have moderate microcytic hypochromic red blood cell indices and normal or borderline HbA_2 levels, or individuals who have normal red cell indices and raised HbA_2 levels. If globin chain biosynthesis gives a biosynthesis ratio for beta thalassaemia trait the diagnosis may be made of ß-thalassaemia trait. Globin chain synthesis is also useful for haemoglobinopathy patients undergoing bone marrow transplant to monitor the ß/α activity following the transplant.

When used for prenatal diagnosis the diagnosis is based on the relative synthesis of ß-globin (representing HbA) and γ-globin (representing HbF). A ß/γ chain synthesis ratio above 0.02 usually indicates an unaffected fetus. However the results should be interpreted with great care as mild ß+-thalassaemia mutations can produce higher levels of ß-globin, which may lead to an incorrect diagnosis of an unaffected fetus (4).

4.1.1 IN VITRO LABELLING OF HAEMOGLOBIN

Reagents: Krebs-Ringer phosphate (KRP) buffer. KRP is a balanced salt solution buffered at a physiological pH, made up from:

a. NaCl 1.5 M
Dissolve 87.66 g NaCl in 1 litre ion-free water.

b. Phosphate buffer pH 7.4
A. 0.1 M $NaH_2PO_4.2H_2O$ (MW 156) = 15.6 g in 1 litre.
B. 0.1 M $Na_2HPO_4.2H_2O$ (MW 178) = 17.8 g in 1 litre.
Add 19 parts of A to 81 parts of B, adjust pH to 7.4.

c. KCl 0.15 M
5.6 g KCl (MW 74.56) in 500 ml distilled water.

d. $MgSO_4$ 0.15 M
3.7 g $MgSO_4.7H_2O$ (MW 246.48) in 100 ml distilled water.

e. $CaCl_2$ 0.11 M
1.62 g $CaCl_2.2H_2O$ (MW 147.02) in 100 ml distilled water.

To make KRP for use:

1. Make up 100 ml of 1.5 M NaCl to 1 litre.
2. Add: 120 ml of phosphate buffer pH 7.4
3. Add: 40 ml of 0.15 M KCl
4. Add: 10 ml of 0.15 M $MgSO_4$
5. Add: 10 ml of 0.11 M $CaCl_2$ – stir carefully to avoid precipitation.
6. Adjust pH to 7.4 with NaOH (1 M)
7. Check osmolarity and adjust with 1.5 M NaCl stock solution or distilled water if necessary, to 280 – 300 m OsM.

Preparation of incubation medium: One pack (300ml) of AB Rhesus negative blood is obtained from the blood bank. The plasma transferrin is saturated with iron by adding 3.0ml ferrous ammonium sulphate solution (10.5 mg/10 ml H_2O) and leaving it to stand for 30 minutes at room temperature. Free amino acids are removed by dialysis at 4°C for 48 hours against KRP using boiled dialysis tubing. The KRP is changed at 1 hour, 6 hours and 24 hours. The calcium in the KRP causes clotting and the fibrin is removed by filtering the dialysed plasma through a nylon mesh. Glucose (6 mg/ml plasma) and leucine free amino acid mix (0.133 ml/ml plasma, details below) are added to the plasma and the pH adjusted to 7.4 with 1 M NaOH. The resulting incubation medium is stored in 0.5 ml aliquots at –40°C until required.

Preparation of the leucine-free amino acid mix: 20 mM (10 ml) of stock solutions of each amino acid are made in water, and stored at –40°C until required. Table 4.1 shows the composition of the amino acid stock solutions. To prepare the leucine-free amino acid mix, 1 ml of each stock solution, except leucine are mixed to give 20 ml of a 1 mM stock solution of all amino acids except leucine.

Table 4.1: Composition of single amino acid stock solutions used for the Weatherall and Clegg method.

	AMINO ACID	MW	mg/10ml
1	Alanine	68	13.6
2	Arginine	210	42.0
3	Aspartic acid	133	26.6
4	Asparagine	150	30.0
5	Cysteine	157	31.4
6	Glutamic acid	147	29.0
7	Glutamine	146	28.0
8	Glycine	75	15.0
9	Histidine	155	31.0
11	Isoleucine	131	26.2
12	Lysine	183	36.6
13	Methionine	149	29.0
14	Phenylalanine	165	33.0
15	Proline	115	23.0
16	Serine	105	21.0
17	Threonine	119	22.8
18	Tryptophan	204	40.8
19	Tyrosine	181	36.2
20	Valine	57	11.4

Preparation of ^{3}H leucine: The ^{3}H Leucine at a concentration of 5 mCi/5 ml aqueous solution containing 2% ethanol is too dilute particularly for the active labelling required for prenatal diagnosis samples. It is concentrated by using a freeze dryer. The ^{3}H Leucine is then taken up in a small measured amount of KRP - usually 0.5 ml/5 mCi ^{3}H Leucine, to give a final concentration of 10 mCi/ml.

When using fetal blood for prenatal diagnosis, the white cell removal and reticulocyte enrichment steps are not required.

Removal of white cells:

1. 10 ml blood is collected in Li-heparin anticoagulant and kept at 4°C.
2. The blood is sedimented by centrifugation at 4000 rpm for 15 minutes at 4°C and the plasma and buffy coat removed.
3. The top 0.5 cm, slightly reticulocyte-enriched portion of cells are resuspended in about 5 ml cold Krebs-Ringer phosphate (KRP) solution and loaded onto a column, 2 cm long x 1cm diameter, containing a mixture of α cellulose (Sigma C 8002) and Sigmacell Type 50 microcrystalline cellulose (Sigma S 5504) (20 g of each in 1 litre of normal saline).
4. The red cells are eluted from the column (the white cells remain behind) with another 5 ml of cold KRP, and washed twice in cold KRP by spinning for 5 minutes.

Radioactive labelling:

1. The red cell pellet is labelled by mixing with 0.5 mCi of ^{3}H Leucine, 0.3 ml of incubation mix (amino acid mix), 0.5 mls KRP, in a 2 hour incubation at 37°C in a shaking water bath.
2. The samples are then washed twice with cold KRP to remove free ^{3}H Leucine. The red cell pellet is either used directly for globin extraction or stored at -80°C.

4.1.2 CARRIER HAEMOGLOBIN

In some circumstances it is necessary to add carrier haemolysate before globin extraction, in order to localise the radioactive globin chains that may be present in very small quantities. This is particularly important for fetal samples, since the fetal ß-globin chains are present in small quantities and do not register on the optical density monitor during carboxymethyl cellulose column chromatography. Carrier haemolysate is a non-radioactive lysate made from an adult blood sample diluted to a Hb concentration of 2 g/dl and stored in aliquots at -40°C. Approximately 0.5 ml of this carrier solution is added to the radioactive test sample prior to globin extraction.

4.1.3 PREPARATION OF GLOBIN

Globin is prepared by acid/acetone precipitation (5). The incubated cells are lysed by freezing and/or by the addition of distilled water to make up a concentration of 2-4 g/100ml. The lysate is then added dropwise with stirring to acetone containing 1.5% (v/v) 11.3 M HCl previously cooled to -20°C. 20 ml of acid/acetone is used for 1 ml of haemoglobin solution. During this procedure the haemoglobin is denatured and the protein moiety precipitates while the haem remains in solution. The globin is then centrifuged at 3000 rpm at -20°C for about 1 minute. The acid/acetone containing the haem is removed by suction and the globin washed twice for 3 minutes with cold acetone that has been cooled to -20°C. A third wash is carried out in diethyl ether, the supernatant discarded and the tubes placed horizontally or slightly inverted to allow the ether to evaporate and drain out. Before the pellet is quite dry it is broken up with a Pasteur pipette so that by the time it dries completely it is clean, white and powdery. If the globin is not to be used immediately it is stored at -40°C.

4.1.4 CARBOXYMETHYL CELLULOSE CHROMATOGRAPHY

The method used is that of Clegg et al (1) with some modifications. The globin chains are separated on carboxymethy cellulose (CM23) column chromatography using a urea-phosphate buffer gradient. Other resins may be used such as CM23. The phosphate buffer pH 6-7 is optimal for the separations of globin chains, but at this pH globins are relatively insoluble, so urea is needed as a solvent. Furthermore a reducing agent (dithiothreitol) must be added to prevent formation of mixed disulphides by the free sulphhydryl groups of the globins. Since the ability to bind to the CM-cellulose depends on the net positive charge of the globins, the affinity of each globin for cellulose at pH 6-7 is α > β > γ. Thus γ-chains are eluted out of the column first, then β, δ and α-chains respectively. The method described is for the preparations of 6 columns.

Reagents:

a. **Preparation of Urea Buffers:**
1440 g of urea is dissolved in distilled water and made up to 3 litres with distilled water to make an 8 M solution. The urea solution is filtered through a column of mixed-bed resin deionizier. The filtered solution is divided into two lots as follows:

b. **Low concentration Buffer:**
1800 ml 8 M urea solution plus 1.05 g of disodium hydrogen orthophosphate (Na_2HPO_4), adjusted to pH 6.4 with 20% orthophosphoric acid and 180 mg dithiothreitol added.

c. **High concentration Buffer:**
1200 ml 8 M urea solution plus 5.1 g of Na_2HPO_4, adjusted to pH 6.4, and 120 mg dithiothreitol added. The Na_2HPO_4 gives a gradient of 0.004 – 0.028 M.

Column chromatography: Figure 4.1 shows the chromatography set up. 15 g Carboxymethyl cellulose (CM23) is suspended in about 200 ml weak buffer for a few minutes and the suspension poured into 6 glass columns (0.5 cm x 15 cm). The columns are allowed to settle partially and then refilled to a level of 12 cm. Each column is then connected via polythene tubing to a Uvicord S11 spectrophotometer and a fraction collector. The optical density of the effluent is monitored continuously at 280 nm, and recorded on a six-channel recorder. One channel is used for recording the number of fractions each time the fraction collector moves, thus the peak optical densities of the α, β & γ fractions can be localised. After the columns are packed they are washed with weak buffer at a flow rate of 0.5 ml/min for 10-15 minutes. The globin samples are then dissolved, 5-10 mg of globin in 2-3 ml weak buffer is applied carefully to the columns, allowed to pass through the resin, and washed with 10 ml weak buffer. The columns are stoppered and joined to 6 peristaltic pumps. Elution with weak buffer is started immediately at a flow rate of 5 ml/12 min.

A sodium phosphate gradient is made with a two-chamber linear gradient maker (each chamber is 9 cm in diameter connected to each other by polythene tubing- (this can be made in house with perspex). In this set-up, the principle compartment that flows out to the columns starts with 1200 ml of a low concentration buffer, the other compartment starts with 1200 ml of high concentration buffer. Prior to starting the run, the compartments are separated by a clip in the connecting

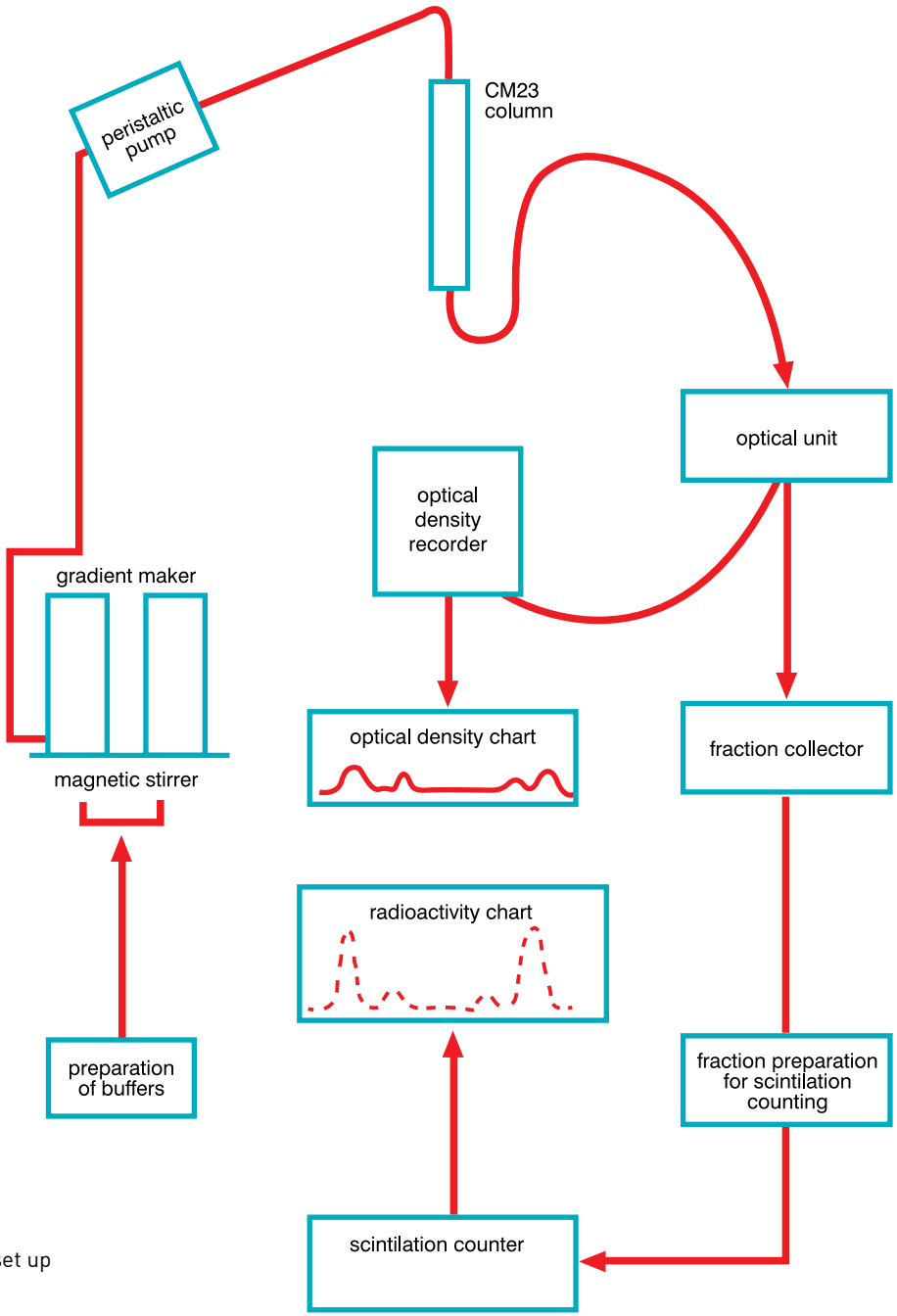

FIG. 4.1
Column set up

tubing. The weaker buffer is first added, this is used to rinse out the tubing between the gradient maker and the fraction collectors. Once the columns are joined to the peristaltic pumps, the level of the low concentration buffer compartment is adjusted to the same level as the high concentration buffer compartment, and the two compartments are then joined by releasing the clip on the connecting polythene tubing.

The flow rate of the peristaltic pumps is regulated to 5 ml/12 min, and the fraction collectors pre-set to collect 12 minute fractions. All the equipment is connected to a timer pre-set for 17 hours, ensuring that at least 80 x 12-minute fractions are collected by the end of the run.

4.1.5 RADIOACTIVITY COUNTING

At the end of the run, the optical density profile from the recorder is examined and aliquots for radioactive counting are taken beginning from the point where the protein starts to be eluted, to the end of the run. 0.5 ml aliquots are taken from the selected fractions into vials and 4.5 ml scintillation fluid is added. The tubes are shaken vigorously and once the gel is homogeneous the vials are placed in a scintillation counter. Each sample is usually counted for 5 minutes.

4.1.6 PROCESSING OF RESULTS

The counts per minute (cpm) for each column is plotted against the fraction numbers and the radioactivity profile compared with the optical density profile, to locate the α, ß and γ-chain peaks. The number of counts under each peak is calculated as shown in the example in Figure 4.2 and Table 4.2, and the biosynthetic ratios of the different chains obtained.

Pre-peaks are included in the calculations. The background is calculated by drawing a line across the two lowest points of the peak. Some centres calculate this by drawing a parallel line from the lowest point of the peak, and drop a perpendicular line to it, from the lowest point. The method chosen here is the previous method, although minimum values are calculated for the globin chains. This is particularly important for prenatal diagnosis samples.

FIG. 4.2
Elution curve of a thalassaemia intermedia individual

The number of counts under each peak were calculated as shown in table 4.2

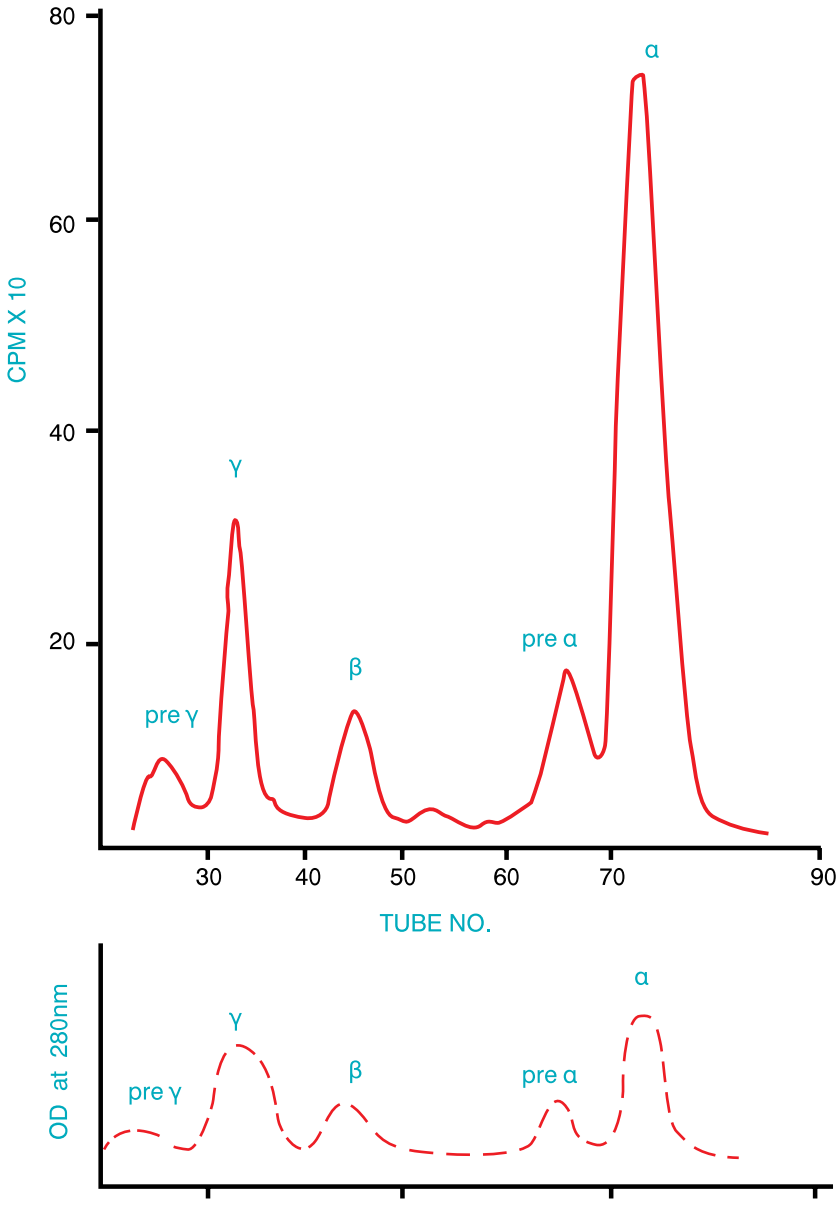

Table 4.2: The calculation of the ß/α, γ/α and non α/α globin chain synthesis ratios for the example shown in FIG. 4.2 (which is from a thalassaemia intermedia patient).

Fraction No.	C.P.M.
23	1801
	5979
	7606
	8619
	7548
	4737
	3557
	3633
	7455
	23810
	32316
	22181
	10164
	4842
	3588
	3251
	2975
40	2816
	2846
	4337
	9363
	13543
	12396
	7961
	4797
	3093
	2723
50	2759
	3307
	3864
	3554
	2845
	2391
	1998
	2340
	2204
	2734
60	3376
	3607
	4582
	7511
	12712
	17404
	17269
	12678
	7826
	9702
70	31566
	60255
	75288
	76211
	63549
	44519
	27189
	14686
	7264
	4028
80	2781

Pre γ + γ

Total counts from fractions 23-39 = 149286

Background counts (fractions 23+39) = $\frac{1801 + 2975}{2} \times 15$

= 35820

Counts under Pre γ + γ peak (total counts minus background) = 149286 - 35820

= 113466

Pre ß ± ß

Total counts for preß + ß = 55490

Background counts = 19492

Counts under peak = 35998

Pre α ± α

Total counts for preα + α = 497845

Background counts = 58492

Counts under peak = 439353

$\frac{\text{Preγ + γ}}{\text{Preα ± α}}$ = 0.26

$\frac{\text{Preß + ß}}{\text{Preα ± α}}$ = 0.08

$\frac{\text{Non α}}{\text{α}}$ = 0.34

4.1.7 STANDARDIZATION OF COLUMNS AND RESULTS

Columns should be standardized using normal adult and normal cord blood samples. Conditions (pH, gradient, temperature) should be adjusted until a balanced non α/α ratio is obtained in these normal samples. Figure 4.3 and Figure 4.4 show elution patterns obtained with standard normal adult and beta thalassaemia trait samples. During a column run, a control sample should be run in parallel. In our centre, a biosynthesis ratio of β/γ above 0.02 indicates an unaffected fetus. For non-fetal samples: Normal β/α 0.96 (range 0.78-1.14); β-thalassaemia trait β/α 0.50 (range 0.38-0.62). Only carriers of mild β^+ promoter mutations are significantly separated from the β^0 and β^+-thalassaemias where they will typically have a biosynthetic ratio well above 0.5. The ratio for α^0-thalassaemia trait is β/α 1.44 (range 1.22-1.82); α^+-thalassamia trait β/α 1.20 (range 0.95-1.39); HbH disease β/α 2.30 (range 1.80-2.95).

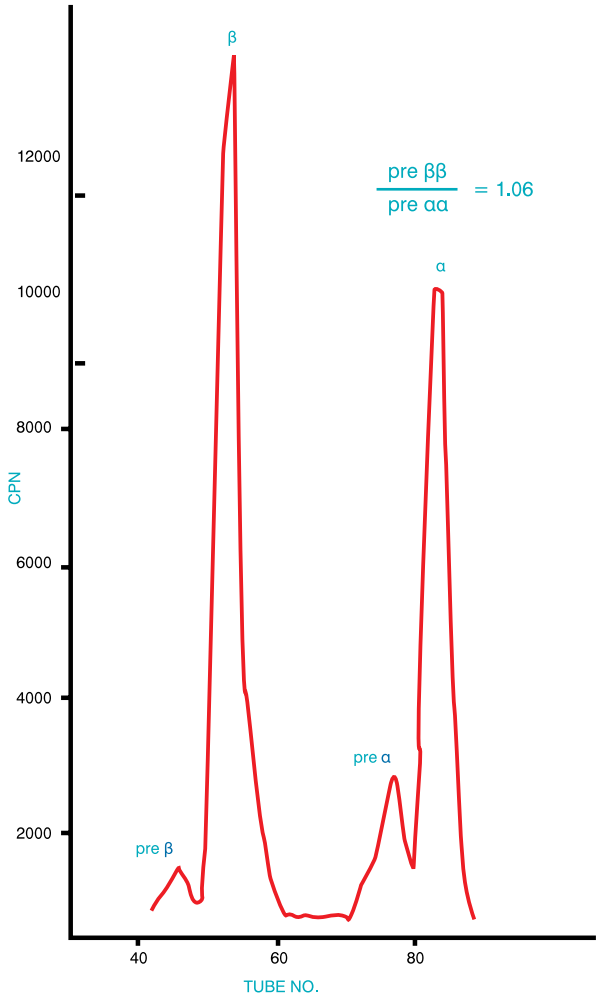

FIG. 4.3
Elution curve
of normal individual

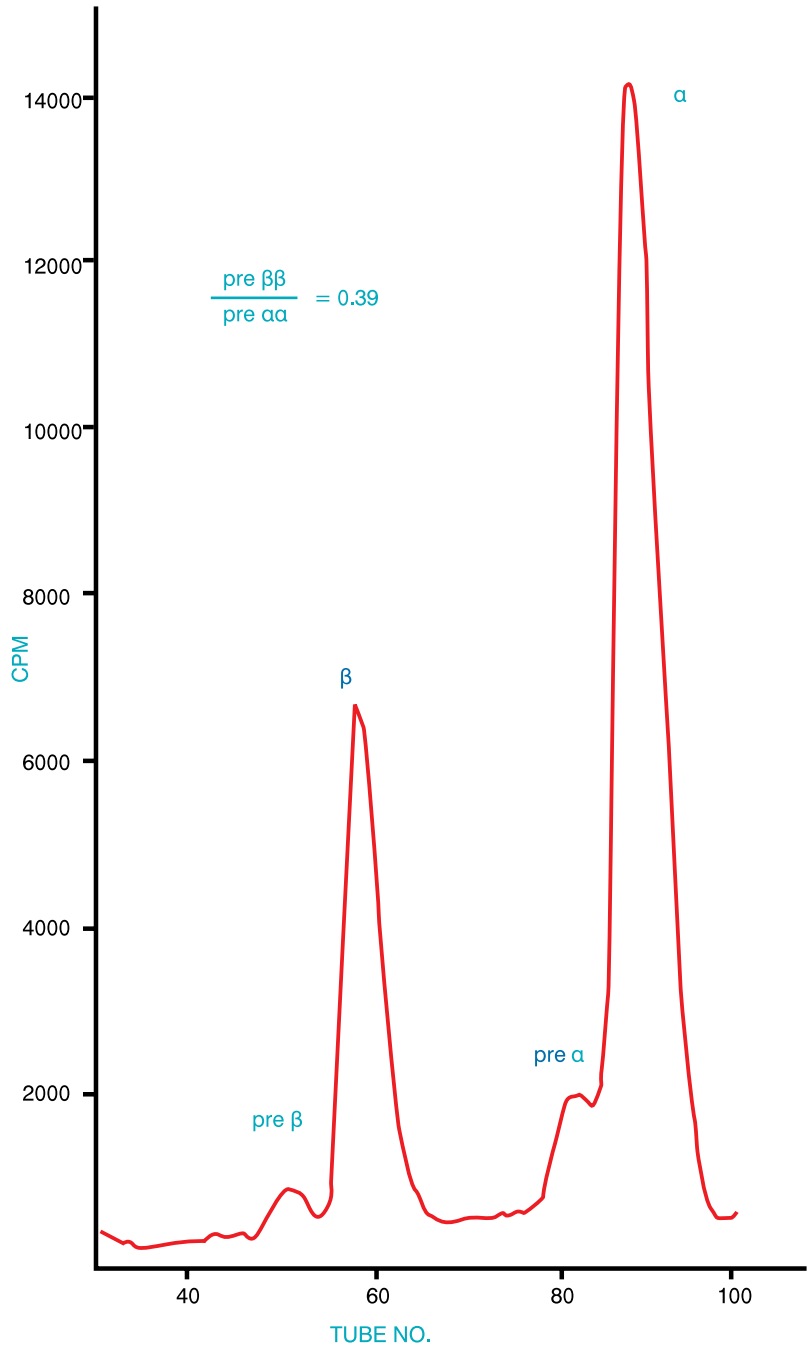

FIG. 4.4
Elution curve of beta thalassaemia trait individual

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CHAPTER 05

MOLECULAR DIAGNOSIS

The haemoglobinopathies are a diverse group of inherited recessive disorders that include the thalassaemias and sickle cell disease. They were the first genetic diseases to be characterised 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 PCR-based techniques that can be used to detect globin gene mutations, including dot blot analysis, reverse dot blot analysis, the amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis (DGGE), mutagenically separated polymerase chain reaction, gap-PCR, restriction endonuclease (RE) analysis, real-time PCR, Sanger sequencing, pyrosequencing, multiplex ligation-dependent probe amplification (MLPA) and gene array systems [1-4].

Each method has its advantages and disadvantages and the particular one chosen by a laboratory for the diagnosis of point mutations depends upon factors such as the technical expertise available in the diagnostic laboratory, the type and variety of the mutations likely to be encountered in the individuals being screened, as well as the budget available.

5.1 DIAGNOSTIC STRATEGIES

Table 5.1 summarises the main diagnostic approaches commonly used for the diagnosis of the haemoglobinopathies. A brief summary of the different categories of globin gene mutations and the main diagnostic approaches is presented here, followed by a section containing detailed protocols.

5.1.1 α-THALASSAEMIA

α-Thalassaemia alleles result from mutations affecting either one α-globin gene (α^+-thalassaemia) or both α-globin genes on the same chromosome (α^0-thalassaemia). The majority of the commonest mutations are gene deletions but a number of point mutations within one of the two α-globin genes resulting in α^+-thalassaemia have been described.

Table 5.1 The principal methods of DNA diagnosis of the haemoglobinopathies.

DISORDER AND MUTATION TYPE	DIAGNOSTIC METHOD
α^0-thalassaemia	Gap-PCR, MLPA
α^+-thalassaemia: deletion non deletion	 Gap-PCR, MLPA ASO, RE, DGGE, Sanger sequencing
ß-thalassaemia: deletion non deletion	 Gap-PCR, MLPA ASO, RDB, ARMS, RE-PCR, Sanger sequencing
δß-thalassaemia	Gap-PCR, MLPA
HPFH deletion non deletion	 Gap-PCR, MLPA ASO, ARMS, RE-PCR, Sanger sequencing
Hb Lepore HbS HbC HbE Hb D-Punjab Hb O-Arab Hb variants	Gap-PCR, MLPA ASO, RDB, ARMS, RE-PCR, pyrosequencing ASO, RDB, ARMS, pyrosequencing ASO, RDB, ARMS, RE-PCR, pyrosequencing ASO, RDB, ARMS, RE-PCR, Sanger sequencing ASO, ARMS, RE-PCR, Sanger sequencing Sanger sequencing

5.1.1.1 DIAGNOSTIC STRATEGY

The strategy for screening for α-thalassemia mutations is directed by the haematological findings and the ethnic origin and of the individual being screened. α^0-Thalassaemia is found in mainly patients of Mediterranean or Southeast Asian in origin, although it may be found at lower frequencies in other ethnic groups such as Syrian and Iranian. Only one or two vary rare α^0-thalassaemia mutations have been described in patients of Asian Indian or African origin, and thus patients with a MCH value below 25 pg will usually have the genotype of homozygous α^+-thalassaemia rather than α^0-thalassaemia trait. α^+-Thalassaemia can reach high gene frequencies in parts of Africa and Asia, with the $-\alpha^{3.7}$ deletion being the predominant mutation in African, Mediterranean and Asian individuals and the $-\alpha^{4.2}$ being more common in Southeast Asian and the Pacific islands populations.

5.1.1.2 DIAGNOSIS OF DELETION MUTATIONS BY GAP-PCR.

Gap-PCR provides a quick diagnostic test for α^+-thalassaemia and α^0-thalassaemia deletion muta-

tions but requires careful application for prenatal diagnosis. Most of the common α-thalassaemia 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-thalassaemia deletions and two α^+-thalassaemia deletions [5-9], as listed in Table 5.2. The α^0-thalassaemia 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 Filipino individuals and finally the $--^{THAI}$ allele, found in Thai individuals. The two α^+-thalassaemia deletion mutations are 3.7 kb and the 4.2 kb single α-gene deletion mutations, designated $-\alpha^{3.7}$ and $-\alpha^{4.2}$.

Table 5.2 Thalassaemia deletion mutations which have been diagnosed by gap-PCR.

DISORDER	DELETION MUTATION	REFERENCE
α^0-thalassaemia	$--^{SEA}$	[6]
	$--^{MED}$	[6]
	$-(\alpha)^{20.5}$	[6]
	$--^{FIL}$	[8,9]
	$--^{THAI}$	[8.9]
α^+-thalassaemia	$-\alpha^{3.7}$	[7]
	$-\alpha^{4.2}$	[7]
β^0-thalassaemia	290 bp deletion	[75]
	532 bp deletion	[76]
	619 bp deletion	[77]
	1393 bp deletion	[78]
	1605 bp deletion	[58]
	3.5 kb deletion	[59]
	10.3 kb deletion	[60]
	45 kb deletion	[61]
$(\delta\beta)^0$-thalassaemia	Hb Lepore	[62]
	Spanish	[62]
	Sicilian	[62]
	Vietnamese	[62]
	Macedonian/Turkish	[20]
$(^A\gamma\delta\beta)^0$-thalassaemia	Indian	[62]
	Chinese	[62]
HPFH	HPFH1 (African)	[62]
	HPFH2 (Ghanaian)	[62]
	HPFH3 (Indian)	[62]

Amplification of sequences in the α-globin gene cluster is technically more difficult than that of the ß-globin gene cluster, requiring more stringent conditions for success due 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 drop out. However the published primers [8,9] seem to be much more robust at amplifying than the earlier published sequences, possibly due to the addition of betaine to the reaction mixture. They are also designed for a multiplex screening test, although in several laboratories they are still used in pairs to test for individual mutations.

5.1.1.3 DIAGNOSIS OF DELETION MUTATIONS BY MLPA.

The rare α^0 and α^+-thalassaemia mutations cannot be diagnosed by gap-PCR because their breakpoint sequences have not been determined. Instead these deletion mutations can be diagnosed by the multiplex ligation-dependent probe amplification assay (MLPA) [10]. This approach is very useful as it permits the diagnosis of any α-thalassaemia deletion (known and unknown) in a single test [11]. The technique will also identify gene rearrangements which lead to the duplication of the α-globin genes in the form of triple and quadruple α–gene alleles [12].

5.1.1.4 DIAGNOSIS OF DELETION MUTATIONS BY OTHER METHODS.

Other approaches have also been developed to provide quick simple, rapid, accurate and cost effective methods of screening for the deletion mutations. These include the use of real-time quantitative PCR analysis for the Southeast Asian α^0-thalassaemia deletion in Taiwan [13], real-time gap-PCR and high resolution melting analysis for the Southeast Asian α^0-thalassaemia deletion [14], denaturing HPLC for the diagnosis the 4.2 kb α^+-thalassaemia deletion gene in Chinese individuals [15], and the use of an oligonucleotide microarray to detect the Southeast Asian α^0-thalassaemia deletion and the 3.7kb and 4.2kb α^+-thalassaemia deletions [16, 17].

5.1.1.5 DIAGNOSIS OF NON-DELETIONA-THALASSAEMIA MUTATIONS.

Non-deletional α^+-thalassaemia is caused by point mutations, small deletions or insertions in one of the two α-globin genes. These 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. For screening unknown mutations, DNA sequence analysis is the gold standard method [18]. For the diagnosis of known mutations, many other methods have been applied. For example, restriction enzyme digestion of amplified product (RE-PCR) has been used for the diagnosis of the mutation for Hb Constant Spring mutation using the enzyme Mse I [19]. In theory any general technique for the direct detection of point mutations such as allele specific oligonucleotide hybridisation or allele specific priming may be used for the diagnosis of non-deletion α^+-thalassaemia mutations. However such strategies have been developed just for the limited range of non-deletion mutations that are found predominantly in a single ethnic group, and no simple strategy to diagnose all the known mutations

has been developed. For example, reverse dot blotting has been applied for diagnosing six Mediterranean α+-thalassaemia point mutations [20], and the amplification refractory mutation system (ARMS) for diagnosing six common Southeast Asian point mutations [21]. The closest approach to a comprehensive screening system is the development of a commercially available strip assay using reverse hybridisation to detect two point mutations in the α1-gene and eleven point mutations in the α2-gene [22]. Thus the gold standard approach in most specialised laboratories to identify any unknown non-deletion α+-thalassaemia mutation is selective amplification of each gene followed by the whole sequencing each α-globin gene in turn, as illustrated by the report of the use of DNA sequence analysis for the molecular prenatal diagnosis of HbH hydrops fetalis caused by non-deletion mutation giving rise to Hb Adana [23].

5.1.2 ß-THALASSAEMIA

Although more than 170 different ß-thalassaemia have been characterised [24], only approximately 30 mutations are found at a frequency of 1% or greater in at risk groups. Most mutations are regionally specific and the spectrum of mutations for has now been determined for most at risk populations in each of the four regions (Mediterranean countries, Asian-Indian, Southeast Asian and African [25]. Countries without a large multi-ethnic immigrant population have just a few of the common mutations together with a larger and more variable number of rare ones. This makes it easy to screen for ß-thalassaemia mutations in most cases if the ethnic origin of the patient is known.

5.1.2.1 DIAGNOSTIC STRATEGY

The diagnostic strategy in many diagnostic laboratories screening for a limited mutation spectrum is to a use a simple and cheap PCR based technique that allows the detection of the common mutations simultaneously [26]. Although a bewildering variety of PCR technologies have been developed and applied for screening ß-globin gene point mutations, most diagnostic laboratories are still using a simple and cheap technique based on allele specific oligonucleotide hybridisation or allele specific priming, eg reverse dot-blotting or ARMS [27]. This approach will identify the mutation in more than 90% of cases and then a further screening for the known rare mutations will identify the defect in most of the remaining cases. Mutations remaining unidentified after this second screening are treated as unknown mutations and then characterised by DNA sequencing.

However, in many European laboratories, the impact of migration by different populations with high frequencies of haemoglobinopathies has led to a significantly enlarged the range of haemoglobinopathy mutations that need to be detected. This has increased the number of possible combinations and interactions of different mutations that need to be analysed for prenatal diagnosis. Molecular diagnostic laboratories in such countries must have the technical expertise, equipment and diagnostic strategy to detect a large variety of mutations quickly for prenatal diagnosis, and these laboratories use DNA sequencing as the main screening method for the diagnosis of ß-thalassaemia point mutations.

5.1.2.2 ALLELE-SPECIFIC OLIGONUCLEOTIDES

The first PCR based method to gain widespread use was the hybridisation of allele-specific oligonucleotide probes (ASOs) to amplified DNA bound to nylon membrane by dot-blotting [28]. Although still in use, the method is limited by the need for separate hybridisation steps to test for multiple mutations. This was overcome by the development of the reverse dot-blotting technique, in which amplified DNA is hybridised to a panel of mutation specific probes fixed to a nylon strip. This technique is compatible with the optimum strategy for screening ß-thalassaemia mutations, using a panel of the commonly found mutations for the first screening and a panel of rare ones for the second screen [29]. It has been applied to the diagnosis of ß-thalassaemia mutations in Mediterranean individuals [30], African-Americans [31] and Thais [32].

Allele specific hybridisation screening is the only technique for the diagnosis of ß-thalassaemia mutations to have been developed commercially with some success and there are currently there are two competing systems on the market. Vienna Lab have a strip assay using allele-specific oligonucleotide probes which reverse-hybridise to biotinylated DNA. The assays cover 21 α-thalassaemia mutations and 22 ß-thalassaemia mutations, the latter optimised in separate strips for the common Mediterranean, Middle Eastern and Indian/Southeast Asian mutations. Bio-Rad Laboratories has developed a different system with the oligonucleotides complementary to mutant and normal sequences immobilised on the wells of a microplate (mDx Gene kits). There are two kits for ß-thalassaemia, one for the eight most common Mediterranean mutations and one for the eight most common Asian ß-thalassaemia mutations (including HbE), plus a kit for alpha thalassaemia mutations (including the anti 3.7 qqq allele) and a variant one for identifying the HbS and HbC alleles.

5.1.2.3 OLIGONUCLEOTIDE MICROARRAYS

The principle of reverse dot blotting has been brought up to date by the development of oligonucleotide microarrays for the simultaneous detection of multiple ß-thalassaemia mutations. This approach promises a one-step strategy for the identification of all possible ß-globin gene mutations that result in both ß-thalassaemia and ß-chain variants. Several groups have now published details of a DNA chip platform which has been used to genotype ß-thalassaemia carriers and patients [17, 33]. The approach of tagged single-based extension and hybridisation to glass or flow-through arrays has been developed for the detection of 17 ß-globin mutations [34] and a similar approach of arrayed primer extension has been used to detect 23 mutations [35]. However the development of single array that will identify any one of the several thousand possible DNA sequence changes in the ß-globin gene simultaneously is still awaited and may not be a feasible option in the low resource setting of some countries [36]. Arrays are discussed in more detail in Chapter 7.

5.1.2.4 PRIMER-SPECIFIC AMPLIFICATION

A number of PCR techniques for detecting ß-thalassaemia mutations have been developed based on the principle of primer-specific amplification. The most widely used method is known as the

amplification refractory mutation system (ARMS)[37]. The method provides a quick screening assay that is cheap and does not require high technology or dedicated instruments [38]. ARMS primers have been developed to screen for the common ß-thalassaemia mutations of all the main ethnic groups [39]. The technique has been established for prenatal diagnosis in countries such as India and Pakistan, because of its rapid and inexpensive properties for screening and prenatal diagnosis [40, 41], and ARMS primers can also can be multiplexed to screen for multiple mutations in a single PCR assay [42].

5.1.2.5 OTHER METHODS FOR POINT MUTATIONS

Denaturing gradient gel electrophoresis (DGGE) is an indirect method which has been widely used to characterise ß-thalassaemia mutations without any prior knowledge of the molecular defect [43]. This detects at least 90% of ß-thalassaemia mutations by a shifted band patterns of homoduplexes and heteroduplexes, and provides an alternative approach to ASO probes or ARMS in countries where a very large spectrum of ß-thalassaemia mutations occur [44]. DGGE has been used for prenatal diagnosis of ß-thalassaemia in India [45] and Greece [46], and also for the analysis of point mutations resulting in δ-thalassaemia and non-deletion hereditary persistence of fetal haemoglobin (HPFH) [47, 48].

Restriction enzyme PCR (RE-PCR) has had a limited diagnostic role because very few ß-thalassaemia mutations create or abolish a restriction endonuclease site, although its use can be widened by the artificial creation of a restriction enzyme site which includes the target mutation [49]. The main use of RE-PCR has been for the analysis of ß-globin gene haplotypes to determine the origin of globin gene mutations in different ethnic groups [50].

Some other techniques have been applied for the diagnosis of known ß-globin gene point mutations. For example, DHPLC has been used for the analysis of polymorphic duplexes created by allele-specific priming [51], the analysis of five common Southeast Asian mutations by multiplex minisequencing [52], multiplex primer extension analysis for 10 Taiwanese mutations [53] and the most common Chinese mutations [54], and the screening for 11 commonest Greek mutations [55]. Real time PCR quantification and melting curve analysis have been used to provide rapid genotyping for a panel of the most frequent Mediterranean mutations [56] and six Lebanese mutations [57]. The DNA ligase method has been updated by the development of a novel piezoelectric method for detection of a single base mutation in codon 17 of the ß-globin gene using nano-gold-amplified DNA probes [58]. All provide rapid and accurate genotyping of the common mutations and are worth considering as alternative diagnostic approaches for point mutations.

5.1.2.6 DELETION MUTATIONS: GAP PCR AND MLPA

The larger $ß^0$-thalassaemia deletion alleles (290 bp to 67 kb) are diagnosed by the techniques of gap PCR [59-62] and/or MLPA analysis [10]. Table 5.2 lists the deletions for which the breakpoint sequences have been characterised and for which gap-PCR assays have been published. All deletion alleles are detectable by MLPA analysis, but unlike gap-PCR, it only provides a diagnosis that is

consistent with a particular known deletion mutation.

5.1.3 δß-THALASSAEMIA AND HPFH

The δß-thalassaemia, HPFH, Hb Lepore and Hb Kenya deletion mutations were characterised originally by restriction enzyme mapping using the Southern blot technique, but gap-PCR and/or MLPA is now used for their detection. Gap-PCR can be used for the diagnosis of most of the common δß-thalassaemia and HPFH alleles, including Hb Kenya as listed in Table 5.2. This technique provides a quick and simple genotype screening method for distinguishing the phenotype of HPFH trait from δß-thalassaemia trait in Asian Indian, African and Mediterranean individuals with raised Hb F levels. [63].

MLPA may be used as the front line screening method, or in combination with gap-PCR for diagnosing those individuals carrying a novel or one of the rarer δß-thalassaemia, εγδß-thalassaemia and HPFH deletion mutations [10]. MLPA is a fast and effective way of screening for the spectrum and frequency of ß-globin gene cluster deletion mutations in a particular population, as has been demonstrated for the UK and Chinese populations [64, 65].

5.1.4 Hb VARIANTS

More than 700 haemoglobin variants have been described to date, most of which were identified by protein analysis and have never been characterised at the DNA level. Positive identification at the DNA level is achieved by selective globin gene amplification and DNA sequence analysis. However the clinically important variants, HbS, HbC, HbE, Hb D-Punjab and Hb O-Arab, can be diagnosed by simpler DNA analysis techniques. All these variants can be diagnosed by ASO hybridisation, the ARMS technique or, except HbC, by restriction endonuclease digestion of the PCR product [66]. 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 for sickle cell disease. Similarly, the mutations giving rise to Hb D-Punjab and Hb O-Arab abolish an EcoR I site at codon 121. However the HbC mutation at codon 6 does not abolish the Dde I site is diagnosed by other methods. The HbE mutation can be diagnosed by ASO hybridisation, ARMS or restriction endonuclease analysis as the mutation abolishes a Mnl I site in the ß-globin gene sequence.

5.2 DNA PREPARATION

To prepare high-quality DNA the blood should be as fresh as possible. The handling of blood samples should be carried out with great care, and they should all be treated as potential sources of infection. As with all laboratory protocols the hazards of storage, handling and disposal of the various reagents must be assessed and addressed properly in the local laboratory standard operating procedure.

The white blood cells (WBC) of peripheral blood are usually the most convenient source of human genomic DNA for DNA analysis with respect to haemoglobinopathies. It is estimated that 10 ml of whole blood yield approximately 250 µg of DNA, more than sufficient for complete analysis of globin genes with the methods that are currently available (ie based on PCR). DNA is an extremely stable molecule, but enzymes that catalyse the breakdown of nucleic acids (nucleases) are found in all cells. In intact cells the DNA is found in the nucleus and thus is protected from the action of nucleases that are abundant in the lysosomes in the cytoplasm. However when cells are lysed, the membranes of the cell compartments are disrupted, allowing nucleases to come in to contact with the DNA. Thus the first stage of DNA extraction uses buffers which contain inhibitors of nuclease activity. Additionally all steps must be carried out at low temperatures (0°C). For long-term storage of samples prior to extraction a temperature of -70°C is recommended. There are many different methods described for DNA extraction from whole blood; the methods described below of salting–out and phenyl chloroform are used in the laboratories of some of the authors. There are also kits on the market for extracting DNA from blood samples that work well, but tend be expensive if used to prepare DNA from 5-10ml blood samples.

5.2.1 DNA EXTRACTION FROM BLOOD: SALTING-OUT METHOD

This method produces good quality high molecular weight DNA, avoiding the use of hazardous organic solvents.

Reagents:

a. Lysis I buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4.
b. Lysis II buffer: 10 mM Tris HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2.
c. 6 M NaCl
d. Proteinase K powder (Roche)
e. 100% Ethanol (store in 'flammables cabinet' with lock)
f. 70% Ethanol

Method:

1. Collect 10 ml whole blood with EDTA as anticoagulant.
2. Preferably freeze the whole blood at -20°C for a few hours to maximize the lysis of red blood cells.
3. Thaw blood, maintaining temperatures as low as possible, and transfer the haemolysate to a centrifuge tube (with cap) that holds at least 40 ml.
4. Add 20-25 ml of cold Lysis I buffer and mix very well. Leave to stand at 4°C for 30 minutes.
5. Centrifuge at 2500-3000g for 15 minutes at 4°C.
6. Carefully remove the supernatant without disturbing the soft pellet and wash the pellet twice more with 20-30 ml cold Lysis I buffer.
7. To the pellet (mainly WBC and cell membranes) add 6 ml of Lysis II buffer. Vortex very well to homogenize pellet, and then add 400 µl 10% SDS and approximately 2 mg proteinase K powder (a tiny sprinkle from the end of an orange stick).
8. Cover each tube, vortex well and incubate in a shaking water-bath for 16 hours (overnight) at 37°C, followed by 1 hour at 55°C.

9. Add 2 ml saturated NaCl (approximately 6 M), vortex very well and spin at 16ºC for 15 minutes at 2500-3000g.
10. Gently remove the supernatant, taking care NOT to take up any of the pellet. Place the supernatant in a CLEAN centrifuge tube and spin again 16ºC for 15 minutes at 2500-3000g.
11. Place the supernatant in a CLEAN centrifuge tube, add 25 ml of cold absolute ethanol, place lid on tube and invert several times, until the strands of DNA become visible.
12. Spool out the DNA using a fine long glass pipette and place in a clean 1.5 ml Eppendorf tube.
13. Rinse the DNA by adding 1 ml of 70% ethanol to the DNA, vortex gently and spin in a microcentrifuge to pellet the DNA.
14. Tip off the 70% ethanol, taking care not to dislodge the DNA pellet, by inverting the Eppendorf tube, and leave the tube inverted so the alcohol drain off and the pellet dries completely (a few hours).
15. Resuspend the DNA pellet in approximately 500 µl of DNAase free water (eg. autoclaved distilled water).
16. Store the DNA at 4ºC; for long-term storage at -20ºC.

5.2.2 DNA EXTRACTION FROM BLOOD: PHENOL-CHLOROFORM METHOD

Reagents:

a. Reticulocyte saline: 5 mM KCl, 0.13 M NaCl, 7.4 mM $MgCl_2$
b. 10x Lysis mix: 0.77 M NH_4Cl, 46 mM $KHCO_3$. Dilute to 2x, to make red blood cell lysing mix.
c. WBC Lysing solution: 100 mM NaCl, 25 mM EDTA
d. 10% SDS [Sodium dodecyl sulphate] (make from 20% stock solution)
e. 10 mg/ml proteinase K [ICN Pharmacueticals}
f. Phenol (saturated with 0.1 M Tris) [Rathburn Chemicals]
g. Chloroform
h. 7.5 M Ammonium acetate
i. 100% Ethanol (store in 'flammables cabinet' with lock)
j. 70% Ethanol

Method:

1. Transfer 2-10 ml blood in EDTA to a 15 ml tube. Spin at 3000 rpm for 10 min.
2. Carefully remove the supernatant (plasma).
3. Fill the tube to 15-30 ml with 2x lysis mix (dependent on quantity of blood material being lysed). Shake well and rotate for 10 min to lyse the red blood cells (the lysis will be complete when the solution is a clearish red).
4. Spin at 3000 rpm for 10 min. A pellet of white cells should form at the bottom of the tube. Carefully tip off the supernatant to waste leaving the pellet stuck to the bottom of the tube. Repeat steps 3 and 4 to get a cleaner/whiter/purer pellet.
5. Add 450 µl WBC lysing solution to an Eppendorf tube labelled with the lab number. Add 30 µl of 10% SDS. Transfer about half of the white pellet (dependent on quantity) to the 450 µl lysing solution and SDS in the labelled Eppendorf tube. Then transfer the rest of the white blood cell pellet to a new Eppendorf together with 200 µl of lysing solution, this is stored as Buffy Coats in the freezer as a back up. Add approximately 200 µl of proteinase K (10 mg/ml) to the WBCs in lysing solution and

SDS (dependent on quantity of WBCs). After pipetting up and down a few times the lysate should be viscous, it may be necessary to add more lysing solution at this stage.

6. Incubate over night at 37°C or at 55°C for 4-5 hours, after which the solution should be less viscous, due to the white blood cells being broken down.
7. Check that the sample has been lysed adequately before proceeding with the phenol-chloroform extraction. If the solution can move easily up and down a Pasteur pipette then lysis is complete. If not add more proteinase K and incubate further.
8. To the sample add an equal volume of phenol (saturated with 0.1 M TrisHCl). 700 µl is usually added. Mix and spin in a microfuge at 3000 rpm for 5 min.
9. Remove the upper aqueous layer to a clean appropriately labelled tube and add another equal volume of phenol.
10. Remove the upper aqueous layer to a clean tube and add an equal volume of chloroform. Spin at 3000 rpm for 5 min.
11. Remove the upper aqueous layer and add another equal volume of chloroform and spin again at 3000 rpm for 5min.
12. Remove the upper aqueous layer to a clean tube and add 1 µl of 4 M Ammonium acetate. Add 2x volume of 100% ethanol and gently shake to precipitate the DNA.
13. Spin at 3000 rpm for 5 min to pellet the DNA.
14. Carefully pour off the 100% ethanol (taking care not to disturb the pelleted DNA) and add 500 µl of 70% ethanol to wash the DNA. Shake well and spin at 3000 rpm for 5 min.
15. Carefully tip off the 70% ethanol wash leaving the DNA stuck to the bottom of the tube. Air-dry the pellet until all the ethanol has evaporated off it should still be clear.
16. Dissolve the pellet in an appropriate amount of sterile water.
17. Leave at 4ºC for 4-5 hours for the DNA to dissolve.
18. The solvent waste from the phenol and chloroform spins should be dispensed into the solvent waste bin and disposed of according to local waste disposal rules. The tubes should then be disposed of in bins for autoclaving.

5.2.3 DNA EXTRACTION FROM CVS: PHENOL-CHLOROFORM METHOD

After cleaning the chorionic villus tissue by microscopic dissection to remove all traces of maternal tissue (see chapter 6), chorionic villi may be transported to the molecular diagnostic laboratory in either tissue culture medium or 0.5 ml of CVS lysis buffer. The latter is better if transportation is expected to take more than 24 hours, as the DNA is perfectly stable at room temperature in CVS lysis buffer for up to two weeks or longer. This phenol extraction method is very quick, consisting of two 30 second phenol/chloroform extractions to remove protein, two chloroform washes to remove the phenol, and ethanol precipitation.

Reagents:

a. CVS Lysing solution: 150 mM NaCl, 25 mM EDTA, 0.1% SDS [Sodium dodecyl sulphate], 50 µg/ml Proteinase K.
b. Phenol (saturated with 0.1 M Tris) [Rathburn Chemicals]
c. Chloroform

d. 7.5 M Ammonium acetate
e. 100% Ethanol (store in 'flammables cabinet' with lock)
f. 70% Ethanol

Method:

1. Transfer cleaned CVS in tissue culture medium to a 1.5 ml Eppendorf tube. Spin at 3000 rpm for 30 seconds and carefully remove the supernatant. If CVS is in lysis solution, go straight to step 4.
2. Wash CVS by resuspension in 0.5 ml 150 mM NaCl, 25 mM EDTA. Spin at 3000 rpm for 30 seconds and carefully remove the supernatant.
3. Add 0.5 ml CVS lysing solution and mix by vortexing.
4. Incubate over night at 37°C or at 55°C for 3 hours.
5. Add 0.25 ml chloroform and 0.25 ml phenol (saturated with 0.1M Tris HCl). Mix and spin in a microfuge at 3000 rpm for 30 seconds.
6. Remove the upper aqueous layer to a clean appropriately labelled tube and carry out second extraction by repeating steps 5.
7. Remove the upper aqueous layer to a clean appropriately labelled tube, add 0.5 ml chloroform and mix thoroughly.
8. Spin at 3000 rpm for 30 seconds and carefully transfer the supernatant to a clean, labelled tube.
9. Mix thoroughly and spin at 3000 rpm for 30 seconds. Carefully transfer the supernatant to a clean labelled tube.
10. Add 250 µl of 7.5 M ammonium acetate and mix. Add 1.5 ml of 100% ethanol and gently mix to precipitate the DNA. This procedure should precipitate just DNA as a few white fibrous strands. If DNA is not observed at this stage, proceed to step 15.
11. Spin at 3000 rpm for 30 seconds to pellet the DNA.
12. Carefully pour off the 100% ethanol (taking care not to disturb the pelleted DNA) and add carefully 500 µl of 70% ethanol to wash the DNA pellet. Spin at 3000 rpm for 30 seconds.
13. Carefully tip off the 70% ethanol wash leaving the DNA stuck to the bottom of the tube. Air-dry the pellet until all the ethanol has evaporated off it should still be clear.
14. Dissolve the pellet in 50 µl of sterile distilled water (use 25 µl for small samples or 100 µl for very large samples).
    If the DNA concentration is too low to see a precipitate at step 10, cool the ethanol mixture by placing it at -20°C for at least 1 hour, or by freezing it by submerging the tube in dry ice pellets for 5 min.
15. Spin the tube at maximum speed in a microfuge for 20 min. Proceed from step 12. Note this procedure will precipitate both RNA and DNA and thus an accurate estimation of the DNA concentration cannot be made unless the RNA is removed by treatment with RNase.

5.2.4 DNA EXTRACTION FROM AMNIOCYTES: PHENOL-CHLOROFORM METHOD

The method of DNA extraction from amniotic fluid cells is essentially the same as for CVS, whether cells are from a direct sample or cultured. It is advisable to always have a culture set up as backup, because sometimes a direct fluid sample fails to provide sufficient amplifiable DNA to achieve a diagnosis. The cultured cells also provide confirmation of the result obtained on the direct cells, which are sometimes blood stained and the result open to possible doubt from maternal contamination.

The yield of DNA is smaller than that obtained from CVS, and therefore the amounts of all solutions used are one fifth of those for the CVS procedure (0.1 ml lysis buffer, 50 µl of chloroform, etc.)

5.2.5 KITS AND AUTOMATED DNA EXTRACTION SYSTEMS

One way to address the potentially limiting step of DNA extraction (purification) in molecular diagnostics is the use of commercially available kits. There are currently many commercial kits available, both manual and automated. Any DNA extraction procedure should ensure sufficient yield and purity of DNA samples. In addition the procedure should preferably be as simple and rapid as possible. Automated DNA extraction systems support more homogeneous sample treatment, and potentially minimize errors (tube-switching) and contamination. They are particularly appropriate for laboratories with a high sample throughput, for example when processing large numbers of samples for definitive molecular diagnosis. To our knowledge all commercially available kits and automated systems are comparable with respect to the purity of DNA samples after extraction, yields and speed, assuming the technical recommendations of each relevant manufacturer are applied at all levels (storage of reagents, protocols, maintenance of equipment etc). Thus each laboratory should select the kits and system most appropriate for their needs and available resources.

5.3 DIAGNOSIS OF KNOWN MUTATIONS

For the analysis of globin gene mutations, it is necessary to perform PCR for the region of DNA in which the particular mutation is located. Because the globin genes are no too large, only one or two fragments usually have to be amplified. This section describes the principal methods of analysis in diagnostic use worldwide:

a. **ASO-probe methods:** The principle of dot blot (DB) and reverse dot blot (RDB) methods is that a single-strand DNA molecule of defined sequence (the "oligoprobe") can hybridize to a second DNA molecule that contains a complementary sequence (the "target") with the stability dependent on the extent of base pairing that can occur. In both methods specific amplified fragments of DNA are need to be hybridized with ASO probes that are fixed on the surface of a membrane (RDB) or by a radioactive-labelled probe to DNA samples fixed to the membrane in the form of dots (DB). DB needs different hybridization and wash temperatures for each probe per mutation, and screens many samples for one mutation per membrane. RDB is a non- radioactive method, which requires the same temperature for hybridisation and washing. RDB can screen one sample for many mutations per membrane.
b. **Amplification refractory mutation system (ARMS):** It is a PCR-based system that discriminates between normal and mutant alleles by selecting primers that have nucleotide at their 3'end corresponding to either normal or mutant sequence. PCR amplifies a DNA fragment only if there is perfect match with the genomic sequence to which the primer is annealing. This is a rapid, simple and non-radioactive method. It can use for screening multiple mutations in one patient but it needs good experience to understand false negatives.

c. **Restriction enzyme analysis:** It is a rapid, simple and non-radioactive method used if a mutation happens to create or abolish a restriction site. The PCR fragment is cut with a restriction enzyme that will cut or not cut when the particular point mutation is present. The limitations of this method that the majority of globin gene mutations do not create or destroy a restriction enzyme site, and some enzymes for those that do are expensive.
d. **Gap-PCR:** This is a rapid, simple and non-radioactive method that allows the identification DNA deletions or gene rearrangements. This method is used to detect α-thalassaemia deletions, δß-thalassaemia deletions, Hb-Lepore rearrangements, etc. The limitation of this method is that the deletion endpoints must be known to design the primers.
e. **Real time PCR:** This is a rapid technique that uses fluorescent probes and dedicated instruments to monitor the accumulation of amplicons produced throughout the progress of a PCR reaction. There are several different systems for genotyping point mutations, each with their own hybridisation and detection chemistries.
f. **Pyrosequencing:** This is a rapid method of DNA sequencing that allows the identification of point mutations. The limitation of the technique is that a sequence run can only read a short stretch of DNA - approximately 30 base pairs.

5.3.1 ALLELE SPECIFIC OLIGONUCLEOTIDE (ASO) DOT BLOT

Dot blot analysis is a technique for immobilizing amplified DNA on a nitrocellulose or nylon membrane. Hybridization analysis can be carried out to determinate the relative target sequence in the blotted DNA preparations. Dot blot analysis was first described by Kafatos et al (67). This procedure was originally used to determinate the relative amount of a target sequences in a series of DNA samples. A large number of samples can be applied simultaneously on the surface of the membrane, enabling many different DNAs to be screened in a single hybridization experiment, or in sequential hybridisation experiments to investigate several different mutations.

Reagents:

a. 20x SSPE, which consists of:
   i. 2 M NaCl
   ii. 0.2 M $NaH_2PO_4.2H_2O$
   iii. 0.02 M Na_2EDTA , pH 7.4 (with 10 M NaOH)
b. b. 50x Denhardt's solution, which consists of:
   i. 1% Ficoll
   ii. 1% Polyvinylpyrrolidene (PVP)
   iii. 1% Bovine serum albumin (BSA)

Method: Samples are usually applied to the membrane using a manifold attached to a suction device especially if a number of blots are to be prepared at any time. Samples of amplified DNA to be transferred are denaturated (by adding 10x volume of 0.4 M NaOH, 25 mM Na_2EDTA) or heat (80ºC for 5 min) and applied to the membrane in a salt buffer. After "blotting", the membrane is treated (washed) with denaturant (0.4 M NaOH) and neutralization solutions (2x SSC) and the DNA immobilized by incubation in vacuum at 80°C (nitrocellulose membrane) or UV irradiation (nylon membrane).

Once the membrane has been prepared with the immobilized denatured amplified DNA, the hybridisation steps can follow. Oligoprobes (ASO's) for each mutation under investigation, (and their wild-type sequence equivalent) are usually designed to be 19bp long, with the expected mutated nucleotide exactly in the middle. The ASO is labelled before hybridisation. One protocol is to label the ASO at the 5' end with γ^{32}ATP as follows: 250 ng of oligoprobe, 125 µCi of radioactivity, 10 units of T4 Kinase and 10x T4 Kinase buffer in a final volume of 20 µl, and incubation at 37ºC for 2 hours. After the reaction 1 µl of 0.5 M EDTA and 480 µl of H_2O are added. The unicorporated radioactive dNTP is removed by passing the reaction through a NAP-5 column:

1. Prepare the column by passing through 3 volumes of H_2O.
2. Load the labelled ASO (500 µl) on to the column and allow it to run in, discarding the initial eluent.
3. Wash the column with 1 ml H_2O and collect the radioactive eluent in a 1.5 ml Eppendorf tube (count if required).
4. For hybridisation, add the 1.5 ml of labelled probe to 8.5 ml of hybridisation buffer (5x SSPE, 0.5% SDS and 5x Denhardt's). (Following the hybridisation step the 10ml of radioactive probe in hybridisation buffer can be frozen at –20ºC for up to 4-6 weeks, or as long as the radioactivity gives a signal).

The hybridisation step is performed for 2 hours at a temperature around 2ºC less than the Tm of the probe (where the Tm is calculated by the simplified formula: Tm = 4x [G+C] + 2x [A+T]). After a suitable incubation, the membrane is washed twice at room temperature (10x SSPE and 0.5% SDS) for 15 minutes and then once at the Tm of the ASO (4x SSPE and 0.1% SDS). Any non-specifically bound probe is removed, leaving only probe that is base-paired to the target DNA.

After hybridization and wash, the membrane is subjected to autoradiography for 12-16 hours. Figure 5.1 shows an example of dot blot analysis (isotopic). The membrane can be stripped (by treating with 0.4 M NaOH at 42ºC for 30 minutes, followed by neutralizing with 0.2 M Tris/HCl, 0.2x SSC and 0.5% SDS) and then re-hybridized with another probe. This method needs one analysis for one mutation. For this reason, you need a lot of the time to screen many mutations. Moreover, the temperature of hybridization and wash for each oligonucleotide is different. In the same procedure it is possible to study many samples for one mutation.

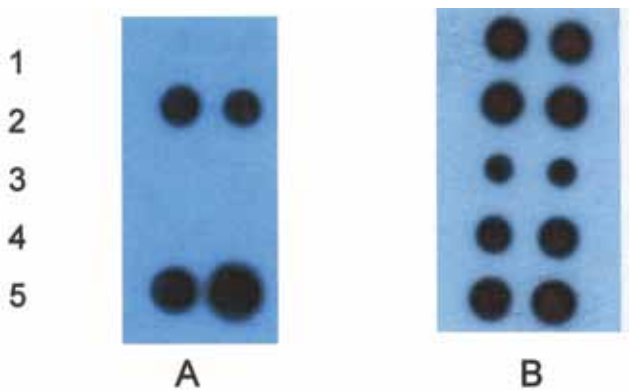


FIG. 5.1
Autoradiogram A was performed with IVS1-110 (G→A) mutant probe.
Autoradiogram B was performed with ISV1-110 (G→A) normal probe.
In autoradiogram A. samples 2 & 5 are positive for ISV1-110 (G→A) mutation.
Individuals 1, 3, 4 are negative for the IVS1-110 (G → A) mutation.
Samples 2 & 5 are heterozygous for IVS1-110 (G→A).

5.3.2 REVERSE DOT BLOT (RDB)

Reverse dot-blot analysis is a technique for immobilizing allele-specific oligonucleotide probes on a nylon membrane rather than the individual DNA samples. This is a non-radioactive method. In this format, multiple pairs of mutant and normal ASO probes are spotted on strips of nylon membranes. For each diagnostic test, a spotted strip containing many normal and mutant oligonucleotides, is hybridized with a specific DNA sample in order to screen many mutations. Reverse dot-blot analysis was first described by Saiki et al (29), and then developed later to screen many ß-thalassaemia mutations in the Sicilian population and for use in prenatal diagnosis (30, 68).

The reverse dot-blot can be theoretically designed to detect any point mutation present in a determined country after the conditions are carefully set-up. RDB is also used in Sicily to analyse α–thalassaemia (68) and δ-thalassaemia point mutations, and for the genotyping of the main ß–globin gene variants (HbS, HbC, HbD, etc). A general outline of the method is described below, for detailed protocols refer to other publications [30, 68, 69].

The amplified DNA is labelled using 5'modified primers with biotin or during the amplification by biotin-16-dUTP. Genomic DNA is amplified in a 50 µl reaction volume contain each 5' biotinylated amplified primer or adding biotin-16-dUTP (Roche) instead of dTTP during PCR reaction. If 5' biotinylated primers are used, a 5' biotin group is introduced during the final coupling step on the DNA synthesizer that can be purchased from company. In the protocol used in the laboratory of Dr. A. Giambona & Prof. A. Maggio, biotin-16-dUTP is introduced during the PCR program.

Method for the amplification and labelling of ß-globin gene

1. The protocol described here uses two pairs of PCR primers for the simultaneous amplification of two ß-globin DNA fragments (duplex PCR) that encompass all the known ß-thalassaemia mutations. These four primers are used simultaneously in the same test tube for duplex PCR in which the leftward pair directs amplification of a 735 bp fragment and the right pair a 526 bp fragment.
2. The leftward primer pair consists of upstream primer 5'GTACGGCTGTCATCACTTAGACCTCA3', and downstream primer 5'TCATTCGTCTGTTTCCCATTCTAAAC3'.
3. The rightward primer pair consists of: upstream primer 5'GGGTTAAGGCAATAGCAAT3', and the downstream primer 5'CTGACCTCCCACATTCCCTT3'.
4. The following amplification buffer and thermal cycling conditions are adopted for ß-globin gene PCR and to label DNA by biotin.
5. In the amplification reaction, target DNA (0.2 µg) is amplified in a 50 µl reaction mixture containing:
   10 pm of each primer
   1x PCR buffer,
   1.5 mM $MgCl_2$,
   100 µM dNTPs,
   10 µM biotin-16-dUTP (Roche)
   2 U recombinant Taq polymerase (Invitrogene).
6. One cycle is performed at 94°C for 5 min, 30 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 45 s, followed by a final extension step at 72°C for 7 min.

Method for the amplification and labelling of α-globin gene:

1. Both α2 and α1 globin gene were amplified selectively by choosing one primer in the 3' region of divergence between the α2 and α1 genes, and a common 5' primer for both genes. The fragments are 923 bp for the α2 and 922 bp for the α1 globin gene.
2. The common 5' α2 and α1 primer is CCAAGCATAAACCCTGGCGCGCT.
3. The primer in 3' region of α2 is AACACCTCCATTGTTGGCACATTCC.
4. The primer in 3' region of α1 is CCATGCTGGCACGTTTCTGAG.
5. Two 50 µl amplification reactions are set up for each DNA sample to be typed, one each for α2 and α1 genes.
6. The amplification buffer consists of:
   10 pm of each primer,
   2.5 mM $MgCl_2$,
   50 mM Tris-HCl pH 8.9,
   100 mM dNTPs,
   10 µM biotin-16-dUTP,
   13% glycerol and
   2 U native Taq Polymerase (Invitrogene).
7. A total of 30 cycles are performed: 5 cycles at 98°C for 45 s, 55°C for 30 s, 72°C for 45 s; and 25 cycles at 96°C for 30 s, 55 °C for 30 s, 75°C for 45 sec; and finally, an additional 72°C for 7 min extension step was included.

Hybridisation of amplified DNA:

1. Oligonucleotide probes are 5' amino-modified. A NH_2 group is added during the last step of the synthesis (MWG-Biotech AG).
2. Table 5.3 lists the sequences and the quantitities of each ß–globin gene applied to filters in picomoles.
3. Table 5.4 lists the sequences and the quantitities of each α2 and α1 globin gene applied to filters in picomoles.
4. Membrane Biodyne-C (PALL-Biosupport) is activated by 16% EDC (1-ethyl-3-dimethylaminopropyl carbodiimide, Sigma) for 15 min. It is rinsed in water and air dried.
5. Oligonucleotide probes are diluted with 0.5 M $NaHCO_3$/Na_2CO_3 buffer pH 8.4 to the concentrations as showed in Tables 5.3 and 5.4 for application onto the membrane.
6. Oligonucleotide probes are spotted on the surface, approximately 1 µl to each spot of the membrane using a 2 µl pipette. On the left and on the right of the same lane are spotted the normal and the mutant oligonucleotides.
7. Membrane is air dried and inactivated in 0.1 M NaOH for 10 min. The membrane is then rinsed thoroughly with water and air-dried. Membrane strips prepared in such a manner can be stored at room temperature in a desiccator for up 6 months.
8. Each filter with bound oligonucleotide probes are placed in 3 ml hybridization solution of 2x SSC and 0.1% SDS.
9. 20 µl amplified DNA is diluted with 0.5 ml of hybridization solution (2x SSC and 0.1% SDS) and denatured at 95°C for 5 min.
10. The denatured DNA is then added immediately to 3 ml hybridization solution and incubated at 45°C for 60 min in a shaker water incubator.
11. The filters are then collectively washed in 2x SSC and 0.1% SDS at 45°C for 15 min two times.

Table 5.3 Oligonucleotide probe sequences and the quantity (pmol of each applied to the filter) used for the diagnosis of Sicilian ß-thalassaemia mutations by Reverse Dot Blot (RDB).

POSITION	PROBE	SEQUENCE	QUANTITY
-101	Normal	ACCTCACCCTGTGGAG	5
-101	C→T	GCTCCACAAGGTGAGGT	5
- 92	Normal	GTGGAGCCACACCCTA	5
- 92	C→T	TAGGGTGTAGCTCCAC	5
- 88	Normal	Same as -87 Normal	5
- 88	C→T	ACCCTAGGATGTGGCT	5
- 87	Normal	GGAGCCACACCCTAG	5
- 87	C→G	ACCCTAGCGTGTGGC	5
- 87	C→T	AA CCCTAGAGTGTGGCT	5
- 30	Normal	CTGACTTTTATGCCCAG	3
- 30	G→C	CTGACTTTTGTGCCCAG	3
Cd 5	Normal	Same as Cd 6 Normal	8
Cd 5	-CT	TTCTCCTCGAGTCAGGT	5
Cd 6	Normal	ACTCCTGAGGAGAAGT	8
Cd 6	-A	TTCTCCCAGGAGTCAG	8
Cd 8	Normal	TGAGGAGAAGTCTGCC	5
Cd 8	-AA	AGGGCAGACCTCCTCA	5
Cd 30	Normal	Same as IVSI.nt1 Normal	3
Cd 30	G→C	CTGGGCACGTTGGTA	3
IVSI.nt 1	Normal	ATACCAACCTGCCCAG	3
IVSI.nt 1	G→A	CTGGGCAGATTGGTAT	3
IVSI.nt 5	Normal	Same as IVSI.nt6 Normal	4
IVSI.nt 5	G→A	GCAGGTTGATATCAAGG	5
IVSI.nt 5	G→C	GCAGGTTGCTATCAAG	5
IVSI.nt 6	Normal	CCTTGATACCAACCTGC	5
IVSI.nt 6	T→C	CAGGTTGGCATCAAGGT	5
IVSI.nt 110	Normal	GAAAATAGACCAATAGGCAGA	7
IVSI.nt 110	G→A	CTGCCTATTAGTCTATTTTC	7
IVSI.nt 130	Normal	ACCCTTAGGCTGCTGG	5
IVSI.nt 130	G→C	ACCCTTACGCTGCTGG	5
Cd 39	Normal	CTTGGACCCAGAGGTTCTT	6
Cd 39	C→T	AGAACCTCTAGGTCCAAGG	4
IVSII.nt 1	G→A	CTTCAGGATGAGTCTATGG	4
IVSII.nt 745	Normal	CAATCCAGCTACCATTC	4
IVSII.nt 745	C→G	GAATGGTACCTGGATTG	4

Table 5.4 Oligonucleotide probe sequences and the quantity (pmol of each applied to the filter) used for the diagnosis of α-thalassaemia mutations by Reverse Dot Blot (RDB).

POSITION	PROBE	SEQUENCE	QUANTITY
α2 IVSI donor site	Normal	GAGGTGAGGCTCCCTC	8
α2 IVSI donor site	-TGAGG	CTGGAGAGCTCCCTCC	8
α2 initiation codon	Normal	ACCCACCATGGTGCTGT	7
α2 initiation codon	CATG→CACG	CCCACCACGGTGCTGT	7
α2 Cd 125	Normal	GCCTCCCTGGACAAG	5
α2 Quong-Sze Cd 125	CTG→CCG	CCTCCCCGGCAAGT	5
α2 Cd 142	Normal	AAATACCGTTAAGCTGGA	5
α2 Constant Spring Cd142	TAA→CAA	AAATACCGTTAAGCTGGA	5
α2 Icaria Cd142	TAA→AAA	AAATAGGCTAAAGCTGGA	5
α2 Seal Rock Cd142	TAA→GAA	AATACCGTGAAGCTGGA	5
α2 Koya Dora Cd142	TAA→TCA	AATACCGTTCAGCTGGA	5
α2 Poly A signal	Normal	GTCTTTGAATAAAGTCTGA	4
α2 T Saudi	AATAAA→AATAAG	TTTGAATAAGGTCTGAGTG	4
α1 initiation codon	Normal	GAGGTGAGGCTCCCTC	7
α1 initiation codon	CATG→CGTG	ACCCACCGTGGTGCTC	7

12. They are then collectively incubated in 20 ml 2x SSC and 0.1% SDS solution containing 5 U of streptavidin-AP conjugation (Roche) for 30 min at room temperature.
13. Membranes are washed 2 times for 15 min at room temperature in 2x SSC and SDS.
14. Colour development is performed by incubating the filters at room temperature in the dark for 30 min in freshly prepared solution containing nitroblue tetrazolium chloride salt (NBT, Roche) and bromo-chloro-indolyl-phosphatase salt (BCIP, Roche) in Tris HCl pH 9.4. Colour is developed in 30-45 min.
15. The filters are washed twice in distilled water by shaking.

Figure 5.2 illustrates the main steps involved in the generation of a Reverse Dot Blot using immobilized ASO probe.

Interpretation of results: It is important to emphasise that there must be a differentiation between false positive due to background signals and the true positive results of the control samples. If in doubt the assay should always be repeated. The strategy, as with ARMS-PCR, is to screen for the common mutations on one membrane and then if no positive result is obtained, the rare ones are screened for on a second membrane. Any mutation remaining unidentified is then investigated by direct sequencing.

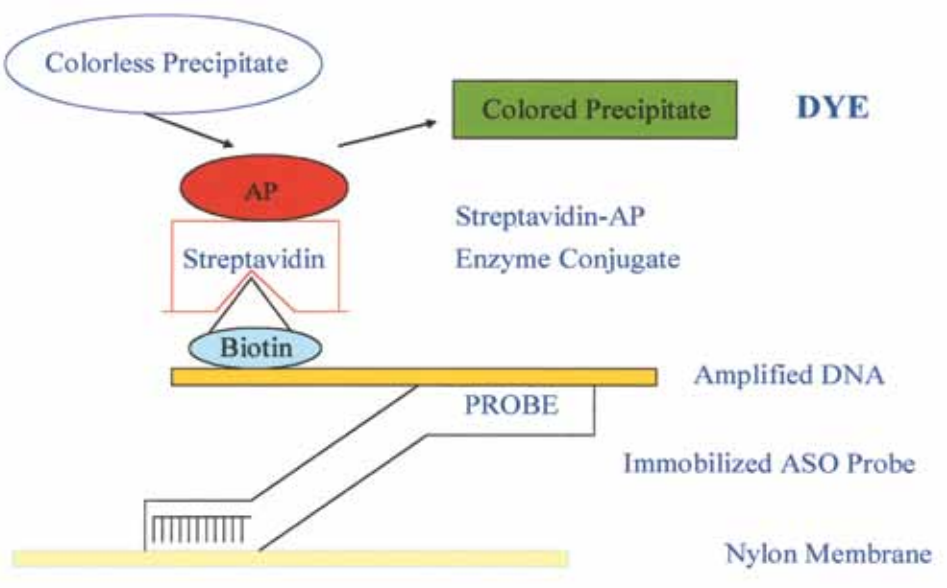
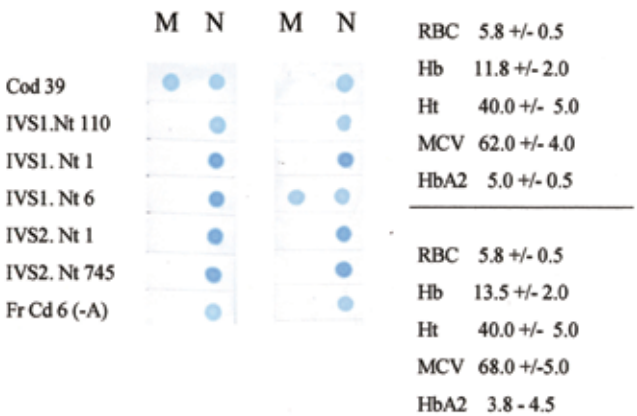


FIG. 5.2
The main steps involved in "Reverse Dot Blot Analysis".

Figures 5.3-5.6 show examples of results of the RDB method obtained from the laboratory of Dr. A. Giambona and Prof. A. Maggio. Figure 5.3 shows RDB being used to screen for the 7 most common mutations found in Sicily. These account for 95% of the ß-thalassaemia defects in Sicily. Figure 5.4 shows RDB being used for prenatal diagnosis of ß-thalassaemia. Figures 5.5 and 5.6 show RDB being used to detect α-thalassaemia and δ-thalassaemia point mutations.

FIG. 5.3
An example of a reverse dot-blot analysis that shows two individuals tested for the presence of the most common Mediterranean mutations. On the left is a carrier positive for the Cd 39 (C→T) point mutation, while on the right a IVSI-6 (T→C) point mutation is identified.
N: normal oligonucleotides;
M: mutant oligonucleotides.



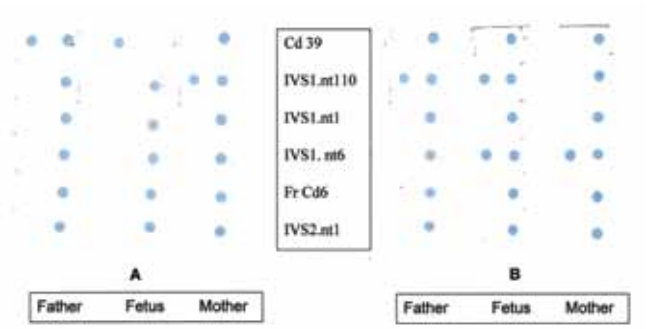


FIG. 5.4
An example of prenatal diagnosis by RDB. On the left (A) a prenatal diagnosis for Cd 39 (C→T) [both parents are carriers for Cd 39]. The fetus is homozygous for the Cd 39 mutation. On the right (B) a prenatal diagnosis for IVSI-110 (G→A) and IVSI-6 (T→C) (the fetus is heterozygous for IVSI-110 and IVSI-6 mutations).

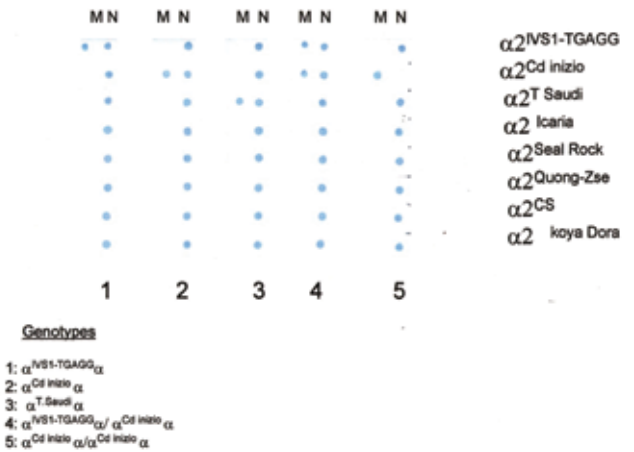


FIG. 5.5
Reverse dot blot strips of non-deletion α+-thalassaemia defects after hybridization and colour development. Hybridization probes complementary to the six common Mediterranean α-thalassaemia mutations and non-Mediterranean mutations are fixed to these reverse dot blot strips. On each filter, the normal probes are on the right and the mutant probes are on the left. The mutations represented by each probe are listed above, and the genotypes of the DNA samples tested are listed below.

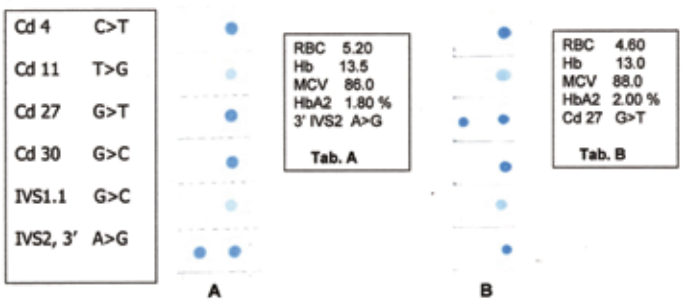


FIG. 5.6
Strip A shows the mutation 3'IVSII (A→G) in the δ-globin gene. Strip B shows the Cd 27 (G→T) mutation of the δ-globin gene, the most frequent mutation in Sicily. Tab A and B are the phenotype of the respective mutations.

5.3.3 AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)

The ARMS technique for detecting known point mutations was first described by Newton et al [37]. It has been developed for the diagnosis of all the common ß-thalassaemia mutations found in all the main ethnic groups [39]. The technique is based on the principle of allele-specific priming of the PCR process, i.e. a specific primer will only permit amplification to take place when its 3' terminal nucleotide matches with its target sequence. Thus to detect the ß-thalassaemia mutation IVSI-5 (G → C), the 3' nucleotide of the ARMS primer is G in order to base pair 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) were found to reduce priming efficiency to zero or below-5%, and to prevent amplification, a further mismatch with the target sequence had to be introduced at the second, third or fourth nucleotide from the 3' end of the primer (70).

As a general rule for ARMS primer design, 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. Usually the mismatch is designed at the second nucleotide in the first instance and the primer then tested for specificity and generation of product. The position of the mismatch can be altered if the primer does not work, or the strength of the mismatch increased if non-specific bands are observed. According to Little in Current Protocols in Human Genetics (71), the strength of mismatch pairings are; maximum, G-A, C-T, T-T; strong, C-C; medium, A-A, G-G; weak, C-A, G-T; none, A-T, G-C.

The mutation-specific ARMS primers used in the Oxford laboratory to diagnose the 25 most common ß-thalassaemia mutations, plus the haemoglobin variants HbS, HbC and HbE, are listed in Table 5.5. All are 30 bases long so that they can all be used at a single high annealing temperature (65°C).

Table 5.5: Primer sequences used for the detection of the common ß-thalassaemia and ß-chain variant mutations by ARMS-PCR.

MUTATION	OLIGONUCLEOTIDE SEQUENCE	SECOND PRIMER	PRODUCT SIZE (bp)
-88 (C→T)	TCACTTAGACCTCACCCTGTGGAGCCTCAT	A	684
-87 (C→G)	CACTTAGACCTCACCCTGTGGAGCCACCCG	A	683
-30 (T→A)	GCAGGGAGGGCAGGAGCCAGGGCTGGGGAA	A	626
-29 (A→G)	CAGGGAGGGCAGGAGCCAGGGCTGGGTATG	A	625
-28 (A→G)	AGGGAGGGCAGGAGCCAGGGCTGGGCTTAG	A	624
CAP+1 (A→G)	AAAAGTCAGGGCAGAGCCATCTATTGGTTC	A	597
Cd 5 (-CT)	TCAAACAGACACCATGGTGCACCTGAGTCG	A	528
Cd 6 (-A)	CCCACAGGGCAGTAACGGCAGACTTCTGCC	B	207
Cd 8 (-AA)	ACACCATGGTGCACCTGACTCCTGAGCAGG	A	520

MUTATION	OLIGONUCLEOTIDE SEQUENCE	SECOND PRIMER	PRODUCT SIZE (bp)
Cd 8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCACACC	B	225
Cd 15 (G→A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	A	500
Cd 16 (-C)	TCACCACCAACTTCATCCACGTTCACGTTC	B	238
Cd 17 (A→T)	CTCACCACCAACTTCATCCACGTTCAGCTA	B	239
Cd 24 (T→A)	CTTGATACCAACCTGCCCAGGGCCTCTCCT	B	262
Cd 39 (C→T)	CAGATCCCCAAAGGACTCAAAGAACCTGTA	B	436
Cd 41/42 (-TCTT)	GAGTGGACAGATCCCCAAAGGACTCAACCT	B	439
Cd 71/72 (+A)	CATGGCAAGAAAGTGCTCGGTGCCTTTAAG	C	241
IVSI-1 (G→A)	TTAAACCTGTCTTGTAACCTTGATACCGAT	B	281
IVSI-1 (G→T)	TTAAACCTGTCTTGTAACCTTGATACGAAA	B	281
IVSI-5 (G→C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	B	285
IVSI-6 (T→C)	TCTCCTTAAACCTGTCTTGTAACCTTCATG	B	286
IVSI-110 (G→A)	ACCAGCAGCCTAAGGGTGGGAAAATAGAGT	B	419
IVSII-1 (G→A)	AAGAAAACATCAAGGGTCCCATAGACTGAT	B	634
IVSII-654 (C→T)	GAATAACAGTGATAATTTCTGGGTTAACGT*	D	829
IVSII-745(C→G)	TCATATTGCTAATAGCAGCTACAATCGAGG*	D	738
β^S Cd 6 (A→T)	CCCACAGGGCAGTAACGGCAGACTTCTGCA	B	207
β^C Cd 6 (G→A)	CCACAGGGCAGTAACGGCAGACTTCTCGTT	B	206
β^E Cd 26 (G→A)	TAACCTTGATACCAACCTGCCCAGGGCGTT	B	236
$\beta^{D\text{-Punjab}}$ Cd 121(G→C)	TCTGTGTGCTGGCCCATCACTTTGGCAAGC	E	250
$\beta^{D\text{-Iran}}$ Cd 22 (G→C)	CAACCTGCCCAGGGCCTCACCACCAACATG	B	255

Legend: The above primers are coupled as indicated with either primer A: CCCCTTCCTATGACATGAACTTAA, B: ACCTCACCCTGTGGAGCCAC;C: TTCGTCTGTTTCCCATTCTAAACT; or D: GAGTCAAGGCTGAGAGATGCAGGA. The control primers used were primers D plus E: CAATGTATCATGCCTCTTTGCACC (which yield a 861 bp product as shown in Figure 1) for all the above mutation specific ARMS primers except the two marked *. Control primers used with these two are the $^G\gamma$-Hind III RFLP primers described in Table 5.9

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 amplified from an unrelated region of the genome under the same PCR conditions. The generation of control product indicates the reaction mixture and thermal cycler is working optimally. Figure 5.7 shows the screening of a DNA sample for a ß-thalassaemia mutation. The strategy is to screen for the common mutations expected in the country of the ethnic origin of the patient first and then to screen for the rarer mutations. After which, uncharacterised mutations are identified by genomic sequencing.

Primers for the diagnosis of the normal alleles for many of these mutations are listed in Table 5.6. These are required when both partners of a couple requesting prenatal diagnosis of ß-thalassaemia carry the same mutation. An example of prenatal diagnosis using mutant and normal ARMS primers is shown in Figure 5.8.

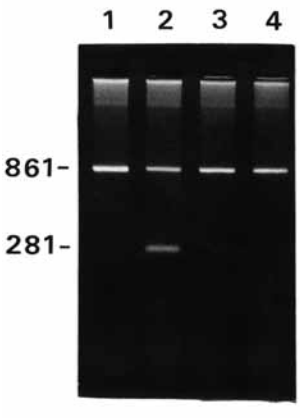


FIG. 5.7
An example of screening for common ß-thalassaemia mutations by ARMS-PCR. Ethidium bromide stained gel showing the screening of a DNA sample for four common Asian Indian ß-thalassaemia mutations by ARMS-PCR: track1, IVSI-5 (G→C); 2, IVSI-1 (G→T); 3, Cd 41/42 (-TCTT); 4, Cd 8/9 (C→T). The results show the patient carries the mutation IVSI-1 (G→T). The control primers D and E produce an 861 bp fragment with normal DNA, present in all four lanes. The primers used are listed in Table 5.4.

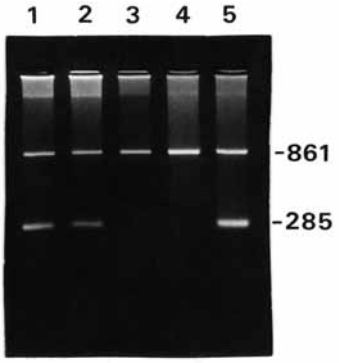


FIG. 5.8
An example of prenatal diagnosis by ARMS-PCR. The ethidium bromide stained gel shows the results of a prenatal diagnosis for ß-thalassaemia for IVSI-5 (G→C) (both parents are carriers for IVSI-5 G→C) using ARMS primers listed in Tables 5.4 and 5.5. Lanes 1 and 2 show paternal and maternal DNA samples tested with mutant primer, lane 3 shows a homozygous IVSI-5 (G→C) control DNA sample tested with normal primer, and lanes 4 and 5 show fetal DNA tested with mutant primer and normal primer respectively. The generation of the ARMS-specific 285 bp product with normal primer and the absence of any 285bp product with mutant primer indicates the fetus is normal.

Table 5.6: Primer sequences used for the detection of normal DNA sequences by ARMS-PCR.

MUTATION	OLIGONUCLEOTIDE SEQUENCE	SECOND PRIMER	PRODUCT SIZE (kp)
-88 (C→T)	TCACTTAGACCTCACCCTGTGGAGCCACTC	A	684
-87 (C→G)	CACTTAGACCTCACCCTGTGGAGCCACCCC	A	683
Cd 5 (-CT)	CAAACAGACACCATGGTGCACCTGACTCCT	A	528
Cd 6 (-A)	CACAGGGCAGTAACGGCAGACTTCTCCTCA	B	207
Cd 8 (-AA)	ACACCATGGTGCACCTGACTCCTGAGCAGA	A	520
Cd 8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCACACT	B	225
Cd 15 (G→A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	A	500
Cd 30 (G→C)	TAAACCTGTCTTGTAACCTTGATACCTACC	B	280
Cd 39 (C→T)	TTAGGCTGCTGGTGGTCTACCCTTGGTCCC	A	299
Cd 41/42 (-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 Cd 6 (A→T)	AACAGACACCATGGTGCACCTGACTCGTGA	A	527
β^E Cd 26 (G→A)	TAACCTTGATACCAACCTGCCCAGGGCGTC	B	236

See Table 5.5 legend for details of primers A-D and control primers.

Reagents:

a. dNTPs: Add together 50 µl of a 100 mM solution of each dNTP (as purchased) and 3.8 ml of distilled water. The 1.25 mM dNTP stock solution should be stored in frozen aliquots.

b. ARMS-PCR buffer: 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at room temperature), 1.5 mM $MgCl_2$, 100 µg/ml 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 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.

c. Taq polymerases: suggested ones are as follows, AmpliTaq Gold (PE Biosystems) works best for ARMS-PCR/RE digestion assays and Platinum Taq (Gibco Life Technologies) for gap-PCR (see Methods).

d. Tris-borate-EDTA (TBE) buffer: 89 mM Tris-borate, 89 mM boric acid,10 mM EDTA (pH 8.0).

Method:

1. Prepare a reaction mixture for 200 reactions (4 ml) comprising of: 0.5 ml of 10x ARMS-PCR buffer;1.25 ml of 1.35 mM dNTP mixture; 2.65 ml of sterile distilled water
2. Pipette 20 µl of PCR reaction mixture into a 0.5 µl tube.
3. Add 1 µl of each primer (1 OD unit/ml).
4. Add 0.05 µl of Taq 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 pipetting errors as larger quantities are used.
6. Add 1 µl of genomic DNA (100 ng/µl).
7. Overlay with 25 µl of mineral oil.
8. Mix, centrifuge and place in thermal cycler.
9. Amplify for 25 cycles as follows: 1 min at 94ºC/1 min at 65ºC/1.5 min at 72ºC with a final extension period of 3 min at 72ºC following the 25th cycle.
10. Remove tubes from 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
12. Stain gel in ethidium bromide solution (0.5 µg/ml) for 15-30 minutes, visualise 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).

5.3.4 RESTRICTION-ENZYME (RE) - PCR

A small number of the ß-thalassaemia 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 ß-thalassaemia mutations, as listed in Table 5.7.

However the main use of this PCR technique is for the diagnosis of the clinically important Hb variants HbS, Hb D-Punjab and Hb O-Arab. Figure 5.9 shows the detection of the HbS mutation by this technique. The primer sequences used in the Oxford laboratory for diagnosing these Hb variants are listed in Table 5.8. However other primer sequences that span the mutation site may be used. Whenever practicable 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 product from both the normal and mutant DNA alleles. This is possible for the HbS and HbE mutations but not for Hb O-Arab and Hb D-Punjab, for which the flanking EcoR I sites are too far away from the one in codon 121.

RE-PCR is also used for ß-globin gene haplotype analysis. At least 18 RFLPs have been characterised within the ß-globin gene cluster. However, most of these RFLP sites are non-randomly associated with each other and thus they combine to produce just a handful of haplotypes [72]. In particular they form a cluster that is 5' to the δ gene and a 3' cluster that extends downstream from the ß-globin gene. The DNA in between the two clusters contains a relative hotspot for meiotic

recombination with a rate of approximately one in 350 meioses [73]. The ß-globin gene cluster haplotype normally consists of 5 RFLP's located in the 5' cluster (Hind II/ε-gene; Hind III/γ-gene; Hind III/γ-gene; Hind II/5'ψß-gene; and Hind II/3'ψß-gene) and two RFLPs in the 3' cluster (Ava II/ß-gene; BamH I/ß-gene) [74].

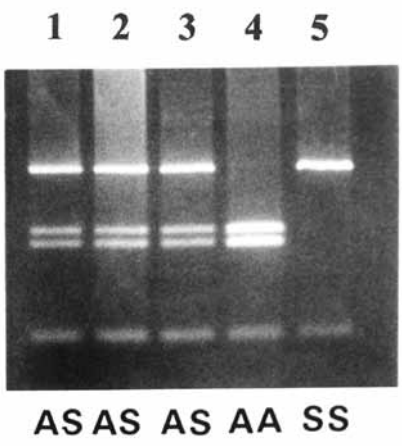


FIG. 5.9
An example of the diagnosis of the sickle cell disease by RE-PCR. The ethidium bromide stained gel shows amplified DNA digested by Dde I using the primers described in Table 5.7. The sickle cell genotype indicated by the pattern of the digested products is given below each lane.

Table 5.7: ß-Thalassaemia mutations detectable by RE-PCR

POSITION	MUTATION	ETHNIC GROUP	AFFECTED SITE
-88	C→T	African/Asian Indian	+ Fok I
-87	C→G	Mediterranean	- Avr II
-87	C→T	Italian	- Avr II
-87	C→A	African/Yugoslavian	- Avr II
-86	C→G	Lebanese	- Avr II
-86	C→A	Italian	- Avr II
-29	A→G	African/Chinese	+ Nla III
+43 to +40	(-AAAC)	Chinese	+ Dde I
Initiation Cd	T→C	Yugoslavian	- Nco I
Initiation Cd	T→G	Chinese	- Nco I
Initiation Cd	A→G	Japanese	- Nco I
Cd 5	(-CT)	Mediterranean	- Dde I
Cd 6	(-A)	Mediterranean	- Dde I
Cd 15	(-T)	Asian Indian	+ Bgl I
Cd 17	A→T	Chinese	+ Mae I
Cd 26	G→T	Thai	- Mnl I
Cd 26	G→A	Southeast Asian (Hb E)	- Mnl I
Cd 27	G→T	Mediterranean (Hb Knossos)	- Sau96 I
Cd 29	C→T	Lebanese	- BspM I
Cd 30	G→C	Tunisian/African	- BspM I
Cd 30	G→A	Bulgarian	- BspM I
IVSI-1	G→A	Mediterranean	- BspM I
IVSI-1	G→T	Asian Indian/Chinese	- BspM I

POSITION	MUTATION	ETHNIC GROUP	AFFECTED SITE
IVSI-2	T→G	Tunisian	- BspM I
IVSI-2	T→C	African	- BspM I
IVSI-2	T→A	Algerian	- BspM I
IVSI-5	G→A	Mediterranean	+ EcoR V
IVSI-6	T→C	Mediterranean	+ SfaN I
IVSI-116	T→G	Mediterranean	+ Mae I
IVSI-130	G→C	Turkish	- Dde I
IVSI-130	G→A	Egyptian	- Dde I
Cd 35	C→A	Thai	- Acc I
Cd 37	G→A	Saudi Arabian	- Ava II
Cd 38/39	(-C)	Czechoslovakian	- Ava II
Cd 37/8/9	(-GACCCAG)	Turkish	- Ava II
Cd 39	C→T	Mediterranean	+ Mae I
Cd 43	G→T	Chinese	- Hinf I
Cd 47	(+A)	Surinamese	- Xho I
Cd 61	A→T	African	- Hph I
Cd 74/75	(-C)	Turkish	- Hae III
Cd 121	G→T	Polish, French, Japanese	- EcoR I
IVSII-1	G→A	Mediterranean	- Hph I
IVSII-4,5	(-AG)	Portuguese	- Hph I
IVSII-745	C→G	Mediterranean	+ Rsa

Table 5.8: Oligonucleotide primers for the detection of β^S, β^E, $\beta^{D\text{-Punjab}}$, and $\beta^{O\text{-Arab}}$ mutations as RFLPs.

POSITION	PRIMER SEQUENCES (5'-3')	PRODUCT SIZE, (bp): ABSENCE OF SITE	PRODUCT SIZE, (bp): PRESENCE OF SITE
HbS: (Dde I)	ACCTCACCCTGTGGAGCCAC GAGTGGACAGATCCCCAAAGGACTCAAGGA	376/67	201/175/67
HbE (Mnl I)	ACCTCACCCTGTGGAGCCAC GAGTGGACAGATCCCCAAAGGACTCAAGGA	231/89/56/35/33	171/89/60/35/33
Hb D-Punjab (EcoR I)	CAATGTATCATGCCTCTTTGCACC GAGTCAAGGCTGAGAGATGCAGGA	861	552/309
Hb O-Arab (EcoR I)	CAATGTATCATGCCTCTTTGCACC GAGTCAAGGCTGAGAGATGCAGGA	861	552/309

The annealing temperature for all primer pairs is 65ºC.

All of the seven RFLPs except BamH I site can be analysed very simply and quickly by PCR. The primer sequences and sizes of the fragments generated are listed in Table 5.9. The BamH I RFLP is located within a L1 repetitive element, creating amplification problems. A Hinf I RFLP located just 3' to the ß-globin gene is used instead, because these two RFLP's have been found to exist in linkage disequilibrium [75].

Table 5.9 Oligonucleotide primer sequences used to analyse ß-globin gene cluster RFLP's.

RFLP	PRIMER SEQUENCES (5'-3')	ANNEALING TEMP (oC)	PRODUCT SIZE, (bp): ABSENCE OF SITE	PRODUCT SIZE, (bp): PRESENCE OF SITE
ε-Hind II	TCTCTGTTTGATGACAAATTC AGTCATTGGTCAAGGCTGACC	55	760	314/446
^{G}γ-Xmn I	AACTGTTGCTTTATAGGATTTT AGGAGCTTATTGATAACTCAGAC	55	650	450/200
^{G}γ-Hind III	AGTGCTGCAAAGAAGAACAACTACC CTCGCATCATGGGCCAGTGAGCCTC	65	323	235/98
^{A}γ-Hind III	ATGCTGCTAATGCTTCATTAC TCATTGTGTGATCTCTCTCAGCAG	55	635	327/308
5'ψß-Hind II	TCCTATCCATTACTGTTCCTTGAA ATTGTCTTATTCTAGAGACGATTT	55	794	687/107
5'ψß-Ava II	TCCTATCCATTACTGTTCCTTGAA ATTGTCTTATTCTAGAGACGATTT	55	794	442/352
3'ψß-Hind II	GTACTCATACTTTAAGTCCTAACT TAAGCAAGATTATTTCTGGTCTCT	55	914	480/434
ß-Rsa I	AGACATAATTTATTAGCATGCATG CCCCTTCCTATGACATGAACTTAA	55	692/413/100	692/331/100/82
ß-Ava II	GTGGTCTACCCTTGGACCCAGAGG TTCGTCTGTTTCCCATTCTAAACT	65	328	227/101
ß-Hinf I	GGAGGTTAAAGTTTTGCTATGCTGTAT GGGCCTATGATAGGGTAAT	55	320/155	219/155/108

Method:

1. Add the following to a 0.5 ml tube: 20 µl PCR reaction mixture (as detailed in the ARMS protocol 5.3.3); 1 µl each primer; 1 µl genomic DNA (100 ng/µl); 2 µl sterile dH_2O; 0.05 µl of AmpliTaq DNA polymerase (5 U/µl).
2. Overlay with 25 µl of mineral oil.
3. Place in 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 units of the appropriate restriction enzyme, plus 2 µl of the corresponding 10x buffer.
5. Incubate at 37°C for a minimum of 1 hour.
6. Add blue dye, mix and spin as in protocol for ARMS-PCR.
7. Load 20 µl aliquot onto a 3% agarose gel consisting of 50% Nusieve GTC agarose and 50% ordinary agarose.
8. Perform electrophoresis, stain and photograph as described for ARMS-PCR.

5.3.5 GAP-PCR

Gene deletion mutations in the ß-globin gene cluster may be detected by PCR using two primers complimentary to the sense and antisense strand in the DNA regions that flank the deletion. For small deletions of less than one kilobase, 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 product is only obtained from the deletion allele. In these cases the normal allele is detected by amplifying across one of the breakpoints, using a primer complimentary to the deleted sequence and one complimentary to the flanking DNA.

Gap-PCR is used for to diagnose some ß-thlassaemia deletions (59-62, 78-79), δß-thlassaemia and HPFH deletions (63), α-thalassaemia deletions (6-9) (Table 5.2) and also the triple α-gene locus generated by the 3.7 kb single α-gene deletion, as listed in Table 5.2. A typical gap-PCR test is illustrated in Figure 5.10 and Figure 5.11. For the diagnosis of α-thalassaemia, the primers can now be multiplexed. The 3.7 kb and 4.2 kb α^+-thalassaemia deletions are detected in one multiplex assay (Table 5.10), the $--^{MED}$ and $-(\alpha)^{20.5}$ α^0-thalassaemia deletions in a second multiplex assay (Table 5.11) and the 3 Southeast Asian α^0-thalassaemia deletions in a third multiplex assay (Table 5.12). The protocols used in the Oxford laboratory for the multiplexing of these primers are given in the tables, but it should be noted that the quantity of each primer pair relative to the others may need adjustment to gain optimum amplification of all the products. PCR diagnosis of the triple α-gene (anti 3.7 allele) requires two separate assays (Tables 5.13 & 5.14). The presence of the allele is diagnosed from a comparison of the results of each assay run side by side and the genotype of the DNA sample can be deduced (Table 5.15) in most instances.

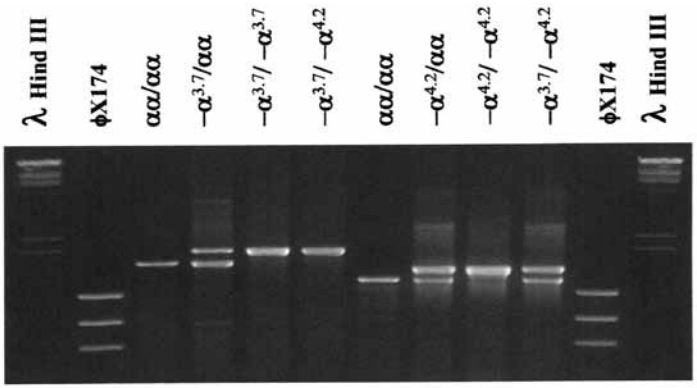


FIG. 5.10
The diagnosis of α^+-thalassaemia deletion mutations by multiplex GAP PCR using the primers described in Table 5.9.

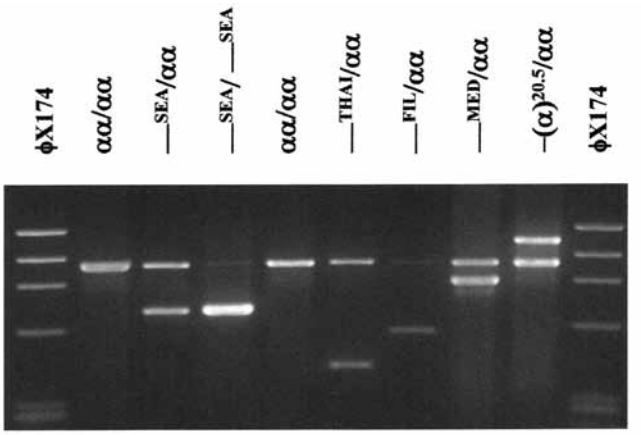


FIG. 5.11
The diagnosis of α⁰-thalassaemia deletion mutations by multiplex GAP PCR using the primers described in Tables 5.10 & 5.11.

For the diagnosis of δß-thalassaemia and HPFH deletions mutations, the assays are set up separately, including those for the two different breakpoints of the Indian and Turkish inversion/deletion δß-thalassaemia alleles. The primer sequences for these assays are detailed in Table 5.16.

Gap-PCR may also be used to check for maternal contamination in the fetal DNA sample by variable tandem repeat (VNTR) polymorphism analysis (80), as detailed in chapter 6.

Reagents:

- **a.** dNTPs: Add together 50 µl of a 100 mM solution of each dNTP (as purchased) and 3.8 ml of distilled water. The 1.25 mM dNTP stock solution should be stored in frozen aliquots.
- **b.** 10x gap-PCR reaction buffer (composition varies according to primers used) see methods and tables.
- **c.** Betaine (Sigma-Aldrich Chemical Co Ltd, England) .
- **d.** Mineral Oil to overlay PCR reactions.
- **e.** PCR primers: dilute aliquots of primer stock solutions to make a working solution of 1 OD unit/ml and store frozen.
- **f.** Ammonium sulphate buffer: 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 0.01% Tween 20, 10% DMSO, 10 mM ß-mercaptoethanol (all final concentrations).
- **g.** Taq polymerases and 10x Taq buffers: in the Oxford laboratory are as follows, AmpliTaq Gold (PE Biosystems) works best for ARMS-PCR/RE digestion assays and Platinum Taq (Gibco Life Technologies) for gap-PCR (see Methods).
- **h.** Tris-borate-EDTA (TBE) buffer : 89 mM Tris-borate, 89 mM boric acid, 10 mM EDTA, pH 8.0.
- **i.** Blue running dye (15% ficoll/0.05% bromophenol blue).
- **j.** UV transilluminator and Polaroid land camera or UV electronic camera system.
- **k.** 0.5 µg/µl Ethidium bromide.

Table 5.10: Multiplex PCR protocol for the diagnosis of $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions.

A. Primer sequences

PRIMER	DESCRIPTION	SEQUENCE	ANNEALING TEMP °C
1	α2/3.7-F	CCCCTCGCCAAGTCCACCC	64
2	3.7/20.5-R	AAAGCACTCTAGGGTCCAGCG	64
3	α2-R	AGACCAGGAAGGGCCGGTG	64
4	4.2-R	CCCGTTGGATCTTCTCATTTCCC	64
5	4.2-F	GGTTTACCCATGTGGTGCCTC	64

B. PCR reaction mix

COMPONET	µl
α2/3.7-F (10 µM)	1.0
α2-R (10 µM)	0.25
α2/20.5-R (10 µM)	1.0
4.2-F (10 µM)	1.0
4.2-R (10 µM)	1.5
10x buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20)	2.5
25 mM $MgC1_2$	1.5
dNTPs (1 mM)	5.0
Betaine (5 M)	3.75
DMSO (10%)	1.25
Platinum Taq (5 units/µl)	0.1
DNA template (100 ng/µl)	1.0
Water	5.2

C. Gel electrophoresis conditions

Run PCR products out on 1.5% (1:1 Nusieve:agarose) gel for 2-3 hours.

D. Interpretation of results

PCR FRAGMENT SIZE (bp)	GENOTYPE	PRODUCT OF PRIMERS
2020	α^+-thalassaemia: $-\alpha^{3.7}$	1 + 2
1800	Normal (αα)	1 + 3
1628	α^+-thalassaemia: $-\alpha^{4.2}$	4 + 5

Table 5.11. Multiplex PCR protocol for the diagnosis of the --^{MED} and -(α)$^{20.5}$ deletions.

A. Primer sequences

PRIMER	DESCRIPTION	SEQUENCE	ANNEALING TEMP °C
1	MED(F)	CGATGAGAACATAGTGAGCAGAATTGCAGG	60
2	MED(R)	ACGCCGACGTTGCTGCCCAGCTTCTTCCAC	60
3	SEA(F)	CTCTGTGTTCTCAGTATTGGAGGGAAGGAG	60
4	SEA(N)	TGAAGAGCCTGCAGGACCAGGTCAGTGACCG	60
5	-(α)$^{20.5}$(F)	GGGCAAGCTGGTGGTGTTACACAGCAACTC	60
6	-(α)$^{20.5}$(R)	CCACGCCCATGCCTGGCACGTTTGCTGACG	60

B. PCR reaction mix

COMPONENT	µl
SEA(F) (10 µM)	1.0
SEA(N) (10 µM)	0.5
MED(F) (10 µM)	0.4
MED(R) (10 µM)	0.4
-(α)$^{20.5}$(F) (10 µM)	0.4
-(α)$^{20.5}$(R) (10 µM)	0.4
10x buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20)	2.5
25 mM $MgC1_2$	1.5
dNTPs (1 mM)	4.0
Betaine (5 M)	3.75
DMSO (10%)	1.25
Platinum Taq (5 units /µl)	0.1
DNA template (100 ng/µl)	1.0
Water	6.2

C. Gel electrophoresis conditions

Run PCR products out on 2% (1:1 Nusieve:agarose) gel for 1-1.5 hours.

D. Interpretation of results

PCR FRAGMENT SIZE (bp)	GENOTYPE	PRODUCT OF PRIMERS
1175	α^{o}-thalassaemia: -(α)$^{20.5}$	5 + 6
1010	Normal (αα)	3 + 4
875	α^{+}-thalassaemia: --^{MED}	1 + 2

Table 5.12. Multiplex PCR protocol for the diagnosis of the $--^{SEA}$ / $--^{FIL}$ / $--^{THAI}$ α^0-thalassaemia deletions.

A. Primer sequences

PRIMER	DESCRIPTION	SEQUENCE	ANNEALING TEMP °C
1	FIL (F)	AAGAGAATAAACCACCCAATTTTTAAATGGGCA	60
2	FIL (R)	GAGATAATAACCTTTATCTGCCACATGTAGCAA	60
3	SEA(F)	CTCTGTGTTCTCAGTATTGGAGGGAAGGAG	60
4	SEA(N)	TGAAGAGCCTGCAGGACCAGGTCAGTGACCG	60
5	SEA(R)	ATATATGGGTCTGGAAGTGTATCCCTCCCA	60
6	THAI(F)	CACGAGTAAAACATCAAGTACACTCCAGCC	60
7	THAI(R)	TGGATCTGCACCTCTGGGTAGGTTCTCTACC	60

B. PCR reaction mix

COMPONENT	µl
SEA(F) (10 µM)	2.0
SEA(N) (10 µM)	1.0
SEA(R) (10 µM)	1.0
FIL (F) (10 µM)	4.0
FIL (R) (10 µM)	4.0
THAI (F) (10 µM)	1.0
THAI (R) (10 µM)	1.0
10x buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20)	2.5
25 mM $MgC1_2$	1.5
dNTPs (1 mM)	4.0
Betaine (5 M)	3.75
DMSO (10%)	1.25
Platinum Taq (5 units /µl)	0.1
DNA template (100 ng/µl)	1.0
Water	0.65

C. Gel electrophoresis conditions

Run PCR products out on 1.5% (1:1 Nusieve:agarose) gel for 2 hours.

D. Interpretation of results

PCR FRAGMENT SIZE (bp)	GENOTYPE	PRODUCT OF PRIMERS
1010	Normal (αα)	3 + 4
660	α^+-thalassaemia: $--^{SEA}$	3 + 5
550	α^+-thalassaemia: $--^{FIL}$	1 + 2
495	α^+-thalassaemia: $--^{THAI}$	6 + 7

Table 5.13. PCR protocol for the diagnosis of the αα (anti 3.7) allele: reaction mix 1.

A. Primer sequences

PRIMER	DESCRIPTION	SEQUENCE	ANNEALING TEMP °C
1	C10	GATGCACCCACTGGACTCCT	55
2	C3	CCATTGTTGGCACATTCCGG	55

B. PCR reaction mix

COMPONENT	µl
C10 (10 µM)	1.0
C3 (10 µM)	1.0
10x buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20)	2.5
25 mM $MgC1_2$	1.5
dNTPs (1 mM)	5.0
Betaine (5 M)	3.75
DMSO (10%)	1.25
Platinum Taq (5 units /µl)	0.1
DNA template (100 ng/µl)	1.0
Water	12.9

C. Gel electrophoresis conditions

Run PCR products of reaction mixture 1 out on 2% (1:1 Nusieve:agarose) gel for 2 hours, in lane next to those of reaction mixture 2. See Table 5.14 for interpretation of results.

D. Interpretation of results

PCR FRAGMENT SIZE (bp)	GENOTYPE	PRODUCT OF PRIMERS
No product	α^+-thalassaemia: $-\alpha^{3.7}$	1 + 2
1900	Normal (αα)	1 + 2
1900	ααα: (anti 3.7)	1 + 2

Table 5.14. PCR protocol for the diagnosis of the αα (anti 3.7) allele: reaction mix 2.

A. Primer sequences

PRIMER	DESCRIPTION	SEQUENCE	ANNEALING TEMP °C
1	C10	GATGCACCCACTGGACTCCT	50
2	C2	CCATGCTGGCACGTTTCTGA	50

B. PCR reaction mix

COMPONENT	µl
C10 (10 µM)	1.0
C2 (10 µM)	1.0
10x buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20)	2.5
25 mM $MgC1_2$	1.5
dNTPs (1 mM)	5.0
Betaine (5 M)	3.75
DMSO (10%)	1.25
Platinum Taq (5 units /µl)	0.1
DNA template (100 ng/µl)	1.0
Water	12.9

C. Gel electrophoresis conditions

Run PCR products of reaction mixture 2 out on 2% (1:1 Nusieve:agarose) gel for 2 hours, in lane next to those of reaction mixture 1. See Table 5.14 for interpretation of results.

D. Interpretation of results

PCR FRAGMENT SIZE (bp)	GENOTYPE	PRODUCT OF PRIMERS
2100	Normal (αα)	1 + 2
2100	ααα: (anti 3.7)	1 + 2
1900	α^+-thalassaemia: $-\alpha^{3.7}$	1 + 2

Table 5.15. Interpretation of the results of reaction mixes 1 & 2 for the diagnosis of the ααα (anti 3.7) allele.

A. Products (bp)

GENOTYPES	PRIMERS: C2+C10	PRIMERS: C3+C10
αα / αα	2100	1900
$-\alpha^{3.7}$/ αα	2100 + 1900	1900
$-\alpha^{3.7}$/ $-\alpha^{3.7}$	1900	
ααα / $-\alpha^{3.7}$	2100 + 1900	2100 + 1900
ααα / αα or ααα / ααα	2100	2100 + 1900

Notes: αα allele: amplifies with C3+C10 (1.9 kb) and C2+C10 (2.1 kb).The -α3.7 allele: amplifies with only C2+C10. Gives a shorter product (1.9 kb) than normal because of the deleted α-gene. The ααα allele: amplifies with C3+C10 (1.9 kb) and twice with C2+C10 (2.1 kb) because of the extra α-gene.

B. Possible Gel Patterns

α-genotypes

αα / αα	$-\alpha^{3.7}$ / αα	$-\alpha^{3.7}$/ $-\alpha^{3.7}$	ααα / $-\alpha^{3.7}$	ααα / αα or ααα / ααα

Primer pairs

C2+C10	C3+C10	C2+C10	C3+C10	C2+C10	C3+C10	C2+C10	C3+C10	C2+C10	C3+C10

Band patterns

___		___				___	___	___	___	2.1 kb
	___	___	___	___		___	___		___	1.9 kb

For the diagnosis of δβ-thalassaemia and HPFH deletions mutations, the assays are set up separately, including those for the two different breakpoints of the Indian and Turkish inversion/deletion δβ-thalassaemia alleles. The primer sequences for these assays are detailed in Table 5.16.

Gap-PCR may also be used to check for maternal contamination in the fetal DNA sample by variable tandem repeat (VNTR) polymorphism analysis (80), as detailed in chapter 6.

Table 5.16. Multiplex gap-PCR protocols for the diagnosis of some δß-thalassaemia and HPFH deletion mutations.

A. Primer sequences:

1. Primers for HPFH1:

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	AGAATGTCACACTTAGAATCTG	54	50769 – 50790
2	CACTTTAATTCTGGTCTACCTGAA	54	52385 – 52362
3	ACTGTGATGTTGGAAATGGAC	54	

2. Primers for HPFH2:

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	GACATGGACTATTGTTCAATGA	52	44592 – 44613
2	TGCTATGCCAACTCACTACC	52	46798 – 46779
3	TTTATATATGAAATGCTACTGATT	52	

3. Primers for HPFH3

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	GACATGGACTATTGTTCAATGA	58	44592 – 44613
2	CTTTGCTGTTCAGGCTTAATTT	58	45433 – 45412
3	GACACAGAGCAGTGATTGGTGCA	58	

4. Primers for Indian deletion/inversion δß-thalassaemia: breakpoint A

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	ATGCCATAAAGCACCTGGATG	58	39809 – 39829
2	GAGCTGAAGAAAATCATGTGTGA	58	41004 – 40982
3	TAACCATATGCATGTATTGCC	58	56093 – 56114

5. Primers for Indian deletion/inversion δß-thalassaemia: Breakpoint B

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	GAGCTGAAGAAAATCATGTGTGA	58	41004 – 40982
2	CAATGTATCATGCCTCTTTGCACC	58	63224 – 63247
3	GCAGCCTCACCTTCTTTCATGG	58	63889 – 63868

6. Primers for Chinese δß-thalassaemia:

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	GGCATATATTGGCTCAGTCA	58	40268 – 40287
2	CTTGCAGAATAAAGCCTATC	58	40948 – 40929
3	TCAACAATTATCAACATTACACC	58	-

7. Primers for Sicilian δß-thalassaemia:

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	GGCATATATTGGCTCAGTCA	60	54954 – 54974
2	CTTGCAGAATAAAGCCTATC	60	56538 – 56515
3	TCAACAATTATCAACATTACACC	60	69482 – 69463

8. Primers for Turkish deletion/inversion δß-thalassaemia: Breakpoint A

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	GACACACATGACAGAACAGCCAAT	65	54568 – 54591
2	GAAGAGCAGGTAGGTAAAAGAACC	65	55310 – 55287
3	CAAAGCAGCAATACTAAACAGGAG	65	73484 – 73507

9. Primers for Turkish deletion/inversion δß-thalassaemia: Breakpoint B

PRIMER	SEQUENCE	ANNEALING TEMP °C	GEN BANK HBB REF
1	TTCCACTATCTTACTTACACAT	65	66422 – 66401
2	TGCTGAGGATTGTTTTAGGTC	65	74903 – 74922
3	GAATAGCAGTGGTGAGAGAG	65	75602 – 75583

B) PCR reaction mix

COMPONENT	µl
Primer 1 (10 µM)	1.0
Primer 2 (10 µM)	1.0
Primer 3 (10 µM)	1.0
ABgene 10x PCR Buffer 1	2.5
ABgene $MgC1_2$ (25 mM)	1.5
Platinum Taq (5 units/µl)	0.1
dNTP mix (25 mM)	0,2
Water (molecular grade)	16.7
Total volume	24.0

C) Gel electrophoresis conditions

Run PCR products out on 1.5% (1:1 Nusieve:agarose) gel for 2 hours.

D) Interpretation of results

DELETION ALLELE	NORMAL FRAGMENT SIZE IN bp (primer pair)	MUTANT FRAGMENT SIZE IN bp (PRIMER PAIR)
HPFH1	1616 (1+2)	1135 (1+3)
HPFH2	2206 (1+2)	1950 (1+3)
HPFH3	841 (1+2)	607 (1+3)
Indian inv/del: A	1195 (1+2)	327 (2+3)
Indian inv/del: B	665 (2+3)	371 (1+3)
Chinese	682 (1+2)	508 (1+3)
Sicilian	1585 (1+2)	1150(1+3)
Turkish inv/del: A	742 (1+2)	432 (1+3)
Turkish inv/del: B	700 (2+3)	489 (1+3)

Reagents:

a. dNTPs: Add together 50 µl of a 100 mM solution of each dNTP (as purchased) and 3.8 ml of distilled water. The 1.25 mM dNTP stock solution should be stored in frozen aliquots.
b. 10x gap-PCR reaction buffer (composition varies according to primers used) see methods and tables.
c. Betaine (Sigma-Aldrich Chemical Co Ltd, England)
d. Mineral Oil to overlay PCR reactions
e. PCR primers: dilute aliquots of primer stock solutions to make a working solution of 1 OD unit/ml and store frozen.
f. Ammonium sulphate buffer: 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$), 2.0 mM $MgCl_2$, 0.01% Tween 20, 10% DMSO, 10 mM ß-mercaptoethanol (all final concentrations).
g. Taq polymerases and 10x Taq buffers: in the Oxford laboratory are as follows, AmpliTaq Gold (PE Biosystems) works best for ARMS-PCR/RE digestion assays and Platinum Taq (Gibco Life Technologies) for gap-PCR (see Methods).
h. Tris-borate-EDTA (TBE) buffer : 89 mM Tris-borate, 89 mM boric acid, 10 mM EDTA, pH 8.0.
i. Blue running dye (15% ficoll/0.05% bromophenol blue).
j. UV transilluminator and Polaroid land camera or UV electronic camera system
k. 0.5 µg/µl Ethidium bromide.

Method:

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 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 mM (dNTP mixture), 2.3 µl of 10x gap-PCR buffer as recommended for the primers in the original reference (see Table 5.2) and below.
2. The buffer recommended for the α-thalassaemia primers is 750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$), 0.1% Tween 20). The buffer for the α-thalassaemia primers should also contains 0.5 M betaine and 0.5% DMSO (this can be achieved by adding 2.5 µl of 5 M betaine and 1.25 µl 10% DMSO).

3. All reactions should be made up to a final volume of 22 µl with the addition of sterile dH_2O.
4. Overlay with 25 µl of mineral oil.
5. Prepare enzyme mixture: 0.2 µl reaction buffer (10x), 0.1 µl AmpliTaq (5U/µl) (PE Biostems) for the ß-gene primers, 0.1 µl Platinum Taq (5U/µl) (Invitrogen) for the α-gene primers, and 2.7 µl sterile dH_2O to a final volume of 3 µl.
6. Mix enzyme mixture and hold on ice.
7. Place reaction mixtures in thermal cycler and perform one cycle as follows, adding 3 µl of the enzyme mix after 2 minutes of the 94ºC denaturation step: 4 min at 94ºC/ 1 min at 55-65^{O}C (as recommended)/ 1.5 min at 72ºC.
8. Continue for 33 cycles with the following steps per cycle: 1 min at 94ºC/1 min at 55-65ºC (as recommended in the published references or in tables 5.10-5.14)/1.5 min at 72ºC.
   Finish with one cycle as follow: 1 min at 94ºC/1 min at 55-65ºC (as recommended)/10 min at 72ºC.
10. Hold at 15ºC until gel electrophoresis.
11. Remove tubes from thermal cycler and add 5 µl of blue dye. Mix and centrifuge.
12. Depending on expected fragment sizes, load a 20 µl aliquot onto a 1-3% agarose gel and run at 100 V for 45 min to 2 hrs in 1x Tris-borate -EDTA buffer (TBE).
13. Stain gel in ethidium bromide solution (0.5 µg/ml) for 15-30 minutes, visualise 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 re interpretation see notes 6,7 and 8.

5.3.6 REAL TIME PCR

This section describes the use of real-time PCR for genotyping point mutations in the ß-globin gene using the LightCyclerTM (system 1.0 or 1.5). Real-time PCR integrates microvolume rapid-cycle PCR with fluorometry, allowing real time fluorescent monitoring of the amplification reaction for quantitative PCR and/or characterization of PCR products for rapid genotyping, precluding any post-PCR sample manipulation.

The LightCyclerTM (Roche Molecular Biochemicals) is one such system. The detection of potential sequence differences for genotyping applications (usually single nucleotide variations), employs the use of two fluorescent probes which hybridize to adjacent internal sequences within target amplified DNA, one of which covers the region expected to contain the mutation(s). Close proximity of annealed probes facilitates fluorescence resonance energy transfer (FRET) between them. The probes are designed to have different melting temperatures (Tm), whereby the probe with the lower Tm lies over the mutation site(s). Monitoring of the emitted fluorescent signals as the temperature increases will detect loss of fluorescence (F) as the lower Tm probe melts off the template. A single base mismatch under this probe results in a Tm shift of 5-10ºC, allowing easy distinction between wild type and mutant alleles. The ability to detect base mismatches under the low Tm probe (allele-specific probe) and the use of two different coloured probes (LightCyclerTM system 1.0 or 1.5) allows more than one mutation to be interrogated in a single PCR reaction.

The protocol described here has been designed for the common ß-thalassaemia mutations in the Mediterranean populations, along with Hb S, but is easily adaptable for most other populations

where the ß-haemoglobinopathies are prevalent. All the allele-specific (mutation-detection) were designed (with the support of TIB MOLBIOL, Berlin, Germany) to be complementary to the wild-type sequence of the ß-globin gene, rather than complementary to each specific mutation (81). This precludes the need to use multiple independent assays using separate mutation-specific detection probes and potentially allows the distinction of any allele with a nucleotide variation located under the length of the probe, minimizing costs and time required to screen a large spectrum of mutations, as is necessary for most populations where ß-thalassaemia is common. Although most mutations have a distinct melting profile and can be implicated by comparison with controls, the definitive characterization of each mutation can be achieved by a second method such as an ARMS-PCR assay (78); as stated in the Best-Practice Guidelines any lab performing DNA diagnostics should have more than one available mutation detection method (82).

The LightCyclerTM systems 1.0 and 1.5 can detect 2 fluorescent labels (LightCyclerTM Red 640 [LC Red 640] and LightCyclerTM Red 705 [LC Red 705]), as well as SYBR® green. The choice of fluorescent label used, for each probe will depend upon the relative frequency of mutations in the population under study and the potential requirement for multiplexed assays when more than one mutation is investigated within a single sample. For example in the Greek population, IVSI-110 G→A is the most common mutation, so the probes for most other mutations encountered in Greece are labeled with the opposite fluorescent marker to that used for IVSI-110 G→A (83).

Equipment and reagents:

a. LightCyclerTM system version 1.0 or 1.5 (Roche).
b. Bench centrifuge for eppendorf tubes (with well depth approximately 4.5 cm) and appropriate for centrifugation around 3000 g.
c. 32 Centrifuge adapters in aluminium cooling Block, LightCyclerTM Centrifuge Adapters (Roche, 1 909 312).
d. LightCyclerTM glass capillary tubes (20 µl), (Roche, 11 909 339 001).
e. Filter tips for maximum volume of 20 µl and 200 µl along with compatible accurate adjustable pipettes.
f. Eppendorf tubes for making the premix.
g. A pair of PCR primers selected according to mutations under study (either LC1F plus LC1R or LC2F plus LC2R as shown in Table 5.17 and Figure 5.12a).
h. Mutation detection probe sets, appropriate for mutations under study (see Table 5.17 and Figure 5.12a).
i. LightCyclerTM -DNA Master Hybridisation probes Kit (Roche, 2 015 102), which also includes $MgCl_2$ (25 mM) and PCR-grade water.

Handling and storage of PCR reagents:

1. All PCR primers to be used on the LightCyclerTM are diluted as stock solutions of 100 µM, divided into aliquots of convenient volume (e.g. 25 µl) and stored at –20ºC. For primer working solutions the stock solutions are diluted to 10 µM and can be subsequently stored at 4ºC for up to 3 months.
2. The LightCyclerTM hybridisation probes are diluted to 3 µM and stored in aliquots of relatively small volume (e.g. 20 µl) at –20ºC. A thawed aliquot should not be refrozen, but can be used up to 1 month when stored at 4ºC.

3. The "Master Mix" from the LightCyclerTM -DNA Master Hybridisation probes Kit should not be refrozen once thawed but can be used for up to 1 month when stored at 4ºC.

Table 5.17 Lightcycler mutation detection probe sets for the most common ß-thalassaemias mutations worldwide (and HbS mutation).

PROBE SET	ACCEPTOR PROBE NAME AND SEQUENCE	DONOR PROBE NAME AND SEQUENCE	BETA-GENE MUTATION(S) DETECTED
Set I	I.1A: 5'-ttc tga cac aac tgt gtt ca tag ca-3' LC Red**	ID: FITC 5'-cct caa aca gac acc atg gtg cac c-3' FITC	CAP+20 (C->T) * CAP+22 (G->A)
	I.2A: LC Red** 5'-gac tcc tga gga gaa gtc tgc-3' P		HbS (Cd 6 A->T) Cd 5 (-CT) Cd 6 (-A) Cd 8 (-AA) Cd 8/9 (+G)
Set II	IIA: LC Red** 5'-tgt aac ctt gat acc aac ctg ccc a-3' P	IID: 5'-tgc cca gtt tct att ggt ctc ctt aaa cct gtc-3' FITC	IVSI-1 (G->A) IVSI-1 (G->T) IVSI-2 (T->G) IVSI-2 (T->C) IVSI-2 (T->A) IVSI-5 (G->A) IVSI-5 (G->C) IVSI-5 (G->T) IVSI-6 (T->C)
Set III	III.1A: 5'-tct gcc tat tgg tct att ttc cc-3' LC Red**	IIID: FITC 5'-ccc tta ggc tgc tgg tgg tc-3' FITC	IVSI-110 (G->A) IVSI-116 (T->G)
	III.2A: LC Red** 5'-acc ctt gga ccc aga ggt tct t-3' P		Cd 39 (C->T) Cd 37 (TGG->TGA) Cd 41/42 (delTTCT)
Set IV	IVA : LC Red** 5'-tct cag gat cca cgt gca gct tg-3'P	VID: 5'-gtc cca tag act cac cct gaa g-3' FITC.	IVSII-1 (G->A)
SetV	VA: LC Red** 5'gct caa ggc cct ttc ata ata tcc cc	VD: 5' ttt ttc att agg cag aat cca ga-3' FITC.	polyA signal mutation AATAAA->AACAAA AATAAA->AATGAA AATAAA->AATAGA AATAAA->AATAAG AATAAA->AA- - AA AATAAA->A- - - - -

The ß-globin gene specific PCR primers include (see Figure 5.12a):
For probes sets I, II, III: LC1F: 5'-GCT GTC ATC ACT TAG ACC TCA-3'; LC1R 5'-CAC AGT GCA GCT CAC TCA G-3';
For probes sets II, III,IV: LC2F 5'-CAA CTG TGT TCA CTA GCA AC-3'; LC2R 5'-AAA CGA TCC TGA GAC TTC CA-3';
For probe set V: LC3F 5'-ATT TCT GAG TCC AAG CTG GGC -3'; LC3R 5'-AAA TGC ACT GAC CTC CCA-3'.
FITC: Fluorescein ; P = Phosphorylated
* = Polymorphism linked with the IVSII-745 (C→G) mutation ** LC Red: The fluorescent label used for each probe will depend upon the relative frequency of mutations in the population under study and the potential requirement of multiplexed.

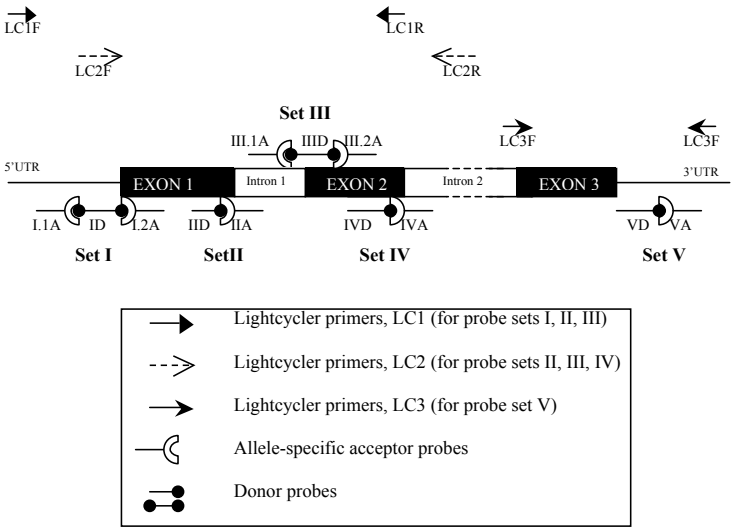


FIG. 5.12a
Primers and probes for the diagnosis of ß-globin gene mutations by real time PCR

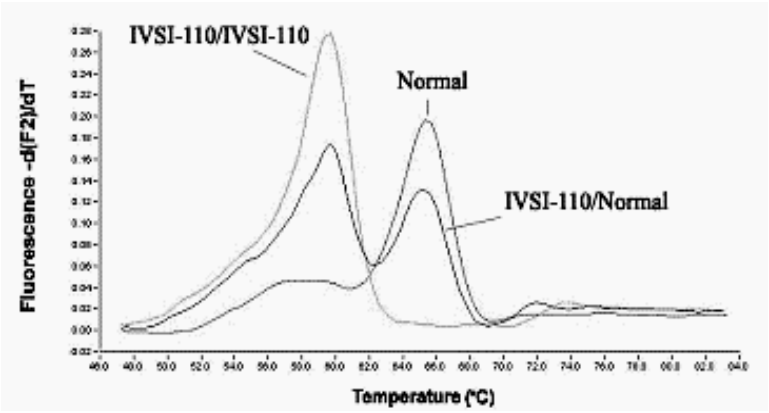


FIG. 5.12b
Example of a melting curve for the common Greek ß-thalassemia mutation Set III using donor with IIIA as mutation detection (acceptor) probe with probe IIID (see Table 5.17);

Real-time PCR reaction set up:

1. In an Eppendorf tube make a premix for the amplification reactions for a total reaction volume of 20 µL per sample. Each reaction should contain the ready-to-use reaction mix provided by the manufacturer (LightCyclerTM DNA Master Hybridization Probes) plus $MgCl_2$, a PCR primer pair (ie LC1, LC2, LCR3) and LightCyclerTM fluorescent probe sets for the relevant mutations. A typical PCR reaction for single colour detection for one sample is shown in Table 5.18.

2. When calculating the premix volume, make premix enough for the number of samples being genotyped, a PCR premix blank plus controls for the mutation(s) under investigation. The controls should include a homozygous wild-type sample (N/N), a sample heterozygous for the mutation (M/N) and, ideally, a sample homozygous for the mutation (M/M).

3. Place the appropriate number of LightCyclerTM glass capillary tubes in the centrifuge adapters in an aluminium-cooling block.

4. Distribute accurately 18 µl of premix in all the capillaries.

5. Add 2 µl genomic DNA (approximately 50 ng) per sample and controls and 2 µl of double-distilled water to the PCR blank.

6. Once the PCR reactions have been set up in the capillaries at 4°C place the caps carefully on each capillary without pressing down yet.

7. Remove the aluminium centrifuge adaptors containing the capillaries from the cooling block and place in a bench centrifuge with well deep enough to hold the aluminium centrifuge adaptors (approximately 4.5 cm).

8. Spin at a maximum of 3000g for 10 sec to pull the 20 µl reaction volume to the base of the glass capillary.

9. Place each glass capillary carefully into the LightCyclerTM carousel by letting it simply "slip" in place. Then press gently the cap fully in to the capillary and simultaneously the glass capillary fully down into position in the LightCyclerTM carousel.

10. Put the loaded carousel in to the LightCyclerTM and initiate the PCR cycles and melting curve protocols using the LightCycler software version 3.5.1, according to the manufacturer's specifications.

Table 5.18. A typical PCR reaction for single color detection for one sample.

	Stock Conc*	Final Conc*	µl/sample
H_2O (PCR grade)			9.6 µl
$MgCl_2$	25 mM	4 mM	2.4 µl
Beta globin forward primer LC1(F) or LC2(F) or LC3(F)	10 µM	0.5 µM	1 µl
Beta globin reverse primer LC1(R) or LC2(R) or LC3(R)	10 µM	0.5 µM	1 µl
LC Red allele-specific probe (640 or 705)	3 µM	0.15 µM	1 µl
FITC donor	3 µM	0.15 µM	1 µl
Master Mix			2 µl
Premix volume			**18 µl**
DNA sample volume			**2 µl**
Total reaction volume			**20 µl**

Conc* = concentration
The volume of water is always adjusted to give final reaction volume of 20µl/sample even when more than one primer or probe set is included in the reaction. For example a PCR reaction with dual color detection using 2 allele-specific LC probes (one labeled with Red 640 and the other with Red 705) and a common (central) doubly labeled FITC probe, or even two sets of LC donor-acceptor probes (ie 4 probes).

Amplification and Melting curve analysis:

1. Preprogram the LightCyclerTM software for the following amplification steps: a first denaturation step of 30 s at 95°C, followed by 40 cycles of 95°C for 3 s, 58°C for 5 s and 72°C for 20 s with a temperature ramp of 20°C /s. During the PCR, emitted fluorescence can be measured at the end of the annealing step of each amplification cycle to monitor amplification.
2. Immediately after the amplification step, the LightCyclerTM is programmed to perform melting curve analysis to determine the genotypes. This involves a momentary rise of temperature to 95°C, cooling to 45°C for 2 min to achieve maximum probe hybridisation, and then heating to 85°C with a rate of 0.4°C/s during which time the melting curve is recorded.
3. Emitted fluorescence is measured continuously (by both channels F2 (640 nm) and F3 (705 nm) if necessary) to monitor the dissociation of the fluorophore-labeled detection probes from the com-

plementary single-stranded DNA (F/T) (F: Fluorescence emitted, T: Temperature). The computer software automatically converts and displays the first negative derivative of fluorescence to temperature vs. temperature (-dF/dT vs. T) and the resulting melting peaks allows easy discrimination between wild type and mutant alleles (Figure 5.12b).

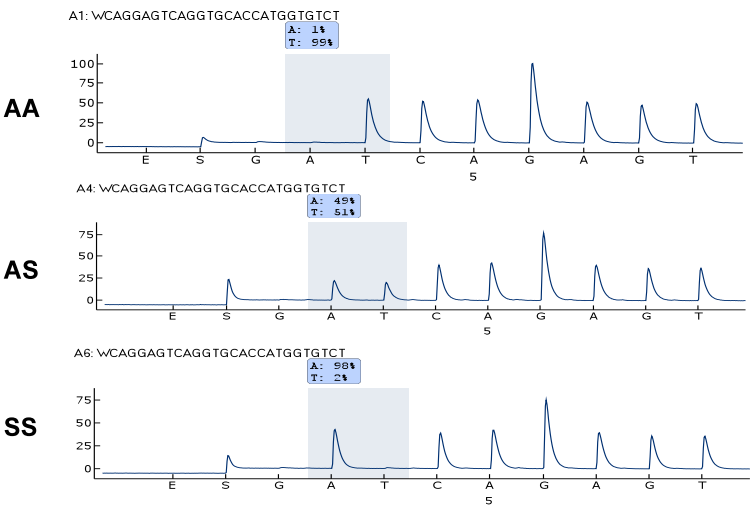
5.3.7 PYROSEQUENCING

Pyrosequencing is a sequencing-by-synthesis technique that enables rapid real-time DNA sequencing. The technique uses sulphurylase to convert the pyrophosphate released following nucleotide incorporation to ATP, which is then used to produce visible light from luciferase. The amount of light generated is measured by a suitable camera and is proportional to the molarity of the integrated deoxynucleotide, allowing a short known DNA sequence read to be constructed (84).

5.3.7.1 AMPLIFICATION PROCEDURE FOR THE DIAGNOSIS OF HbS AND HbC MUTATIONS

A protocol for the diagnosis of the HbS and HbC mutations by pyrosequencing is described below. This protocol is being used routinely by the Oxford laboratory for the prenatal diagnosis of sickle cell disease (Figure 5.13). The method has been also been developed for the detection of a number of clinically significant α-globin variants (85), and the application of pyrosequencing for the prenatal diagnosis of the common ß-thalassaemia mutations found in the UK is in current development.

FIG. 5.13
Diagnosis of Hb AA, AS and SS genotypes by pyrosequencing. The results show the % incorporation of A and T at the sickle site mutation (Cd 6 A →T)



Primer sequences (5' to 3'):

1. ß-gene PCR (Forward): TAGACCTCACCCTGTGGAGCCA (biotinylated at 5' end)
2. ß-gene - PCR (Reverse): TCGTAACGACTCAAAGAACCTCTGGG
3. Pyro - ß-gene Cd 6 SC: ACGGCAGACTTCTCC

Method:

1. Program the Biometra Thermoblock for a single step cycle to activate the Qiagen Mastermix solution at 95°C for 15 minutes linked to an amplification cycling program of 1 minute at 95°C (denaturation), 1 minute at 65°C (annealing) and 1 minutes at 72°C (extension) for 40 cycles. This should then be linked to a 10 minute final extension step at 72°C and finally paused at 15°C.
2. At least 2 DNA controls are included in each PCR run. A blank (Sigma water) is also included in each PCR run.
3. Thaw all the frozen reagents and mix gently by vortexing. Centrifuge the vials briefly.
4. Fill in the pyrosequencing record sheet.
5. Prepare sufficient reaction mix for the number of samples and controls to be tested. The formulation for the reaction mix is as follows:

REAGENTS	1 REACTION	10 REACTION
Qiagen Mastermix	12.5 µl	125 µl
ß-gene PCR Forward primer (10 µg/µl)	1.0 µl	10 µl
ß-gene PCR Reverse primer (10 µg/µl)	1.0 µl	10 µl
Sigma water	9.5 µl	95 µl
Total volume	24.0 µl	240 µl

6. Mix the reaction mix thoroughly by vortexing. Dispense 24.0 µl of reaction mix into the bottom of each thin-walled PCR vial.
7. Label each PCR vial for each sample or control.
8. Using separate pipette tips each time add 1 µl of test or control sample to each vial. Add 1 drop of mineral oil and cap the tubes firmly. Do not add DNA to the PCR vial for the blank control.
9. Centrifuge the PCR vials for a few seconds.
10. Place all the vials firmly in the thermal cycler block of the Biometra thermoblock. Initiate the 95°C single step cycle followed by the amplification cycling program.
11. Fill in the batch number details as well as the PCR machine used on the Pyosequencing record sheet.
12. Discard the remaining unused reaction mix.
13. On completion of the amplification cycling program, the samples may be stored at 2-8°C for up to 7 days before analysis by Pyrosequencing.

Agarose gel electrophoresis:

1. Clean and dry a plastic gel-casting tray.
2. Use masking or coloured plastic tape to seal the free ends of the plastic tray (20 x 20 cm). Folding

about 3-4 mm of tape to the under surface of the tape makes a good seal. Place a plastic comb (24 teeth and 1.5 mm wide) in a slot in the gel tray.

3. Dissolve 3 g of NuSieve GTG agarose and 3 g of LE agarose in 200 ml of 1xTBE electrophoresis buffer in a glass conical flask (for a 3% gel). Use a flask sufficiently large to prevent the agarose from boiling over. Boil the mixture in the microwave oven at setting 7 for 5 minutes and after mixing for further 1-minute intervals. Check that no solid particles of agarose remain.
4. Allow the agarose gel to cool down to 65ºC (just bearable to the hand) while mixing gently on a magnetic stirrer. Pour the agarose into the former set on a level surface. Allow the gel to completely solidify (about 20 minutes).
5. Carefully remove the tape and place the gel in an electrophoresis tank and cover it with 1xTBE electrophoresis buffer to a depth over the gel surface of 1mm. Remove the comb out of the agarose gel, taking care not to break the agarose. The gel origin must be at the negative (black) electrode end of the tank.
6. Transfer 20 µl of PCR product samples to a PCR plate being sure to mark the A1 position to use for Pyrosequencing.
7. Prepare the remaining PCR product for electrophoresis by adding 4 µl loading dye to each sample. Pipette mix the sample and spin the tubes briefly.
8. The 100 bp molecular size ladder is used as a standard control and loaded in the last well of each gel.
9. Carefully load the samples in the sample wells of the gel using a Gilson pipette.
10. Place the lid on the tank and connect the electrical leads to the power supply. Turn the power supply on. Run the gel at 100-120 V for 1-2 hours. Check that current is flowing by looking for electrolysis at the electrodes and that the electrical cables have been connected to correct terminals of the power supply.
11. When the run has finished switch off the power supply. Check that the current is off before disconnecting the leads. Then remove the lid of the tank and carefully lift out the gel (beware gels can slip off the former very easily). Always wear gloves when handling gels. Use a plastic tray to carry the gel to prevent accidental loss.
12. The gel is stained in a 0.5 µg/ml solution of ethidium bromide, made up with water, and rocked gently for 5 minutes and then de-stained in water for 10 minutes. The gel is given one final rinse and then taken to the darkroom.
13. Photograph the gel using the Gel DOC system and attach the photo to the relevant beta PCR record sheet. Write the gel number on the photo and number the lanes.

5.3.7.2 PYROSEQUENCING PROCEDURE FOR THE DIAGNOSIS OF HbS AND HbC MUTATIONS

Computer preparation:

1. Open the Pyromark Q24 software by double-clicking the icon.
2. Select File, followed by New Run.
3. In the Instrument Method, select Pyromark Q24 Method 001 Rev.A from the dropdown window.
4. Add the SC Assay method to the appropriate wells by highlighting the A1 square by clicking the bottom right-hand corner, and then drag the mouse to highlight the desired number of wells.
5. On each individual well, highlight the middle row and fill in the lab number and surname.

6. Select Tools, then Run Information to obtain the volumes of reagents to add to the cartridge. This will open a new window.
7. Select the printer icon on the top left of the new window to print the Run Information. This sheet can also act as a header sheet for the run.
8. Save the file to a USB stick by selecting File, Save As. This will open another window. In this window select Removable Disk from the drop-down menu and give the file a name. Ensure that the file has saved as a Run File (*.pyrorun)

Protocol:

1. Place the troughs in the workstation and add the correct reagent to fill each one over the lip on the trough.
2. Preheat the hotblock to 80°C
3. Prepare the binding buffer mix as follows:

REAGENTS	1 REACTION	10 REACTION
Binding Buffer	40 µl	1000 µl
Sigma water	18 µl	450 µl
Streptavidin Beads	2 µl	50 µl
Total	60 µl	150 µl

4. Add the binding buffer mix to the PCR product. If using less than 20 µl of PCR product make the total mix up to 80 µl using Sigma water.
5. Seal with a foil lid and place on the plate shaker for a minimum of 5mins.
6. Prepare the annealing mix as follows:

REAGENTS	1 REACTION	10 REACTION
Pyro - ß-gene Cd 6 SC primer (10 pmol/ µl)	0.75 µl	19.5 µl
Annealing buffer	24.25 µl	630.5 µl
Total	**25.00 µl**	**650.0 µl**

7. Dispense 25 µl of annealing mix into the Q24 plate and place the plate onto the workstation.
8. Turn on the pump.
9. Prime the tool by turning the switch to On and drawing through around 70 ml of distilled water from the trough to the left of the home station.
10. Remove the PCR plate from the shaker and quickly pipette mix to ensure the beads are dispersed throughout the wells.
11. Transfer the tool to the PCR plate and allow it to draw up the liquid in the wells (around 15 seconds).
12. Place the tool into the ethanol trough; wait for liquid to be drawn into the hosing then leave for 5 seconds.

13. Move the tool to the denaturation trough; wait for liquid to be drawn into the hosing then leave for 5 seconds.
14. Move the tool to the washing trough; wait for liquid to be drawn into the hosing then leave for 10 seconds.
15. Remove the tool from the trough and tilt back to beyond 90°C for around 15 seconds.
16. Flick the switch to Off and place the tool onto Q24 plate and agitate it to release the beads.
17. Once the beads have been removed, place the Q24 plate on the 80°C hotblock for 2 min.
18. Transfer the Q24 plate to the pyrosequencer and secure with the safety bar. Leave the plate to cool for 5 min before starting the analyser.
19. Add the required volume of reagents to the cartridge and place into the pyrosequencer with the label facing forwards. Secure with safety bar.
20. Select Run on the pyrosequencer.
21. Select the desired run, and then select Yes to start the run.
22. After the run the results will be saved to the USB stick and can be interpreted using the Pyromark Q24 software.

Cleaning:

The Pyrosequencing tool and cartridge need to be cleaned immediately following the run to prevent the probes and cartridge becoming blocked.

1. Place the tool in the water trough to the right of the Q24 plate station and agitate it to remove any remaining beads.
2. Transfer the tool to the next water trough and rinse with around 70 ml of water.
3. Place a 2nd 70% ethanol trough in the Parking well and place the tool in this trough.
4. Leave for 5 min, and then rinse with around 70 ml of 70% ethanol.
5. Remove the 70% ethanol trough from the Parking well and leave the tool to dry.

5.3.7.3 INTERPRETATION OF PYROSEQUENCING RESULTS

It is necessary to analyse the HbC mutation and Hb S mutation independently using the P Pyromark Q24 software, by by clicking on Analyse All Wells on the right-hand side of the screen.

HbC mutation analysis:

1. To analyse the HbC mutation, click the Analysis Setup tab. In the Sequence to Analysis box, change the sequence to TYAGGAGTCAGGTGCACCATGGTGTCT then click Apply. This will open another window entitled Apply Analysis Setup. Within this box click Apply to All.

2. Each well in the sample plate in the Overview tab should now be allocated a colour. Blue is a pass, yellow should be interpreted with caution due to low peak heights and red is a fail.

3. Selecting each well brings up a more detailed result showing both the raw data and the histograms. It is possible to place histograms on the peak trace by right clicking and selecting Show Histogram.

4. On each well window it shows the percentage of the nucleotides in the region of interest.

Results are as follows:
- Normal samples should have >95% C at the first region.
- Heterozygous HbC samples will have around 50% T and 50% C.
- Homozygous HbC samples will have >95% T.
- HbSC samples have around 70% C and 30% T. These are also shown as a fail by the software as the dispensation does not match the reference due to the HbS mutation.

5. There should be no peaks in the blank well.

6. The pyrograms can be printed by selecting the wells of interested then clicking Reports and then AQ Pyrogram Reports. This opens a new window. Within this window it is possible to choose the orientation of the pyrograms as well as the number of pyrograms per page. The page can be viewed as a PDF using Preview and then printed.

Hb S MUTATION ANALYSIS:

1. To analyse the HbS mutation, click the analysis setup tab. In the Sequence to Analysis box, change the sequence to WCAGGAGTCAGGTGCACCATGGTGTCT then click Apply. This will open another window entitled Apply Analysis Setup. Within this box click Apply to All.

2. Each well in the sample plate in the Overview tab should now be allocated a colour. Blue is a pass, yellow should be interpreted with caution due to low peak heights and red is a fail.

3. Selecting each well brings up a more detailed result showing both the raw data and the histograms. It is possible to place histograms on the peak trace by right clicking and selecting Show Histogram.

4. On each well window it shows the percentage of the nucleotides in the region of interest.
Results are as follows:
- Normal samples should have >95% T at the first region.
- Heterozygous HbS samples will have around 50% A and 50% T.
- Homozygous HbS samples will have >95% A.
- HbSC samples have around 70% T and 30% A. These are also shown as a fail by the software as the dispensation does not match the reference due to the HbC mutation.

5. There should be no peaks in the blank well.

6. The pyrograms can be printed by selecting the wells of interested then clicking Reports and then AQ Pyrogram Reports. This opens a new window. Within this window it is possible to choose the orientation of the pyrograms as well as the number of pyrograms per page. The page can be viewed as a PDF using Preview and then printed.

5.3.8 EVALUATION OF METHODS

The advantages and disadvantages of each method of analysis for known mutations are summarised below.

ADVANTAGES	DISADVANTAGES
ASO dot blot hybridisation • Widely applicable and reliable	ASO dot blot hybridisation • Traditional protocols use radioactively labelled probes • Time consuming and can only screen one mutation at a time • Expensive
Reverse dot blot hybridisation (RDB) • Simultaneous screening for many mutations • Usually no radioactivity • Relatively inexpensive • Simple, rapid and reliable	Reverse dot blot hybridisation (RDB) • Need sample controls to standardize new mutations • Need good technical expertise in the laboratory to set up and validate RDB • May be expensive if use commercial kits
ARMS-PCR • Simple, rapid and inexpensive • Suitable for technical modification • Can be multiplexed to detect mutation	ARMS-PCR • Need control DNA to validate test and some rare mutations unavailable in homozygous state • Primers can degrade, giving non-specific signal
Restriction enzyme (RE)-PCR • Simple and rapid • Reliable	Restriction enzyme (RE)-PCR • Limited to few mutations • Need care to avoid partial digestion problems • 'Frequent cutter' enzymes not very useful • Some enzymes costly
Real Time PCR • Very quick • No post-PCR processing	Real Time PCR • Quite costly per analysis • Equipment is expensive
Pyrosequencing • Cheap and rapid technique for the diagnosis of a known point mutation • Results are quantitative.	Pyrosequencing • Can only be used for screening known predicted mutations.
Gap-PCR • Simple, rapid and inexpensive • Can be multiplexed to detect mutation	Gap-PCR • Need control DNA to validate test • Limited to diagnosis of deletions with known DNA breakpoint sequences • Amplification of α-gene region technically difficult - possibility of allele drop-out.

5.4 DIAGNOSIS OF UNKNOWN MUTATIONS

5.4.1 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Denaturing Gradient Gel Electrophoresis or DGGE, as originally described by Myers et al, 1987 (86), allows the separation, and thus detection, of DNA molecules that differ by as little as a single nucleotide. The electrophoretic separation is based upon the melting properties of the double-stranded DNA molecule. The two complimentary strands of DNA separate (melt-out) under conditions of increased temperature or under the influence of certain chemicals (termed denaturants). An increase in the temperature and/or concentration of chemical denaturant around a DNA molecule will cause it to melt out along its length in discrete segments or regions, called melting domains. Melting domains vary in length between about 25 up to several hundred bases (bp), and each melts cooperatively at a distinct temperature called a Tm. The Tm of a melting domain is highly dependant on its nucleotide sequence and even a single-base substitution is sufficient to alter the Tm of a domain.

DGGE involves the electrophoresis of double stranded DNA molecules (up to 400-500 bp for efficient analysis) through a vertical polyacrylamide gel that contains a linear gradient (from top to bottom) of increasing chemical denaturant concentration (as opposed to temperature, which is practically more difficult to control). As a DNA fragment enters the concentration of denaturant where its lowest-temperature melting domain begins to denature, the domain 'opens' forming a DNA molecule with a branched structure. This branched DNA has a greatly retarded mobility in the gel matrix. If a DNA sample contains a point mutation in this domain which differs from the normal sequence, the Tm of the domain will be different, the DNA molecule will melt out at a slightly different Tm and thus will have an altered mobility in the gel compared to control DNA. DGGE can be used to detect single base changes in all but the highest-temperature melting domain of a DNA fragment. To facilitate the detection of mutations along the complete length of a DNA fragment under study, a GC-rich segment may be attached to one end of the fragment during the PCR reaction (by the addition of a GC-rich sequence to the 5'end of one of the primer pair). This GC-rich segment, or GC-clamp (usually 30-60 bp long) has a very high Tm, and in comparison the remaining DNA fragment will have a single and lower Tm (87), as shown in Figure 5.14.

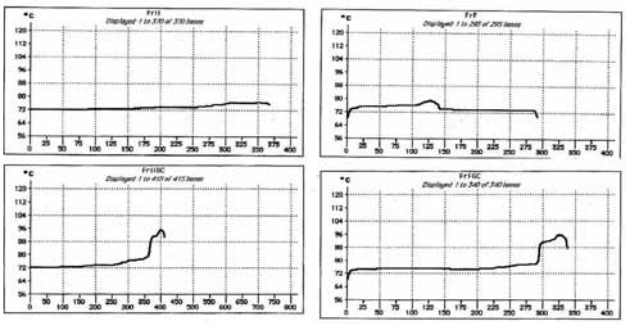


FIG. 5.14
Examples of meltmaps:
A. Fragment B without (i) and with (ii) GC-clamp;
B. Fragment F without (i) and with (ii) GC-clamp.

Prior to the discovery of PCR, DNA fragments for DGGE analysis were generated by cloning. Now with appropriate PCR primers it is possible to generate fragments of any gene of interest. If a DNA sample is heterozygous for a point mutation, the PCR reaction will generate a mixture of double-stranded molecules and up to four bands will be visible after running the DGGE gel. There will be homoduplexes from the wild and the mutant sequences and in addition there will be two types of heteroduplex DNA molecules (which migrate at a slower rate), representing hybridised mismatched wild-type and mutant DNA strands, generated by re-assorting of DNA strands during the PCR reaction. If the sample is either homozygous for the normal (wild) sequence or for a mutation, it will only have one homoduplex molecule (Figure 5.15).

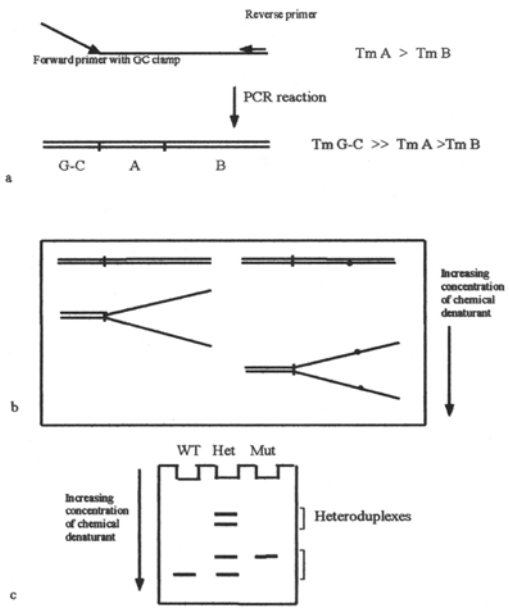


FIG. 5.15
The principle of DGGE: **A.** DNA fragment with a high melting domain A and a low melting domain. B. Electrophoresis of 2 PCR products, which differ by just a single nucleotide substitution in domain. **B.** through a gel containing increasing concentration of chemical denaturant from top to bottom will show "melting-out" at a different a concentration of denaturant ie will have different electrophoretic mobility. **C.** Theoretical example of bands visualized after DGGE for samples which are homozygous wild-type in the region under evaluation (WT), heterozygous for wild-type and mutant allele (Het) or homozygous for the mutant allele (Mut).

In order to select the optimal position for the primers used to amplify a region of DNA for DGGE analysis, and to estimate the range of chemical denaturant required, it is useful to have prior knowledge of the melting behaviour of the fragment under study. This can be done using the computer algorithm (Melt '87) developed by Lerman and Silverstein, 1987 (88). The chemical gradient most appropriate for the fragment is calculated as the Tm of the lowest (preferably main) domain ±10°C whereby 1°C is equivalent to 3% denaturant (see calculations).

If this is not possible, the chemical gradient can be analysed empirically by running the fragment on a gel that has a gradient perpendicular to the direction of electrophoresis (Figure 5.16).

Much of the equipment that is required for making and running of DGGE gels is quite specialized. A gradient maker for small volumes is necessary for creating the vertical chemical gradient in the

gel (Figure 5.17). Furthermore the gels have to be run at high temperature (usually 60°C) for >12 hours, which requires equipment that can heat the electrophoresis enivironment (buffer), maintain a steady temperature and recirculate the buffer to replenish that at the cathode during the run. Thus the electrophoresis has to be carried out in a tank large enough to fit the gel and a heater, with an external pump buffer for recirculation (Figure 5.18). There are a few commercially available DGGE systems, the most comprehensive being the D-Gene system from Biorad.

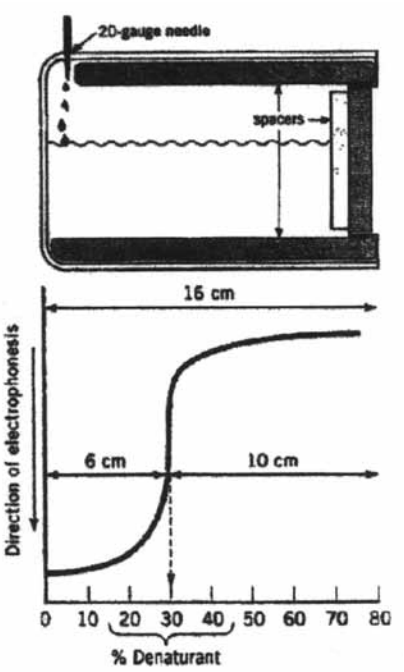


FIG. 5.16
A. Making a perpendicular DGGE gel; the top spacer becomes one long well for loading the sample along the entire width of the gel. **B.** Drawing of the ethidium-bromide stained perpendicular gel after electrophoresis. The steep decrease in mobility in the middle of the gel is due to the cooperative melting of the domain with the lower Tm. A ruler is placed across the top or bottom of the gel when it is photographed, allowing the relative position of the mobility transition midpoint (dotted line) to be determined. The fraction of the left-to-right distance of the transition corresponds to the % denaturant at which the domain melts out. In this example 6/16 or 0.375 is the fraction of the total distance, ie 0.375 x 80% = 30%. Thus a parallel denaturant gel with a range of approximately 15-45% would be optimal for analysing this fragment. (Adapted from Myers RM, Hedrick Ellenson L, Hayashi K, in Genome Analysis, A Laboratory Manual, Vol 2, ed Birren B, Green ED, Klaholz S, Myers RM, Roskans J, Cold Spring Harbor Laboratory Press, 1998).

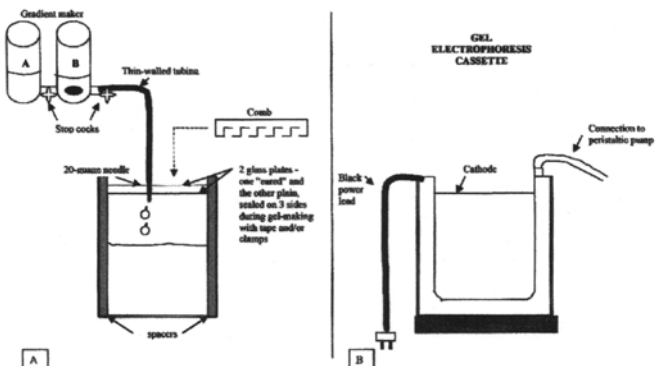


FIG. 5.17
A. Making a DGGE gel.
B. The gel electrophoresis cassette in which gel is placed for electrophoresis. (Adapted from Myers RM, Hedrick Ellenson L, Hayashi K, in Genome Analysis, A Laboratory Manual, Vol 2, ed Birren B, Green ED, Klaholz S, Myers RM, Roskans J, Cold Spring Harbor Laboratory Press, 1998).

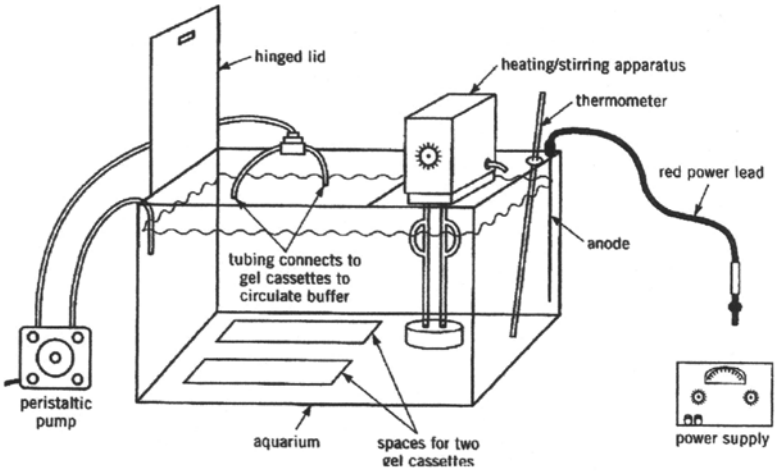


FIG. 5.18
The electrophoresis tank and additional equipment required for electrophoresis. (Adapted from Myers RM, Hedrick Ellenson L, Hayashi K, in Genome Analysis, A Laboratory Manual, Vol 2, ed Birren B, Green ED, Klaholz S, Myers RM, Roskans J, Cold Spring Harbor Laboratory Press, 1998).

It must be noted that DGGE is not a method for direct characterization of point mutations. However, it is extremely useful for:

a. screening gene regions (of up to several hundred base pairs) in order to localise a potential mutation(s), simultaneously excluding regions without a mutation,
b. directing the use of direct mutation assays, such as ARMS or RE-PCR, for those mutations known to occur within the targeted gene region, since most nucleotide changes are associated with a distinct DGGE banding pattern.
c. investigating the genotype status in a prenatal diagnosis sample, alongside positive and negative controls for the parental mutations.

5.4.1.1 DGGE ANALYSIS STEPS

Protocols for mutation detection throughout the length of most of the globin genes have been described (44, 88-93). Here we will focus on a protocol appropriate for the ß-globin gene.

The steps of DGGE analysis include:

1. PCR of the fragment(s) for analysis
2. gel preparation
3. gel electrophoresis
4. gel staining and viewing.

Exact details of each step will vary slightly according to the equipment available in the lab, but the description here is a solid guideline.

5.4.1.2 PCR AMPLIFICATION OF ß-GLOBIN GENE REGIONS

The PCR protocols in this manual are based on those described by Losekoot et al, 1990 (44) and Kleiman et al, 1994 (91), and are suitable for the detection of the majority of common point mutations underlying ß-thalassaemia throughout the world. The ß-globin gene has been divided into 8 different fragments (named A-H), which span the complete coding sequence and the flanking regions at the 5' and 3' end of the gene (Figure 5.19). The optimal position of the primers used to amplify each fragment and the position and length of the GC-clamp was achieved by predicting the fragment melt-map using the computer algorithm (87), examples of which are depicted in Figure 5.14. The sequence of some PCR primer have been modified by using the Amplify version 2.0 computer software (Bill Engels, 1992-1995, Freeware, US), in order to minimize non-specific PCR products (92). The primer sequences for the amplification of fragments A-H are listed in Table 5.19. The fragments should be screened in the order most appropriate for the frequency of mutations in the population under investigation. Fragment E is a region that does not contain many common mutations, but it contains 3 of the 5 polymorphic sites that predict the framework of the ß-globin gene (Figure 5.20), which are potentially useful for linkage analysis when appropriate (93). See example in Figure 5.21.

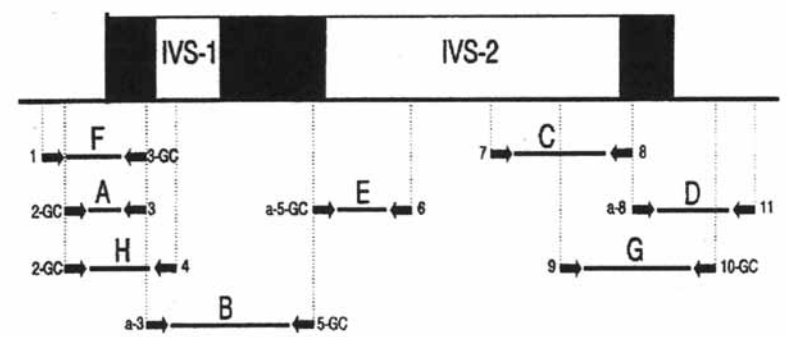
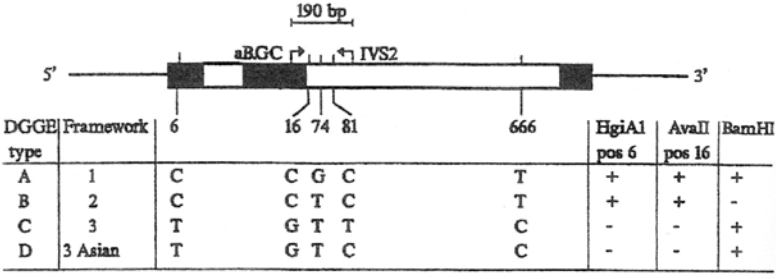


FIG. 5.19
The beta globin genes showing the gene regions amplified for DGGE analysis. Black boxes represent exons; white boxes represent introns.



DGGE type	Framework	6	16 74 81	666	HgiA1 pos 6	AvaII pos 16	BamHI
A	1	C	C G C	T	+	+	+
B	2	C	C T C	T	+	+	-
C	3	T	G T T	C	-	-	+
D	3 Asian	T	G T C	C	-	-	+

FIG. 5.20
The beta globin gene with the position and base substitutions of the framework polymorphisms, especially those detected by analysis of fragment E. Black boxes represent exons; white boxes represent introns.

Table 5.19: Sequences of primers for amplifying fragments of ß-globin gene for DGGE analysis into 8 different fragments (A-H in fig 5.19).

OLIGO NAME	SEQUENCE (5' TO 3')	GC-CLAMP
1	TGAAGTCCAACTCCTAAGCCA	No
2-GC	GTACGGCTGTCATCACTTAGACCTCA	GC-45*
3	Caacttcatccacgttcacc	No
3-GC	Caacttcatccacgttcacc	GC-45*
a-3	GGTGAACGTGGATGAAGTT	No
4	ACCTTGATACCAACCTGCC	No
5-GC*	TGCAGCTTGTCACAGTGCAGCTCACT	GC-45*
a-5-GC	AGTGAGCTGCACTGTGACAAGCTGCA	GC-45*
6	AAACGATCCTGAGACTTCCA	No
7	GTGTACACATATTGACCAAA	No
8	AGCACACAGACCAGCACGTT	No
a-8	AACGTGCTGGTCTGTGTGCT	No
9	ATTCTGAGTCCAAGCTAGGC	No
10-GC	CTTAGGGAACAAAGGAACCTT	GC-57*
11	AAATGCACTGACCTCCCACA	No
GC-45	CGCCCGCCCCGCCCCCGTGCCCCCCGC GCCGCCCGCCCCGCCCCC	
GC-57	CCCGCCCCGCCCGCCCCCGCCCCGCCC GCCGCGCCCCCCGTGCCCCCGCCCCGCCCG	

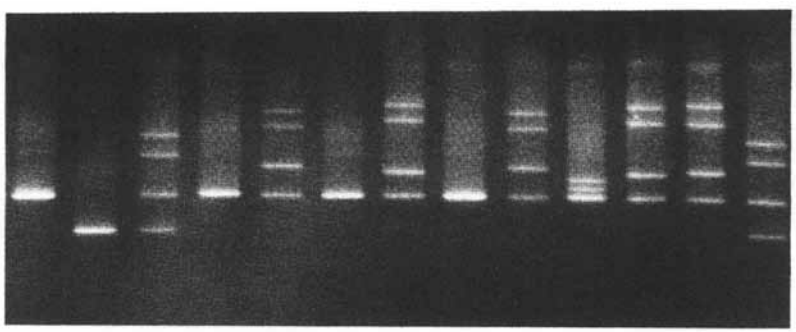


Figure 5.21
Photograph of a DGGE gel stained with ethidium bromide and viewed with UV-light. Analysis of Fragment B. Lanes 1, 4, 6, 8: no mutation; lane 2: Homozygous IVSI-6 (T→C); Lane 3, 13: heterozygote IVSI-6 (T→C); Lanes 5, 9: heterozygote IVSI-110 (G→A); Lanes 7, 11: heterozygote IVSI-5 (G→A); Lane10: heterozygote codon 39 (C→T); Lane 12: heterozygote IVSI-1 (G→A).

Reagents:
For PCR of each ß-globin gene fragment, using a 50 µl reaction, add the following to an Eppendorf tube, on ice:

a. 0.5 µg genomic DNA
b. 15-20 pmol of each primer (forward and reverse for relevant fragment)
c. H_2O up to 50 µl final volume
d. 5 µl of 10x reaction buffer (usually provided with the Taq polymerase by the manufacturer, containing 500 mM KCl, 100 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 2 mg/ml BSA)
e. 2 mM dNTP's
f. 1 unit of Taq polymerase
g. Overlay with 50 µl paraffin oil.

PCR conditions: The following cycling conditions are appropriate for the majority of PCR machines for all fragments, although some minor adjustments may be required to optimise the PCR reaction:

a. Denaturation at 95ºC for 60 s.
b. Annealing at 58-60ºC for 60 s (may differ between PCR machines)
c. Extension at 72ºC for 90 s.
d. Repeated for 35-40 cycles.

Following the PCR reaction, 5 µl may be run on a 1.5% agarose gel to check efficiency and specificity. The expected sizes of fragments A-H are included in Table 5.20. To prepare samples for loading on to the DGGE gel, mix approximately 10 µl of PCR product with 5 µl of loading dye.

Table 5.20: DGGE gel running conditions for fragments A-H.

Fragment	AMPLI-PRIMERS	PCR PRODUCT SIZE* (bp)	GRADIENT	% ACRYLAMIDE
A	2-GC and 3	252	45-75	6
B	a-3 and 5-GC	370	40-70	6
C	7 and 8	423	25-55	6
D	a-8 and 11	317	25-55	6
E	a-5-GC and 6	194	35-55	8
F	1 and 3-GC	295	45-75	6
G	9 and 10-GC	243	40-70	6
H	2-GC and 4	285	40-70	6

* size excludes length of GC clamps

5.4.1.3 DGGE GEL PREPARATION

Equipment:

a. Two glass plates approximately 18 x 20 cm, one "eared" and the other plain.
b. Spacers and combs 1mm thick.
c. DGGE holder for stabilizing glass plates during gel making.
d. Gradient maker, for small volumes (up to approximately 25 ml/chamber), and appropriate tubing.
e. Magnetic stirrer and small magnets to fit diameter of gradient maker chambers.
f. Tape.
g. DGGE holder with electrodes (gel electrophoresis cassette).
h. Transparent aquarium with lid (to avoid excessive evaporation of buffer during electrophoresis).
i. Heating element with thermostat and circulating ability.
j. Thermometer to monitor temperature during electrophoresis.
k. Power supply.
l. Syringe and needles to flush out wells prior to sample loading .
m. Fine-ended (duck-billed) tips for sample loading.
n. Staining tray.
o. UV transilluminator, 256 nm wavelength.
p. Camera.

Reagents:

a. 40% Acrylamide, which consists of:

i. acrylamide bis-acrylamide 37.5:1	100 g
ii. H_2O	up to 250 ml

b. 20x TAE, pH 8.0, which consists of:

i. Tris-HCl	800 mM	96.912 g
ii. Na_2EDTA	20 mM	7.445 g
iii. Na Acetate	400 mM	54.432 g
Adjust pH with acetic acid		(about 36 ml)

c. 80% Denaturant / 6% acrylamide, which consists of:

i. 75 ml of 40% stock / 6% acrylamide	
ii. 32% formamide	
iii. 160 ml of 100% stock 5.6 M Urea	170 g
iv. 1x TAE: 25 ml of 20x TAE stock	
v. H_2O	up to 500 ml

d. 0% Denaturant/6% acrylamide, which consists of:

i. 75ml of 40% stock 6% acrylamide	
ii. 1x TAE: 25 ml of 20x TAE stock	
iii. H_2O	up to 500ml

e. 80% Denaturant/8% acrylamide
i. 100 ml of 40% stock 8% acrylamide
ii. 32% formamide 160 ml of 100% stock
iii. 5.6 M Urea 170 g
iv. 1x TAE: 25 ml of 20x TAE stock
v. H_2O up to 500 ml

f. 10% Ammonium peroxydedipersulphate
5 g per 50 ml water
Store in 1ml aliquots at –20ºC. Discard surplus after use.

g. TEMED: N,N,N',N'-Tetramethylethylenediamine.
Store at 4ºC

h. Loading Buffer
i. 0.25% bromophenol blue, which consists of:
ii. 0.25% xylene cyanol FF
iii. 15% Ficoll-isopaque (6.1%-18.6%)

i. Ethidium bromide
10 mg/ml (store in dark bottle)

Preparation of DGGE gel:

1. Clean glass plates well, using in succession strong detergent, water and finally acetone or alcohol. Dry well.
2. Place spacers between the plates and tape the sides and bottom, sealing very well to prevent leakage (see Figure 5.17).
3. Place the comb in between the glass plates and put the construction into a "holder" that stabilizes the plates in a vertical position. NOTE: Suitable size of glass plates is approximately 18-20 cm, with spacers and combs 1 mm thick. The volume of gel required is approximately 32 ml.
4. Connect the tubing to a clean and dry gradient maker, closing the communicating channel between the 2 chambers and also the tubing that leads from the gradient maker to the glass plates, with the use of stop-cock or clamps. The tubing that leads from the gradient maker to the glass plates should be about 25-26 cm long.
5. Position the magnetic stirrer about 25 cm above the glass plates and place the gradient maker on top, securing well.
6. In two separate test tubes prepare two denaturing solutions (representing the high and low of the range appropriate for the fragment) of 16 ml* each, and add 10µl TEMED and 130 µl of fresh ammonium peroxydedipersulphate (10% APS). Mix well.
   - For glass plates sized approximately 18 cm x 20 cm with 1mm width spacers; the relative volume of 0% and 80% acrylamides to achieve the upper and lower limits of the range should be calculated as follows: (% denaturant/80% denaturant) x volume.

For example, for a 70% denaturant solution, calculate 70/80 x16, ie 14 ml 80% solution + 2 ml 0%

solution. For gradient to be as precise as possible the volume of acrylamide should be the volume between plates plus 1-2 cm spare in case of slight leakage compensation.

7. Without delay, put the solution with the lowest denaturant concentration in the chamber of the gradient maker furthest from the glass plates (chamber A in Figure 5.17). Allow a minimal volume to flow through the connecting channel to avoid blockage by air, and then place the solution with the highest denaturant concentration in the chamber nearest the glass plates (chamber B in Figure 5.17).
8. Put the magnet in this chamber, turn stirrer on and open the connecting tube between chambers to begin mixing the two solutions. Immediately open the connection on the tubing leading to the glass plates and the acrylmide will begin to flow steadily under the force of gravity.
9. As soon as the solution has reached the comb (and slightly overflowed), stop the flow and remove the tubing. Wash the tubing and gradient maker well under running water.
10. The gel will polymerise within 30-45 minutes, depending upon ambient temperature (the warmer the temperature, the faster the polymerisation).

5.4.1.4 DGGE GEL ELECTROPHORESIS

1. Fill the electrophoresis tank with running buffer (1x TAE, about 15-20 litres depending on apparatus). Heat buffer to 60ºC. (Note: The running buffer can be re-used about 5 times).
2. Once the gel has polymerised, carefully remove the comb and clean away excess acrylamide, taking care not to spoil the wells. Remove the tape from bottom of plates.
3. Place the gel in the gel electrophoresis cassette (Figure 5.17B) and submerge in the buffer in the tank (Figure 5.18). Attach the electrophoresis cables (cathode at top) and pre-run gel for about 30 min at 40 volts (about 40 mA).
4. Flush the wells with a fine syringe and load about 10-15 µl of each sample, containing loading dye, using thin-ended or duck-billed tip.
5. Run gel about 16 hours (overnight) at 40-50 volts. The gel is ready for viewing when the bromophenol blue dye has run completely out of the gel.

5.4.1.5 DGGE GEL STAINING AND VIEWING

1. After the run, turn off power supply and remove gel from tank and holder.
2. Unseal the sides of the glass plates and gently remove one of the glass plates, leaving the gel to rest on the other.
3. Place the gel (on the glass plate) in a container with approximately 250 ml of 1x TAE (or water) containing 0.5 µg/ml ethidium bromide and stain for about 15 minutes. (Note: The container should be covered to protect the gel from light).
4. Destain the gel for 5 minutes in water.
5. Place the gel onto a UV transilluminator, sliding it off the plate carefully so it does not fold over or split.
6. Examine the gel under UV light (256 nm wavelength) and photograph.

5.4.1.6 TROUBLESHOOTING AND PRECAUTIONS

DGGE is a technically demanding method, and the following points must be noted: All chemicals used especially the components of the gel (acrylamide, formamide and urea) should be of highest quality and as fresh as possible. Acrylamide solution is not very stable and thus acrylamide powder is recommended which should be made into solution not too long before use. Many of the chemicals used in DGGE are highly toxic (eg acrylamide, formamide) and should be handled with extreme care. The interpretation of DGGE patterns should be done with careful comparison to controls since any nucleotide change, including polymorphisms, may be potentially detected. It must be noted that DGGE is not a method for direct characterization of point mutations, and any mutation detected should be confirmed by a second direct mutation assay.

5.4.1.7 CALCULATIONS FOR CONVERSION OF TEMPERATURE TO DENATURANT CONCENTRATION AND CALCULATION OF DGGE GRADIENT RANGE

1. 1.100% denaturant = 7 M urea plus 40% formamide.
   (Stock solutions of 80% denaturant and 0% denaturant are satisfactory for making most gradient ranges, see Table 5.20).
2. The gradient range should be calculated as the Tm of main melting domain ±10ºC.
3. 1ºC is equivalent to 3% denaturant.
   Thus for gels run at 60ºC calculate denaturant concentration % as 3 x (Tm-60ºC).
   Eg for a fragment with main (lowest) melting domain of 72ºC, the gradient range should be 62ºC – 82ºC.

   Where 62ºC = 3 x (62-60) = 6%.
   And 82ºC = 3 x (82-60) = 66%.

5.4.2 DNA SEQUENCING - SANGER METHOD

DNA sequencing allows the analysis of the exact nucleotide sequence of the gene, or area of gene, under study. The method most commonly used is based on that first described by Sanger et al, 1977 (95). The method depends on the *in vitro* synthesis of DNA strands copied from a DNA template (usually generated using PCR nowadays), during which the synthesis of each nascent DNA chain is stopped at every point along the strand, following the random incorporation of a dideoxynucleotide triphosphate (ddNTP). ddNTP's can be incorporated by DNA polymerase into the growing chain, but the absence of a hydroxyl residues at the 3' position prevents the extension of the DNA chain.

The basic reaction includes the template DNA (single-stranded), of which the nascent sequencing strand will be a complementary copy, a synthetic oligonucleotide or 'primer' about 20 bp long which is complementary to a small region of the template DNA close to the target area for sequencing, a specialized DNA polymerase enzyme (T7, or a thermostable DNA polymerase) and free nucleotides, most of which are deoxynucleotide triphosphates (dNTP's), with a small fraction of dideoxynucleotide triphosphates (ddNTP's).

There are variations of the Sanger protocol, which differ relative to the DNA polymerase employed for strand synthesis and/or the detection system used to detect the newly synthesised DNA strands. For the so-called manual sequencing method the product of the sequencing reaction is usually radioactively labelled and is separated on a vertical acrylamide gel, which is then exposed to produce an autoradiograph for manual reading (Figure 5.22). More recent modifications to the Sanger protocol include cycle-sequencing (exploiting the use of a thermal stable DNA polymerase) and detection of fluorescently labelled nascent DNA using an automatic sequencer (96). Following the cycling reactions, the products are separated according to size on a polyacrylamide gel (or capillary), which is monitored by a laser to detect the fluorescently labelled nascent strands. There are two main fluorescent dye chemistries to label the nascent strand, involving either a fluorescent primer or fluorescent dNTP's.

In this chapter the basic steps of 2 general sequencing protocols are outlined: Manual sequencing using single-stranded DNA template, T7 polymerase and radioactive label. Cycle sequencing with use of fluorescent labels and analysis on automatic sequencer.

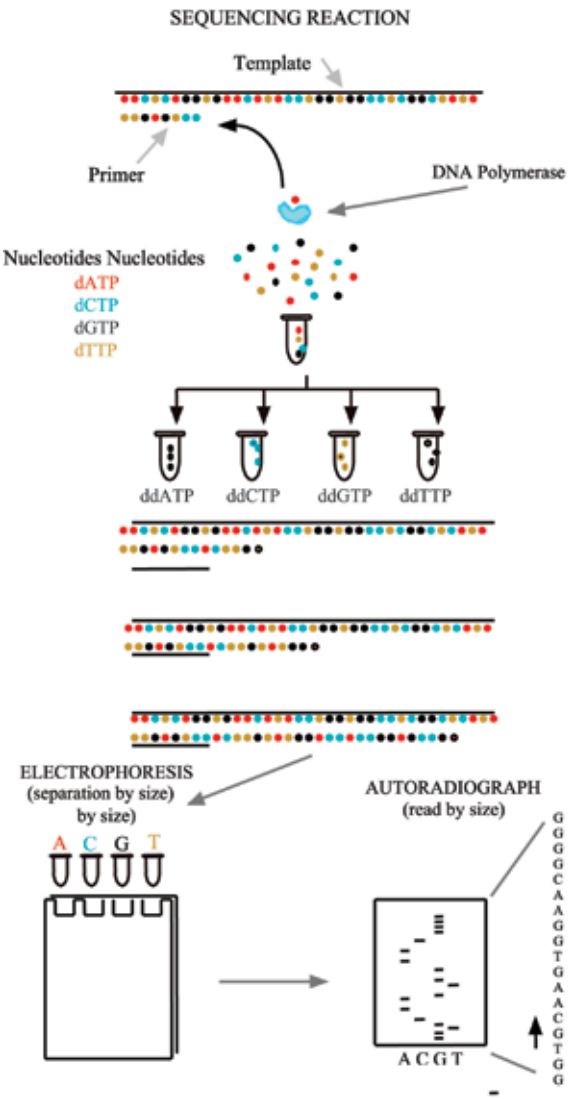


Figure 5.22
Figure depicting the principle of a manual sequencing reaction, followed by electrophoresis and autoradiography

5.4.2.1 PREPARATION OF SINGLE-STRANDED DNA TEMPLATE BY ASYMMETRIC PCR (MANUAL SEQUENCING)

This method involves a first amplification to produce double-stranded DNA. For a 50 µl reaction, add the following to an Eppendorf tube, on ice:

a. 0.1-0.5 µg genomic DNA
b. 20 pmol of each primer (forward and reverse for relevant fragment)
c. H_2O up to 50 µl final volume
d. 5 µl of 10x reaction buffer (usually provided with the Taq polymerase by the manufacturer, containing 500 mM KCl, 100 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 2 mg/ml BSA)
e. 2 mM dNTP's
f. 1 unit of Taq polymerase
g. Overlay with 50 µl paraffin oil.

Protocol for PCR:

1. With 30 cycles of: denaturation at 95ºC for 60 s, annealing at a temperature appropriate for Tm of primer pair for 60 s, and extension at 72ºC for 90 s.
2. A second PCR reaction follows using 2 µl of the first PCR and only ONE of the 2 primers in a100 µl reaction volume.
3. Following the second PCR, the product is cleaned to remove excess dNTP's etc:
4. Add 1 volume of chloroform, mix and spin in microcentrifuge at 10,000 rpm for 5 minutes.
5. Remove supernatant to a clean eppendorf tube and add 100 µl of 5 M ammonium acetate and 200 µl isopropanol.
6. Leave to stand for 10 min, then spin at 10,000 rpm for 10 minutes.
7. Discard supernatant, wash pellet with 70% ethanol and dry. Dissolve single-stranded DNA pellet in 15 µl of distilled water.
8. Check 3-5 µl of product on a 1% agarose gel in TBE, stain with ethidium bromide and view under UV light. (Single-stranded DNA product usually runs slower than equivalent double-stranded product).

Sequencing reaction: This forsees the use of one of the many available kits which provide reaction buffers, labelling mixes (including dNTP's), termination mixes (including ddNTP's), T7 DNA polymerase and enzyme dilution buffer. The user has to purchase separately the radioactively labelled dNTP (usually either [α-35-S]-dATP or [α-35-S]-dCTP.

1. Take 10 µl single-stranded DNA product, and add 2 µl sequencing primer.
2. Incubate at 37ºC for 10-20 minutes and then leave at room temperature for a further 10-20 minutes to ensure primer-template annealing.
3. To the annealed primer-template mix add 3 µl labeling mix, 1µl radioactive dNTP (10 µCi) and 2 µl T7 polymerase (usually diluted just prior to use with enzyme dilution buffer).
4. Mix gently and leave at 37ºC for 5 minutes.
5. During this time label 4 Eppendorf tubes (1 each for A, C, G and T), add 2 µl of the appropriate ddNTP termination mix to each and warm at 37ºC for a few minutes.
6. Distribute 4.5 µl of completed labelling reaction to each of the 4 ddNTP termination mixes and incubate for a further 5 µl.

7. Add 5 µl stop solution and place on ice.
8. Immediately before loading, heat samples to 80ºC for 2 minutes and place on ice.

Polyacrylamide gel, electrophoresis and autoradiography:
There are many types of electrophoresis equipment available commercially, and the arrangement of glass plates and spacers may vary. Standard sequencing gels are usually about 40 cm long with spacers and combs that are 0.4 mm thick. All sequencing electrophoresis apparatus includes a heatable "plate" to warm the surface of the gel during the run.

The concentration of acrylamide in the gel depends upon the size of the DNA fragments to be analysed. Sequences between 20-250 nucleotides from the primer can be read from 40 cm length gels and 6% acrylamide with a single loading.

The following solution containing 6 or 8% acrylamide can be prepared as follows:

a. 6% acrylamide:

Acrylamide	17.1 g
Bis-acrylamide	0.9 g
Urea	150 g
10x TBE*	30 ml
Distilled H_2O to	300 ml

b. 8% acrylamide:

Acrylamide	22.8 g
Bis-acrylamide	1.2 g
Urea	150 g
10x TBE*	30 ml
Distilled H_2O to	300 ml

(*10x TBE: 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA).
The solution is stable at 4ºC for several months.

1. Sequencing gels should be made at least 2 hours before loading.
2. For plates 40 cm long, 20 cm wide and 0.4 mm wide, about 40 ml of gel is required, as follows: 40 ml of 6% or 7% acrylamide/8M urea, 300 µl 10% ammonium persulphate and 30 µl TEMED. The mixture will polymerise within 1 hour.
3. Once polymerised, remove the well-former from the top, clean away excess acrylamide and place in electrophoresis apparatus. Fill upper and lower buffer reservoirs with 1x TBE and pre-run gel for about 30-60 minutes to reach a temperature of about 50ºC.
4. Place a sharks-tooth comb on top of gel. Flush out wells and load 2-3 µl of each dNTP from each sample per lane (ie one sample is 4 lanes, A, C, G and T).
5. Electrophoresis is performed at 1500-2000 volts for 2-3 hours depending upon the distance from the primer to be analysed.
6. After electrophoresis, turn off the power, remove one glass plate and place a piece of Whatman paper (35 x 42 cm) to stick over the entire surface of the gel, taking care to avoid formation of bub-

bles and not to split the gel.

7. Carefully cover the gel side with Saran-wrap (or equivalent) and place paper side down on a gel dryer and dry under vacuum for about 90 min at 80ºC.
8. Remove wrap from gel and in a dark room place a 35 x 42 cm X-ray film (Kodak XAR-2 or equivalent) and place in an X-ray cassette. Perform autoradiography overnight, and read the sequence of nucleotides according to size (Figure 5.22).

5.4.2.2 CYCLE SEQUENCING

Since the details of the method for an automatic sequencer depends upon the particular system used, a general protocol will be outlined. Most of the preparation steps are supported by a wide range of commercially available kits for template preparation and sequencing. Some machines are able to detect only a single fluorescent label, and others have the ability to detect multiple fluorescent labels. The level of automation relative to gel (or capillary) preparation and sample loading may vary considerably between machines. Additionally each system will have particular software for controlling running conditions and analysing data. The quality of template and precision of the sequencing reactions are paramount to the generation of good data for base calling.

Preparation of double-stranded DNA template and purification:
For a 50 µl PCR reaction to produce double-stranded DNA, add the following to an Eppendorf tube, on ice:

a. 0.1-0.5 µg genomic DNA.
b. 20 pmol of each primer (forward and reverse for relevant fragment).
c. H_2O up to 50 µl final volume.
d. 5 µl of 10x reaction buffer (usually provided with the Taq polymerase by the manufacturer, containing 500 mM KCl, 100 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 2 mg/ml BSA).
e. 2 mM dNTP's.
f. 1 unit of Taq polymerase.
g. Overlay with 50 µl paraffin oil.
h. With 30 cycles of: denaturation at 95ºC for 60 s, annealing at a temperature appropriate for Tm of primer pair for 60 sec, and extension at 72ºC for 90 sec.
i. Following the amplification reaction, a 5 µl aliquot can be checked on a 1% agarose gel in 1x TBE. The template may then be purified by using a commercially available PCR-product purification kit (eg by Qiagen).

PCR reaction: There are several commercially available cycle-sequencing kits and most manufacturers will recommend that (or those) most suited to the automatic sequencer to be used. Additionally there are two main fluorescent dye chemistries used to label the nascent strand, involving either a fluorescent primer or fluorescent dNTP's. Essentially cycle sequencing is a PCR reaction which involves making a template-primer mix which is then distributed amongst 4 tubes containing dNTP's and very small quantities of either ddATP or ddCTP or ddGTP or ddTTP, an appropriate buffer and a thermostable polymerase.

5.4.3 MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA)

Multiplex Ligation-dependent Probe Amplification (MLPA) is a technology based on ligation of multiple probe-pairs hybridised across a (usually large) region of interest, followed by quantitative PCR using universal-tag PCR primers for all ligated probe-pairs and subsequently fragment analysis. In this way MLPA can detect deletions or duplications across the locus analysed and represents a valuable alternative or supplementary method to gap-PCR when investigating known and unknown deletions underlying α-, ß- or δß-thalassaemia (10, 97-98).

The MLPA methods described here refer to two types of (commercially available) MLPA assays for each of the α- and the ß-globin gene clusters. The MLPA-kit from MRC-Holland makes use of cloned cosmid probes, which are restricted to the α- and ß-gene clusters and in the regulatory elements. The MLPA assay as described by Harteveld et al. (2005) [10] makes use of oligonucleotide probes designed over a larger region allowing the detection of both smaller and more extensive deletions involving the α- and ß-globin gene cluster and neighbouring regions (Figures 5.23 -5.26). These MLPA probes are available from the Leiden Genome Technology Center in Leiden, NL (www.lgtc.nl). The major experimental differences are the probe length, and therefore the optimal separation region on the fragment analyser, the location of the probes and the use of additional fluorescent dyes. The MRC-Holland probes range in length from approximately 100-480 bp using FAM-labelled primers, while the oligonucleotide probes (LGTC) have their optimal separation between 40-120 bp using ROX- and FAM labelled primers.

Reagents:

a. MRC-Holland kit: SALSA P140B (www.MRC-Holland.com) for the detection of deletions/duplications in the α-globin gene cluster and Multi Species Conserved regulatory element (also known as HS-40 region). Reagents included in the kit (SALSA Probe mix, SALSA MLPA buffer, Ligase-65 buffer A and B, Ligase-65, 10x SALSA PCR buffer, MLPA-Primer mix, SALSA Enzyme Dilution buffer and SALSA Polymerase).
b. MRC-Holland kit: SALSA P102 for the detection of deletions/duplications in the ß-globin gene cluster and Locus Control Region (ß-LCR) .
c. Size standard: GeneScan, LIZ-500 (Applied Biosystems) The formamide/size standard mix is prepared by adding 0.25 µl LIZ-500 size standard to 15.75 µl (de-ionized) formamide per reaction before loading on the capillary fragment analyser.
d. LGTC HBA Alfa GlobinMLPA kit (www.LGTC.nl) for the detection of large deletions/duplications involving the α-globin gene cluster and beyond. Reagents included in the kit.
e. LGTC HBB Beta GlobinMLPA kit (www.LGTC.nl) for the detection of large deletions/duplications involving the ß-globin gene cluster and beyond.

Sample preparation and conditions: MRC-Holland kit.

1. Prepare the DNA-samples for analysis by diluting to 50 ng/µl; include also the appropriate normal and positive control samples to be analysed in parallel.
2. Put 1 µl of the diluted DNA-sample in labelled 0.2 ml PCR tubes and heat for 5 min. at 98°C to denature and spin down to collect the DNA-sample at the bottom of the tube.

FIG. 5.23
Schematic presentation of the alpha-globin gene cluster with the positions of the MLPA probes MRC-Holland kit P140B2 (not on scale).

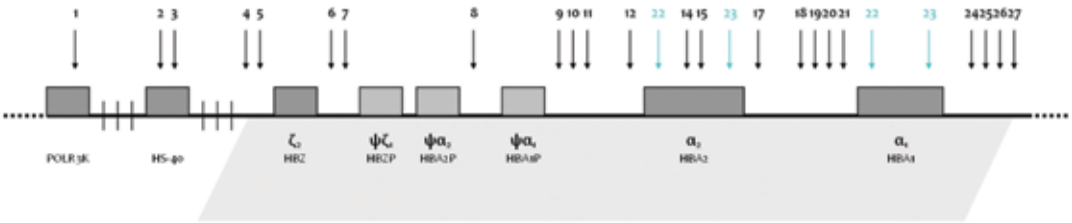


FIG. 5.24
Schematic presentation of the alpha-globine locus with the positions of MLPA probes LGTC HBA – kit (Harteveld et al. 2005).

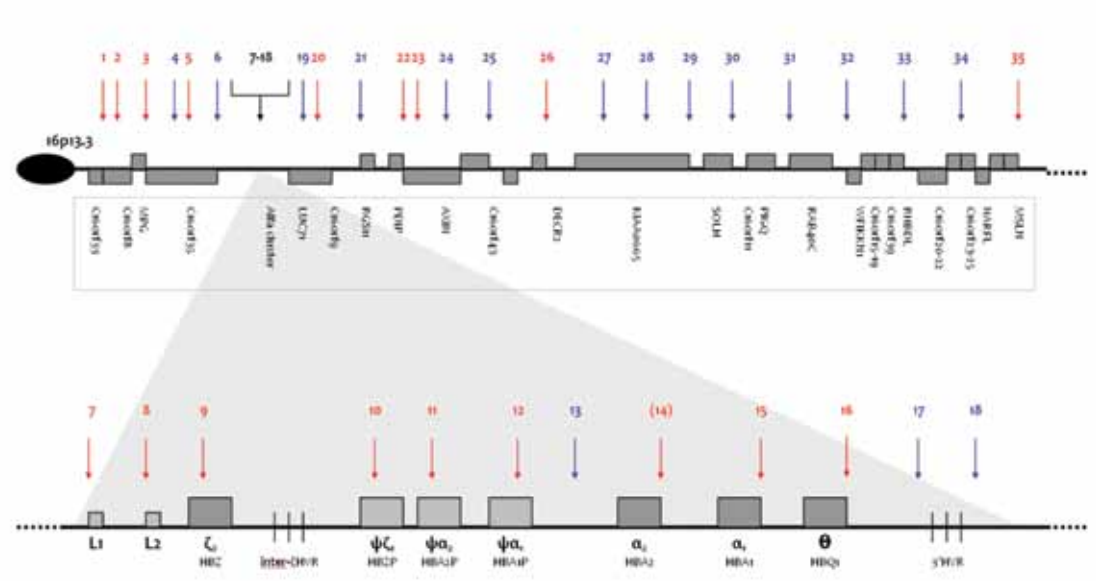


FIG. 5.25
Schematic presentation of the beta-globin gene cluster with the positions of the MLPA probes MRC-Holland kit P102 (not on scale).

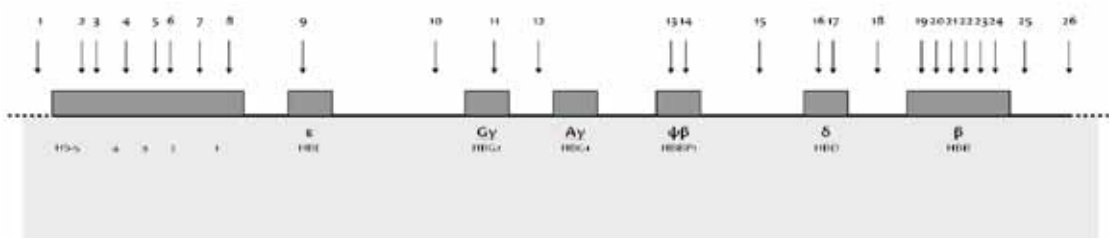
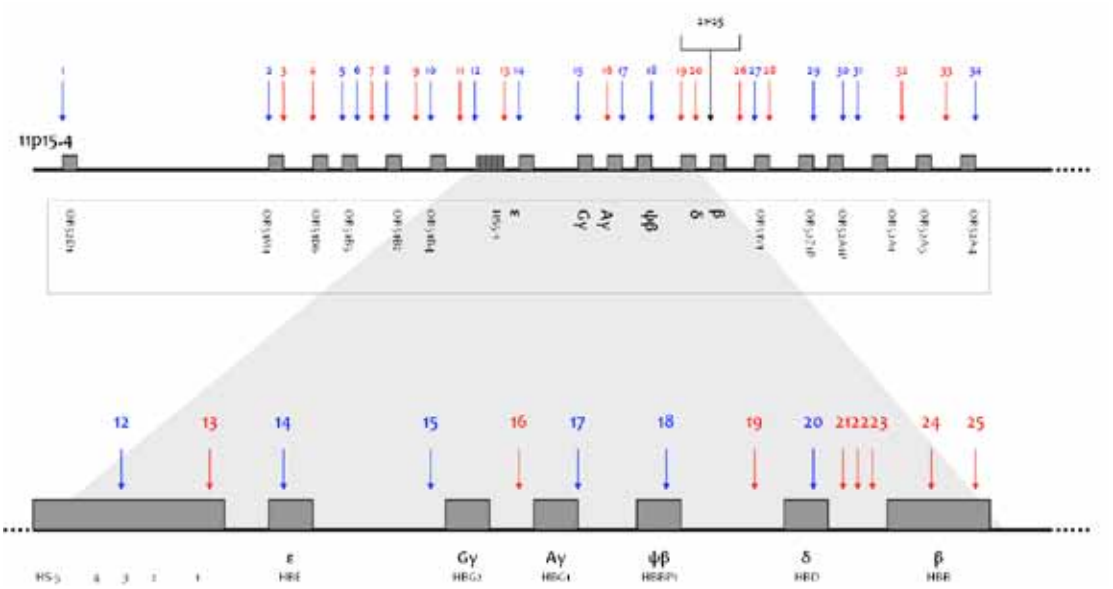


FIG. 5.26
Schematic presentation of the beta-globine locus with the positions of MLPA probes LGTC HBA – kit (Harteveld et al. 2005).



3. Prepare a Hybridisation Master Mix for all reactions containing per reaction: 0.375 µl SALSA Probe mix, 0.375 µl SALSA MLPA buffer and 0.25 µl water, and keep at room temperature to prevent precipitation. Add 1 µl to the DNA-sample and mix by pipetting up and down several times.
4. Heat the sample for 1 min at 95°C, and hybridise for 3 hours at 60°C.
5. Prepare a Ligase Master Mix containing per reaction: 0.75 µl Ligase-65 buffer A, 0.75 µl Ligase-65 buffer B, 6.25 µl water and 0.25 µl Ligase-65. While maintaining the reaction at a temperature of

54°C, add to each tube 8 µl of this Ligase Master Mix and mix by pipetting up and down. Incubate 15 min at 54°C, followed by a ligase inactivation step of 5 min at 98°C. Cool down to 4°C. Samples can be stored for 48 hours or at -20°C for longer periods, however, the best results are obtained when the PCR reaction is done immediately after ligase inactivation.

6. Prepare a PCR Master Mix containing per reaction: 2 µl 10x SALSA PCR buffer, 15.75 µl water, 1 µl MLPA-Primer mix, 1 µl SALSA Enzyme Dilution buffer and 0.25 µl SALSA Polymerase. This mix needs to be made less than 1 hour in advance and stored on ice.
7. The tubes containing the ligated products should be preheated at 60°C, subsequently 20 µl of the PCR Master Mix is added to each sample and amplification initiated: 20 s at 95°C, 30 s at 60°C and 1 min at 72°C for 33 cycles, 20 min at 72°C and hold at 15°C. The PCR products can be stored at 4°C for at least 48 hours.
8. The sample for loading contains 1 µl undiluted PCR product added to 16 µl of formamide/size standard mix.
9. Fragment analysis can be performed according to the manufacturer's instructions on an ABI 3130, 3730 or an equivalent apparatus.
10. Data-analysis is performed by using the Genemarker software of Softgenetics

Sample preparation and conditions: LGTC- kit.

1. The reaction conditions and pipetting schemes are similar to the MRC-Holland kit as both kits contain the same components.
2. The major difference however is the use of a different Probe-mix (the ones included in the LGTC kit) in the Hybridisation Master Mix and the use of two differently labelled MLPA-primer mixes in the PCR reaction Master Mix. The Hybridisation Master Mix is prepared in double amount for 2 µl of
3. DNA dilution and split in two after the ligase inactivation step.
One half is amplified with the universal MLPA-primer set for PCR labelled with FAM, the other with the ROX labelled primer set both included in the LGTC kit. Of each reaction 1 µl is pooled in 16 µl of formamide/size standard mix before loading on the ABI for fragment analysis in two colours.
4. For fragment separation on the ABI 3130, 3730 or other it is important to realize that probe lengths are different, the MLPA-probes are oligonucleotide probes, which differ in length between 40 and 120 bp.

Interpretation of results: Originally for quantitative analysis, trace data from GeneScan (Applied Biosystems) were exported to Excel (Microsoft; www.microsoft.com) to calculate allelic loss in the patient samples tested [98]. However, more advanced software is available now, like for instance GeneMarker software from Softgenetics® (www.softgenetics.com). An example is shown in Figure 5.27.

In brief, two probes for unlinked loci were included per probe set as a reference in each sample. The height of each α- (or ß-) globin cluster specific probe peak was divided by the sum of the heights of the two reference probe peaks to give a ratio. The median ratio for each probe across all samples was calculated and this value was used to normalize each probe to 1.0, which corresponds to a copy number of two. The upper threshold for deletions was set at 0.75 and the lower threshold for duplications at 1.25. The normalizing factor for each sample was calculated as the mean value of the unaffected probes within a sample (defined as falling between 0.8 and 1.2) and dividing all values within that sample by this value (Tables 5.21-5.24). Detection of deletions is simplified by the fact

that a series of flanking probes all generate a decreased signal. In cases of unlinked or single probe deletions the region covering the MLPA probes is amplified and sequenced to rule out the presence of rare sequence variants under the ligation site.

Trouble shooting: The DNA quality and concentration are both very critical in order to obtain reliable MLPA results. For the DNA isolation procedure the salting-out procedure is preferred over phenol extraction. Automated DNA isolation (Gentra) or column based DNA isolations give the most reliable results. From experience it was noted that concentrations of DNA-stock lower than 40 ng/µl are likely to give poor peak ratios, and it is preferable to prepare a DNA dilution of 50 ng/µl from a higher concentrated stock solution of DNA (100-500 ng/µl). It was noted recently that the presence of RNA may disturb the ratios of certain probes (see MRC-Holland web-site for comments), because of competition between probe and DNA or RNA as a template, which gives the impression that certain probes are deleted. In most DNA isolation procedures, however, the unstable RNA is virtually absent due to degradation. If odd ratios seem to disturb the normal pattern it may be advisable to perform an RNase treatment after the DNA-isolation.

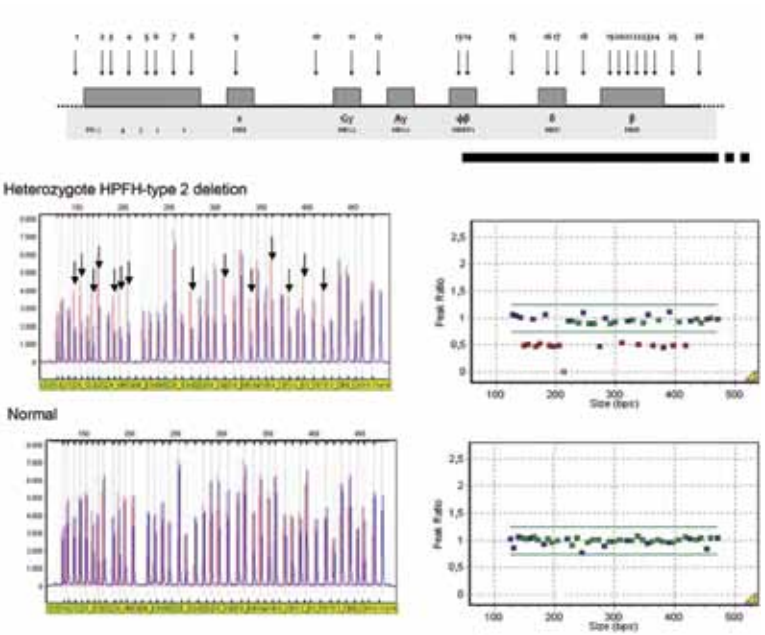


FIG. 5.27
Schematic presentation of the beta-globin gene cluster showing the positions of the MLPA probes (MRC-Holland P102). The black bar indicates the deletion type HPFH-2. The stippled line on the right indicates that the deletion length can not be determined using this kit as the most 3'probe is deleted. In this case the LGTC-kit could be used as more probes are available in the region flanking the beta-globin gene cluster. The upper graph shows the peak pattern of the HPFH deletion carrier on the left and the plot of the ratio's in comparison to the normal control on the right (GeneMarker). Vertical arrows indicate which peaks show approximately half the intensity. These coincide with the deleted area. The lower graph shows a healthy individual for comparison.

5.4.4 EVALUATION OF METHODS

The advantages and disadvantages of the most commonly used methods for characterizing unknown mutations are listed below.

ADVANTAGES	DISADVANTAGES
DGGE • Relatively cheap • Suitable for large scale screening • Characteristic patterns due to heteroduplexes • Predictive computer programs make it easier to optimize	DGGE • Experience required to interpret results as DGGE detects polymorphic as well as disease-causing mutations • CG-rich regions difficult to investigate • Sometimes laborious to optimise conditions • Overall DGGE is technically demanding
Direct sequencing (Automated) • All mutations identified • Use of automated sequencers makes it more rapid and easier. • In some systems ddNTP's can be labelled, precluding use of modified primers.	Direct sequencing (Automated) • Expensive equipment • PCR-products need to be purified.
Pyrosequencing • Cheap and rapid technique for the diagnosis of a known point mutation. • Results are quantitative.	Pyrosequencing • Cannot be used for screening unknown mutations.
MLPA • Detects all deletion and duplication mutations (common, rare and novel) compared to gap-PCR • Simple and rapid	MLPA • Does not provide a definitive diagnosis, result is always consistent with an estimated deletion length and location of breakpoints, which generally is sufficient for diagnostics. • Requires automated DNA sequencer

Table 5.21 Interpretation heterozygotes known alpha-thalassaemia deletions (MRC-Holland P140B2)

Probe name	Ratio SEA	ratio wt	het BW	hom BW	het SW	alfa-thal	het SEA	het MED1	het (a)3.5	het FIL	het THAI	het Dutch/ MedII	BW/SEA	african pahse	del HS-40
01_HS-40 PROBE	1,36	1	1	1	1	1	1	1	1	1	1	1	1	1	1
02_HS-40 (1)	1,28	1	1	1	1	1	1	1	1	1	1	1	1	1	0,5
03_HS-40 (2)	1,31	1	1	1	1	1	1	1	1	1	1	1	1	1	0,5
04_9.4kb up HBZ	1,64	1	1	1	1	1	1	1	1	1	1	1	1	1	1
05_3.6kb up HBZ	1,46	1	1	1	1	1	1	1	1	1	1	0,5	1	1	1
06_HBZ/HBZP(1)	1,02	1	1	1	1	1	1	1	0,5	0,5	0,5	0,5	1	1	1
07_HBZ/HBZP(2)	1,18	1	1	1	1	1	1	1	0,5	0,5	0,5	0,5	1	1	1
08_HBA2/HBAP	1,84	1	1	1	1	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1
09_HBAP/HBA2(1)	1,73	1	1	1	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1
10_HBAP/HBA2(2)	1,01	1	1	1	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1
11_HBAP/HBA1(3)	1,14	1	1	1	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1
12_HBA2/HBA1(4)	1,10	1	1	1	0,5	1,4	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1
13_HBA2 IVS1(1)	1,60	1	0,5	0	0,5	1,2 of 1,4	0,5	0,5	0,5	0,5	0,5	0,5	0	0,5	1
14_HBA2 IVS1(2)	1,40	1	0,5	0	0,5	1,2 of 1,4	0,5	0,5	0,5	0,5	0,5	0,5	0	0,5	1
15_HBA2/HBA1 ex3	1,96	1	0,5	0	0,5	1,4	0,5	0,5	0,5	0,5	0,5	0,5	0	1	1
16_HBA2/HBA1(1)	1,90	1	0,5	0	1	1,4	0,5	0,5	0,5	0,5	0,5	0,5	0	1	1
17_HBA1/HBA1(2)	2,10	1	0,5	0	1	1,4	0,5	0,5	0,5	0,5	0,5	0,5	0	1	1
18_HBA1/HBA1(3)	2,56	1	0,5	0	1	1,4	0,5	0,5	0,5	0,5	0,5	0,5	0	1	1
19_HBA2/HBA1(4)	1,99	1	0,5	0	1	1,4	0,5	0,5	0,5	0,5	0,5	0,5	0	1	1
20_HBA1 ex1	1,42	1	0,75	0,5	0,75	1,2	0,5	0,5	0,5	0,5	0,5	0,5	0,25	1	1
21_HBA1 ex3	1,03	1	0,75	0,5	0,75	1,2	0,5	0,5	0,75	0,5	0,5	0,5	0,25	1	1
22_3.4kb d HBA1	1,18	1	1	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	1	1
23_3.7kb d HBA1	1,83	1	1	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	1	1
24_4.2kb d HBA1	1,10	1	1	1	1	1	0,5	0,5	1	0,5	0,5	1	0,5	1	1
25_4.7kb d HBA1	1,03	1	1	1	1	1	0,5	1	1	0,5	0,5	1	0,5	1	1
Constant Spacing	1,36														

Table 5.22 heterozygotes known alpha-thalassaemia deletions (LGTC HBA -kit)

Probe name	ratio wt	$(\alpha\alpha)^{L}$	Dutch II	de novo _AB	_42	$(\alpha\alpha)^{SW}$	de novo _MK	_QH	_DUTCH	_THAI	_FIL	$-(\alpha)^{20.5}$	_MedI	_SEA	$-\alpha^{7.9}$	$-\alpha^{4.2}$	-α 3.7
01_c16orf3	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
02_c16orf3	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
03_MPG	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
04_c16orf35(2)	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
05_c16orf3	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1
06_c16orf35(3)	1	0,5	0,5	0,5	0,5	1	0,5	1	1	1	1	1	1	1	1	1	1
07_L1	1	0,5	0,5	0,5	0,5	1	0,5	1	1	1	1	1	1	1	1	1	1
08_L0	1	0,5	0,5	0,5	0,5	1	0,5	1	0,5	1	1	1	1	1	1	1	1
09_HBZ	1	0,5	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1
10_HBZP	1	1	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1	1	1
11_HBAP2	1	1	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1	1
12_HBAP1	1	1	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1
13_HBAP1-A2	1	1	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1
14_HBA2	1	1	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5
15_HBA1	1	1	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	1	0,5	0,5	1	1	1
16_HBQ	1	1	0,5	0,5	0,5	1	0,5	0,5	1	0,5	0,5	1	1	0,5	1	1	1
17_3HVR(1)	1	1	0,5	0,5	0,5	1	0,5	0,5	1	1	1	1	1	1	1	1	1
18_3HVR	1	1	0,5	0,5	0,5	1	0,5	0,5	1	1	1	1	1	1	1	1	1
19_LUC7L(2)	1	1	0,5	0,5	0,5	1	0,5	0,5	1	1	1	1	1	1	1	1	1
20_LUC7L	1	1	0,5	0,5	0,5	1	0,5	0,5	1	1	1	1	1	1	1	1	1
21_c16orf9	1	1	0,5	0,5	0,5	1	0,5	0,5	1	1	1	1	1	1	1	1	1
22_PDIP	1	1	0,5	0,5	0,5	1	0,5	1	1	1	1	1	1	1	1	1	1
23_AXIN1	1	1	1	0,5	0,5	1	0,5	1	1	1	1	1	1	1	1	1	1
24_AXIN1(2)	1	1	1	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
25_c16orf43	1	1	1	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
26_DECR2	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
27_KIAA0665(1)	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
28_KIAA0665(2)	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
29_KIAA0665(3)	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
30_SOLH	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
31_RAB40C	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
32_WFIKKN1	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
33_RHBDL1	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
34_c16orf25	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1
35_MSLN	1	1	1	1	0,5	1	1	1	1	1	1	1	1	1	1	1	1

Table 5.23 heterozygotes known beta-thalassaemia deletions (MRC-Holland P102).

Probe name	bin size	ratio wt	Dutch IV (εγ)δβ°-thal	Dutch V εγδβ°-thal	Dutch II/VI (εγδβ)°-thal	Dutch III 111 kb	Belgian 50 kb	26-41 kb εγ-γ(δβ)°-Indian	HPFH 2	Filipino β°-thal	Chinese β°-thal 25-30 kb	13.4-22 kb Sicilian	Dutch I 12.6 kb	1.4 kb Black	Indian (-619 bp)
01_IP5	356	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
02_HS4(1)	301	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
03_HS4(2)	286	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
04_HS3(1)	328	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
05_HS3(2)	346	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
06_HS3(3)	210	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
07_HS2	292	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
08_HS1	248	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	1	1	1
09_HBE1 exon	463	1	0.5	1	0.5	0.5	1	1	1	1	1	1	1	1	1
10_HBG2 prom	374	1	0.5	1	0.5	0.5	1	1	1	1	1	1	1	1	1
11_HBG1 ex3	436	1	0.5	1	0.5	0.5	1	1	1	1	1	1	1	1	1
12_HBG1 region	405	1	0.5	1	0.5	0.5	1	1	1	1	1	1	1	1	1
13_HBBP1 ex1	445	1	1	1	0.5	1	0.5	0.5	1	1	1	1	1	1	1
14_HBBP1 ex3	381	1	1	1	0.5	1	0.5	0.5	0.5	1	1	1	1	1	1
15_HBD region	420	1	1	1	0.5	1	0.5	0.5	0.5	1	1	1	1	1	1
16_HBD ex1	290	1	1	1	0.5	1	0.5	0.5	0.5	1	1	1	1	1	1
17_HBD ex3	399	1	1	1	0.5	1	0.5	0.5	0.5	1	1	0.5	1	1	1
18_HBB region	356	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1
19_HBB prom	148	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
20_HBB ex1	360	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
21_HBB IVS1	154	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
22_HBB IVS2	360	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
23_HBB ex1(3)	364	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
24_HBB ex2(1)	210	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
25_0.5 kb 5' HBB	173	1	1	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1

Table 5.24 heterozygotes known beta-thalassaemia deletions (LGTC HBB -kit)

Probename	bin size	ratio wt	Dutch IV (εγ)δβ°-thal	Dutch V εγδβ°-thal	Dutch II (εγδβ)°-thal	Dutch VI (εγδβ)°-thal	Dutch III 111 kb	Croatian (εγδβ)°-thal	Belgian 05 kb	26-41 kb εγ-γ(δβ)°-Indian	HPFH-2	Filipino β°-thal	Chinese β°-thal 25-30 kb	13.4-22 kb Sicilian	Dutch I 12.6 kb	1.4 kb Black	Indian (-619 bp)
01_OR52D1	124	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
02_OR51M1	57	1	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
03_Probe 79	78	1	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1	1	1
04_Probe 45	44	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
05_OR51B6	47	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
06_OR51B5	71	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
07_OR51B5-B2	80	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
08_OR51B2	77	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
09_OR51B3P	57	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
10_OR51B4	55	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
11_OR51B4-HS4	76	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1	1
12_HS-4	43	1	0,5	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1
13_HS-1	46	1	0,5	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1
14_Hb E	51	1	0,5	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1
15_Hb GG	59	1	0,5	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1
16_HbGG_HbGA	62	1	0,5	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1	1
17_Hb GA	61	1	0,5	1	0,5	0,5	0,5	0,5	0,5	1	1	1	1	1	1	1	1
18_Hb BP	99	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1
19_HbG-HbD	48	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	1	1	1	1	1	1
20_Hb D	75	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	1	1	0,5	1	1	1
21_Hb B (1)	66	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1	1
22_Hb B (2)	68	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	1
23_Hb B (3)	54	1	1	1	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1
24_Hb B (4)	56	1	1	1	0,5	1	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1
25_Hb B (5)	70	1	1	1	0,5	1	1	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	1	0,5
26_Hb B (6)	59	1	1	1	0,5	1	1	0,5	0,5	1	0,5	0,5	0,5	1	1	1	1
27_OR51V1	41	1	1	1	0,5	1	1	0,5	0,5	1	0,5	0,5	1	1	1	1	1
28_OR52Z1P-V	72	1	1	1	0,5	1	1	0,5	1	1	0,5	0,5	1	1	1	1	1
29_OR52Z1P	63	1	1	1	0,5	1	1	0,5	1	1	0,5	0,5	1	1	1	1	1
30_OR51A1P	69	1	1	1	0,5	1	1	0,5	1	1	0,5	0,5	1	1	1	1	1
31_OR52A1	65	1	1	1	0,5	1	1	1	1	1	1	0,5	1	1	1	1	1
32_OR52A5-A1	74	1	1	1	0,5	1	1	1	1	1	1	0,5	1	1	1	1	1
33_OR52A5-A4	64	1	1	1	0,5	1	1	1	1	1	1	0,5	1	1	1	1	1
34_OR52A4	67	1	1	1	0,5	1	1	1	1	1	1	0,5	1	1	1	1	1

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CHAPTER 06

FETAL DNA ANALYSIS

The methods of DNA analysis used for prenatal diagnosis are the same ones used for mutation screening described in chapter 5. The main difference with prenatal diagnosis is that the normal DNA sequence at the mutation sites needs to be analyzed in those cases where both partners carry the same mutation. When the parents carry different mutations then simple mutation screening for each is required. However whatever mutations the parents may carry, analysis of DNA polymorphisms/short tandem repeats is required in every case, or at least when the fetal diagnosis result is identical to the maternal result, to identify any maternal DNA contamination present. When a prenatal diagnosis, chorionic villus sample is received in the laboratory, it is essential that any maternal tissue present in the fetal sample is carefully removed. The most common cause of error in a haemoglobinopathy fetal diagnosis, is likely due to maternal contamination.

6.1 CLEANING CHORIONIC VILLI BY MICROSCOPIC DISSECTION

Protocol:

1. The tissue sample usually arrives in the lab in culture medium. Transfer the tissue sample into a Petri dish and add clean culture medium.
2. Wash the tissue by gentle agitating the tissue to remove any maternal blood present.
3. Discard the blood stained culture medium with a Pasteur pipette.
4. If the sample is heavily blood stained repeat this procedure 3-4 times until no blood is visible in the Petri dish.
5. Place the Petri dish under a dissecting microscope and visualize for the presence of maternal decidua. Turn the tissue over to ensure you have visualized both sides of the tissue. Any maternal tissue should be carefully removed using either two small clean forceps or two needles, hold down the tissue with one needle/forceps and gently pull away any maternal tissue with the other.
6. While viewing the chorionic villi under the microscope carefully select the fetal tissue and transfer to another Petri dish in clean culture medium.
7. Transfer the clean chorionic villus tissue to a clean tube ready for digestion.

A method of DNA preparation from cleaned chorionic villi samples of amniotic fluid cell pellets is described in chapter 5.

6.2 CHECKING FOR MATERNAL DNA CONTAMINATION

Routine examination of short tandem repeats (STR's) or Variable Number of Tandem Repeats (VNTR's) in fetal and parental DNA samples by PCR analysis is essential to rule out maternal contamination.

The tandem repeated "minisatellite" regions of DNA are an ideal method to identify maternal contamination. These highly polymorphic DNA regions show allelic variation in the number of repeated units. Because of the large number of different alleles these repetitive DNA areas provide informative genetic markers. Since these variations are inherited according to Mendelian genetics, they can be used to test for maternal contamination and indeed will also identify non-paternity. Trisomies may also be detected using the quantative fluorecesent (QF-PCR) technique described below, particularly tri-allelic patterns (3).

The choice of polymorphic markers available is wide, including the short tandem repeat (STR) markers; D21S11, D21S1414, D18S535 (1). However commercial kits such as the AmpF lSTR Identifiler Kit (ABI) are now available which multiplex at least 16 polymorphic markers. If laboratories do no have access to an automated fluorescent gene scanner then the Variable Number Tandem Repeat (VNTR) markers method such as for ApoB, IgJH and Has-ras may be used (2), although this method is not as sensitive as the automated STR methods.

6.2.1 SHORT TANDEM REPEAT (STR) ANALYSIS

Protocol: With the use of unique fluorescent oligonucleotide primers flanking the repeat sequence, the polymorphic regions on the chromosomes can be amplified using gap-PCR. The PCR products are separated and analysed using an automated laser DNA analyser such as the ABI 3100), and the appropriate Genescan software.

Reagents:

a. PCR mix (8 ml):
1 ml 10 x cetus buffer
5.4 ml sterile distilled H_20
1.6 ml 1.25 mM NTPs
8 µl spermidine 1 M
Final concentration of PCR reaction: 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each dNTP
Store frozen in 1 ml aliquots

b. 10 x Cetus buffer:
1.25 ml 2 M KCl (stock solution)
0.5 ml 1 M Tris pH 8.3 (stock solution)
75 µl 1 M $MgCl_2$ (stock solution)
5 mg gelatin (300 bloom)

3.2 ml sterile distilled water
Place at 37°C to dissolve gelatin
Store frozen

c. Stock solutions:

2 M KCl	14.91 g/100 ml
1 M Tris	12.11 g/100 ml (adjust pH to 8.0 with concentrated HCl)
1 M $MgCl_2$ $6H_20$	20.33 g/100 ml

Store at 4°C

d. dNTPs 1.25 mM - 100 mM concentration)
60 µl of each dNTPs (dATP, dCTP, dGTP, dTTP)
4760 µl sterile distilled water
Store frozen

e. 1 M Spermidine
1.452 g spermidine/10 ml distilled wateer
Store frozen

f. 0.5 M Di-sodium EDTA solution pH 8.0
May be purchased directly from Applied Biosystems, or made up as follows. Dissolve 93.1 g di-sodium EDTA in 200 ml distilled water. Add pellets of sodium hydroxide, adjust to pH 8.0. Make up to 500 ml

g. TE buffer:
10 mM Tris-HCl pH 8.0 (10 mM Tris, 0.1 mM EDTA)
10 ml 1 M Tris, pH 8.0; 200 µl 0.5 M, pH 8.0. Make up to 1 litre

Primers: Primers are diluted to an O.D. of 1 (ie. approximately 10 pg/µl) and stored frozen. Table 6.1 shows the primers used in multiplex QF-PCR. Seven STR markers are described here. In each case the forward primer from each set is labelled with a fluorescent dye (3,4). The primers are optimised to ensure comparable amounts of PCR products. In addition, markers on the gender determing Amelogenin gene (AMDXY) can be used. The use of these markers enables sexing of the fetus. The Y chromosome produces a fragment of 252 bp and the X chromosome a fragment of 432 bp (5,6).

a. **Fluorescent PCR (QF-PCR) Method:** The protocol for seven tetranucleotide STR markers and the Amelogenin (AMXY) gender determining gene, shown in Table 6.1, is described here.

1. Make up 25 µl 1 PCR mix as follows:
2 µl Forward labeled primer (10 pmol/µl)
2 µl Reverse primer (10 pmol/µl)
0.1 µl - 0.5 units taq polymerase
1 µl DNA diluted 1 in 10 (if originally approximately 0.5 mg/µl)
1 drop of mineral oil if required depending on the thermal cycler being used
Make up to 25 µl with de-ionised distilled water

Table 6.1: Primers used in the multiplex QF-PCR.

MARKER	SEQUENCE OF PRIMERS 5'-3'	CHROMOSOME LOCATION	FLUORESCENT LABEL	SIZE OF PRODUCT (bp)
D21S11(F) D21S11 (R)	TAT GTG AGT CAA TTC CCC AAG TGA GTT GTA TTA GTC AAT GTT CTC C	21q21	6-FAM none	172-264
D21S1411(F) D21S1411®	TAT GTG AGT CAA TTC CCC AAG TGA GTT GTA TTA GTC AAT GTT CTC C	21q22.3	HEX None	-250
D21S1414(F) D21S1414(R)	GGC ACC CAG TAA AAA ATT ACT CTG TCT GTC TGT CTG TCT ATC	21q21	6-FAM none	330-380
D18S535 (F) D18S535 (R)	TCA TGT GAC AAA AGC CAC AC AGA CAG AAA TAT AGA TGA GAA TGC A	18q12.2-12.3	NED None	-150
D13S631(F) D13S631 (R)	GGC AAC AAG AGC AAA ACT CT TAG CCC TCA CCA TGA TTG G	13q31-32	HEX None	-220
AMXY (F) AMXY (R)	CTG ATG GTT GGC CTC AAG CCT ATG AGG AAA CCA GGG TTC CA	X and Y	HEX None	Y252 X432
D21S1442(F) D21S1442(R)	CTC CTC CCC ACT GCA GAC TCT CCA GAA TCA CAT GAG CC	21q21.1-q21.2	HEX None	237-296
D21S1435(F) D21S1435(R)	CCC TCT CAA TTG TTT GTC TAC C GCA AGA GAT TTC AGT GCC AT	21q21.1-q21.2	NED None	131-155

2. Place in thermal cycler programmed as follows:
   93°C for 48 seconds
   60°C for 48 seconds
   72°C for 1 minute
   For 25 cycles
   72°C for 3 minutes
   Refrigerate

Analysis on ABI 3100

1. Pool the amplified products by adding 5µl of amplified product from each of the above amplification products.
2. Add 10 µl HiDi formamide and 0.5 µl size GeneScan Rox (red) 500 size standard to the wells of a 96 well MicroAmp Optical Density plate.
3. Add 1.3 µl pooled amplified product to the appropriate well.
4. Place at 94°C for 4 minutes to denature the products.
5. The PCR products are run through capillaries where the specific PCR products are sized and the amount of each PCR product evaluated by the extent of fluorescent activity, equal to the area of the fluorescent peak generated.

b. Protocol for multiplex QF-PCR method:

A multiplex assay can also be used instead of the above method; the protocol described below is for the multiplex of D21S11, D13S631, D18S535 and AMDXY short tandem repeat markers in a single PCR amplification.

1. **Make up 25 µl 1PCR mix as follows:**
   2.5 µl 10 x PCR (supplied with Amplitaq).
   1.5 µl 25 mM $MgCl_2$ (supplied with Amplitaq).
   4 µl 1.25 mM dNTPs.
   2 µl of each primer: D21S11 F and R, D13S631 F and R, D18S535 F and R, AMDXY F and R.
   0.2 µl -1 unit taq polymerase .
   1 µl 1/5 dilution DNA (if originally approximately 0.5 mg/µl).
   1 drop mineral oil if required.
2. Amplify as above but with an extension time of 30 minutes at 60°C after 25 cycles.
3. Analyse on ABI 3100 as above, analyze the red size standard and the other relevant colors relating to the STR markers used.

c. Protocol for Setting Up PCR using AmpF lSTR Identifiler Kit (ABI)

The method described below is for the commercially available kit from ABI. Clear instructions are given in the kit instructions. The AmpFlSTR Identifier PCR amplification Kit is a short tandem repeat multiplex assay that amplifies 15 tetranucleotide repeat loci and the Amelogenin gender determining marker in a single PCR amplification. The kit uses a five-dye fluorescent system for automated DNA fragment analysis; 6-FAM, VIC, NED, PET, LIZ.

Table 6.2: List of the16 alleles detected by primers included in the primer mix:

MARKER	CHROMOSOME LOCATION	*FLUORESCENT LABEL	PRODUCT SIZE (bp)
D8S1179	8	6-FAM	122-170
D21S11	21q11.2-q21	6-FAM	184-243
D7S820	7q11.21-22	6-FAM	258-293
CSF1PO	5q33.3-34	6-FAM	304-342
D3S1358	3p	VIC	111-141
THO1	11p15.5	VIC	162-202
D13S317	13q22-31	VIC	218-248
D16S539	16q24-qter	VIC	254-294
D2S1338	2q35-37.1	VIC	307-360
D19S433	13q12-13.1	NED	101-136
vWA	12p12-pter	NED	154-208
TPOX	2p23-2per	NED	222-254
D18S51	18q21.3	NED	264-345
Amelogenin	X:p22.1-22.3 Y:p11.2	PET	X-106 Y-112
D5S818	5q21-31	PET	133-172
FGA	4q28	PET	214-355

Notes: Fluorescent colour. FAM: Blue, HEX: Green, NED: Yellow, PET: Red)

1. Prepare a master mix containing:
   10.5 µl AmpFlSTR PCR Reaction Mix
   0.5 µl Amplitaq Gold Polymerase
   5.5 µl AmpFlSTR Identifiler Primer Set
   Vortex and add 15 µl to each labeled tube.
2. Add 10 µl of the diluted DNA (Diluted to 0.05-0.125ng/µl with TE buffer).
3. Add 1 drop of mineral oil if required depending on your thermocycler.
4. Also set up positive control sample supplied with the kit and the appropriate reagent blank controls.
5. Place tubes in a thermocycler and amplify using the following programme:
   95°C for 11 minutes
   94°C for 1 minute
   59°C for 1 minute
   72°C for 1 minute
   For 28 cycles
   60°C for 60 minutes
   Refrigerate at 4°C

Analysis on ABI3100 (AmpFlSTR kit):

1. The allelic ladder supplied with the kit must be run on the ABI 3100.with the samples being analysed.
2. Use 10 µl of HiDi Formamide and 0.5 µl of GeneScan LIZ 500 size standard and add 10.5 µl to the appropriate number of wells of a 96 well MicroAmp Optical Density plate.
3. Add 1.3 µl of amplified product to the appropriate well.
4. Add 1.3 µl of allelic ladder supplied with the kit to the appropriate well.
   Place at 94°C for 4 minutes to denature the products.
   Run on the ABI 3100 to size the products.

Interpretation of results:

Prior to analysing the results check that the positive control sample supplied with the kit gives the expected size for the respective STR as shown in the kit instructions. Also check the blank control samples are negative. The fetus will inherit an allele from the father and the mother and therefore have 2 alleles. If the alleles of each parent are different in size (i.e. each parent has different peak sizes) then that particular STR can be used to assess maternal contamination. If the parents share one common allele then this may be informative for maternal contamination. Maternal contamination is excluded when the fetal banding pattern differs from the maternal sample by one allele. If the fetal and maternal allele sizes are identical then maternal contamination cannot be excluded. If maternal contamination is present then there is a third peak in the fetal sample corresponding to the maternal second allele.

Note: These analyses can also identify chromosomal trisomies.

The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter than the corresponding main allele peak. This is referred to as the stutter peak. Se-

quence analysis of stutter products at tetranucleotide STR loci has shown that the stutter product is missing a single tetranucleotide core repeat unit relative to the main allele (7). This is usually due to slippage of the enzyme during amplification. When stutters occur in the fetal sample which corresponds to the maternal second allele, maternal contamination cannot be excluded. If there is no informative marker, but there is however a clear paternal allele present in the fetal sample, which is of expected peak area, then maternal contamination is unlikely to be present. Peak heights should be >100. If lower, the amplification can be repeated using a lower dilution of the DNA.

Figure 1 shows an example of D21S11 STR analysis of (a) paternal alleles 215 bp and 223 bp; (b) fetal alleles 223 bp and 227 bp; (c) maternal alleles 227 bp and 237 bp. Therefore the fetus has inherited the 223 bp allele from the father and the 227 bp allele from the mother. We therefore can conclude that there is no maternal contamination in the fetal sample as the 237 bp maternal allele is not present. Figure 2 shows contamination during fetal sampling in a twin pregnancy.

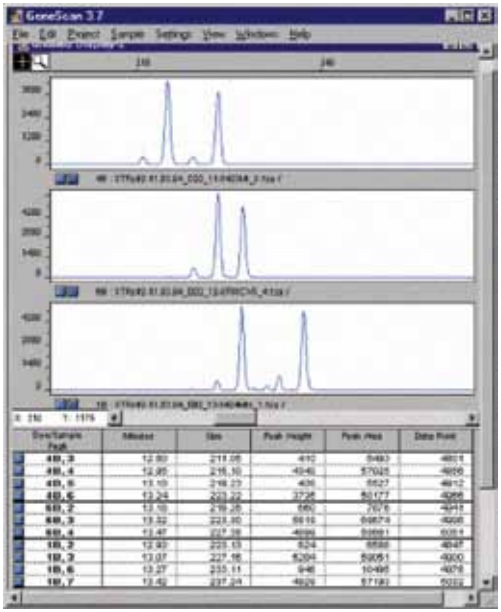

FIG. 6.1
An example of a check for maternal DNA contamination using the STR D21S11. The gel shows STR analysis of (a) paternal alleles 215 bp and 223 bp; (b) fetal alleles 223 bp and 227 bp; (c) maternal alleles 227 bp and 237 bp. The results show the fetus has inherited a 223 bp allele from the father and a 227 bp allele from the mother. We can therefore conclude that there is no maternal contamination in the fetal sample as the 237bp maternal allele is not present.

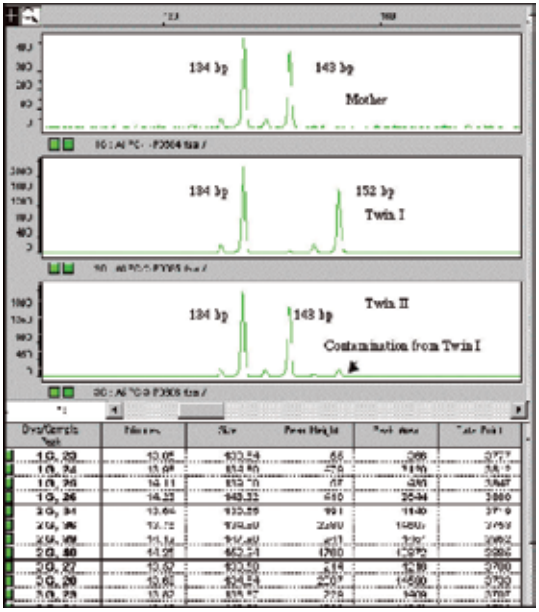

FIG. 6.2
Results showing contamination during fetal sampling in a twin pregnancy.

6.2.2 VARIABLE NUMBER TANDEM REPEAT (VNTR) ANALYSIS

PCR can also be used to check for maternal contamination in the fetal DNA sample by variable tandem repeat (VNTR) polymorphism analysis, using the procedure of gap-PCR. The reagents and method of this non fluorecent PCR is detailed in chapter 5. The method uses simple technology in the form of agarose gels and ethidium bromide staining, the disadvantages are that the low resolution of agarose gel electrophoresis means that only VNTRS with a repeat size of 30 nucleotides or above are useful. This non fluorescent method is not as sensitive as the fluorescent STR method in identifying small amounts of contamination.

The primer sequences required for the analysis of the Apo B, IgJh, Co121A1 and D4S95 VNTR polymorphisms are detailed in Table 6.3. An informative test is when both parents have two different size alleles and the fetus can clearly be seen to have inherited one of the maternal and one of the paternal alleles. If a faint positive signal for the second maternal allele is seen in the fetal DNA sample (which shows three bands in total) then this indicates maternal DNA contamination.

Table 6.3: Primers used to check for maternal contamination by VNTR analysis.

VNTR	PRIMER PAIR	ANNEALING TEMP (°C)	REPEAT LENGTH (bp)	PRODUCT SIZE (bp)
ApoB	GAAACGGAGAAATTATGGAGGG	55	30	541-871
	TCCTGAGATCAATAACCTCG			
IgJh	GGGCCCTGTCTCAGCTGGGGA	68	50	520-1720
	TGGCCTGGCTGCCCTGAGCAG			
Col2A1	CCAGGTTAAGGTTGACAGCT	55	34 & 31	584-779
	GTCATGAACTAGCTCTGGTG			
D4S95	GCATAAAATGGGGATAACAGTAC	60	39	900-1600
	GACATTGCTTTATAGCTGTGCCTCAGTTT			

All except the IgJh primers use the standard PCR buffer. The IgJh primers require a $(NH_4)_2SO_4$ buffer as detailed in the methods in chapter 5.

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CHAPTER 07

EMERGING TECHNOLOGIES FOR HAEMOGLOBINOPATHY SCREENING AND DIAGNOSIS

This chapter will outline some of the newest methods for detecting DNA mutations, although with the speed that technologies are being developed and improving it will probably soon be out-dated! At the present time there is limited experience with their application for clinical diagnostics, and so we are not able to recommend the most reliable and cost-effective methods for diagnosing the disorders of haemoglobin at the DNA level, nor to describe specific procedures for most of the protocols. All the methods are based on the analysis of PCR-generated amplicons from the gene region(s) of interest and involve the use of equipment with high purchase cost, although subsequent sample analysis may be cost-effective in most cases.

7.1 MICROARRAY (GENE-CHIP) ANALYSIS

Genechip or array technology can be applied for gene discovery, gene expression, gene mapping, and mutation detection. Microarrays typically consist of an ordered collection of microspots on a surface matrix, where each microspot contains a single defined sequence of nucleic acid (probes), which may either be large DNA fragments (cDNA's or PCR amplicons) or allele-specific oligonucleotides, the latter being either pre-synthesised, or in some cases, synthesised in-situ (on-chip). The microarray technique is based on hybridisation of nucleic acids, whereby sequence complementarities leads to the hybridisation between two single-stranded nucleic acid sequences, one of which (usually the allele-specific oligonucleotide) is immobilized on the surface matrix. In this section, the state-of-the-art with respect to microarrays technologies for the characterization of single nucleotide DNA variations (mutations) and deletions associated with the haemoglobinopathies will be presented [1].

7.1.1 MICROARRAY ANALYSIS FOR POINT MUTATIONS

Microarray analysis is potentially appropriate for characterizing point mutations within the globin genes associated with the disorders of haemoglobin synthesis, representing an approach for rapid genotyping, potentially interrogating a large number of samples for a large numbers of mutations simultaneously. The probes used for genotyping applications are usually allele-specific oligonucle-

otides. In some protocols the sample-probe hybridization step is followed by enzymatic mismatch discrimination to enhance allelic specificity. The detection of sample-probe hybridisation on the array requires that samples or probes are labelled, and this is usually achieved with a fluorescent moiety. However, the development of reliable microarrays or gene-chips for detecting disease-associated mutations within a diagnostic setting has been hindered by difficulties in optimizing homogeneous stringency conditions for a potentially wide and varied range of DNA sequences necessary for the simultaneous analysis of multiple mutations. This criteria is exacerbated by the requirement to discriminate alleles within samples which may differ by only a single nucleotide (eg homozygous wild-type or homozygous mutant versus heterozygote at a particular nucleotide position).

During the last decade there was considerable effort directed towards the standardization and validation of genotyping microarrays. Amongst the more successful examples is an arrayed primer extension-based system which involves the extension of oligonucleotides probes that are designed to be a single nucleotide short relative to the base of the allele variant, called Arrayed Primer EXtension (APEX) technology (APEX, Asper Biotech, Tartu, Estonia; http://www.asperbio.com) [2]. In this method the probes, designed to specifically interrogate known mutations and single-nucleotide polymorphisms in the gene of interest, are immobilized via their 5'end on a glass surface, while its 3' end is free for enzymatic extension. Following an initial step based on the polymerase chain reaction (PCR), an amplicon from a DNA containing the region which harbours the mutation(s) or Single Nucleotide Polymorphisms (SNPs) under investigation is hybridized to the complementary probe sequence that is immobilized on the microarray chip. A single on-the-chip base extension of the probe is performed by incorporation of the appropriate dye-labeled dideoxynucleotide which is complementary to the variant base, followed by termination of the reaction. An automatic laser imaging system is used to read the APEX slide and detect which of the four fluorescent dideoxynucleotide labels (Fluorescein, Cy3, Cy5 and Texas Red) have been incorporated at each oligo-probe site on the microarray.

The APEX method has been adapted for large scale ß-globin mutation and polymorphism detection (amongst other disease-gene applications), [3, 4], The updated validated version, known as ThalassoChip is a ß-thalassaemia genetic diagnostic tool based on Array Primer Extension (APEX) technology which has the ability to detect over 60 ß-globin gene mutations and polymorphisms in a single step [5]. The optimized APEX reaction conditions are entirely reproducible, as long as prerequisites, such as good quality human genomic DNA at the appropriate concentration, a successful DNA fragmentation step and optimized PCR amplification conditions are fulfilled. A commercially available service for the diagnosis of ß-thalassaemia mutations is available from Asper Biotech.

Although microarrays for point mutations provide diagnostic tool with potentially relatively low running costs along with the possibility to determine a wide spectrum of mutations and polymorphisms in a single experiment, with a very few exceptions, many genotyping microarray technologies have not evolved beyond the prototype stage, or have proved to be economically unsustainable (eg the Nanogen Nanochip®microelectronic microarray system) [6].

A major disadvantage of microarrays for point mutation detection compared to DNA sequencing

is that the microarray chip is limited for detecting only known nucleotide changes for which the probes have been designed. Previously undetected polymorphisms and new mutations will be missed, and furthermore may even lead to false results through mismatched bases under the probes. Finally microarray platforms are technically quite demanding with respect to both sample processing and data interpretation, and thus require highly trained operators. High-throughput sample preparation is also an advantage and microarray systems are generally not supported by integrated automated DNA extraction and PCR preparation systems. Finally, the costs of most platforms developed to date are higher than in-house technologies.

7.1.2 MICROARRAY ANALYSIS FOR DELETIONS/DUPLICATIONS OF CHROMOSOMAL REGIONS

The array comparative genomic hybridization (aCGH) technology is a method for copy number variation across chromosomal regions and has become a valuable routine diagnostic tool in genetics. The high resolution, simplicity, high reproducibility and precise mapping of imbalances are the most significant advantages of aCGH over traditional cytogenetic methods.

Many studies have used fine-tiling oligonucleotide arrays for breakpoint analysis of deletions underlying several genetic diseases such as neurofibromatosis (7), Wilms tumor (8) or breast cancer (9). These studies not only confirm the power of the fine-tiling arrays to find breakpoint regions, but also underline the increasing importance of fine tiling array technology as a follow up after MLPA for the delineation of deletions and breakpoints in common and rare rearrangements. Similarly a custom fine-tiling array has been developed recently for high-resolution determination of deletion breakpoints in the α- and ß-globin gene clusters (10). This array has been used to fine-map the positions of breakpoint junctions supporting the design of gap-PCR primers for sequencing analysis in order to determine the exact deletion breakpoints. The design of gap-PCR assays for deletions characterized by aCGH, has an important diagnostic application, by providing a simple screening method in laboratories where MLPA is not available, especially appropriate if the specific deletions reach high frequencies in a local population.

Array design, experimental conditions and data analysis: Two custom fine tiling arrays covering the α- and ß-globin gene clusters plus surrounding areas have been developed (Roche NimbleGen, Madison, WI, USA) (10). Design of the array was based on NCBI Build 36.1 (hg18). (NimbleGen Arrays User's Guide: CGH Analysis v4.0). These customized fine-tiling arrays included 135,000 probes (12 x 135K format), with 12 identical sub-arrays per slide, allowing for simultaneous analysis of up to 12 different samples. The coverage of the probes on the Nimblegen fine-tiling array is shown in Figure 7.1. The probes on the α-globin gene cluster array cover the 2 Mb telomeric region of chromosome 16p, including the α-globin gene cluster (position 1-2,000,000, according to UCSC Genome Browser, March 2006, hg18). The array for the ß-globin gene cluster on chromosome 11p and surrounding region covers 0.6 Mb (position 4,900,000-5,500,000). The average spacing on each array is 20 bp, and the oligonucleotide probes have a length of 60-80 bp, involving an overlap between probes and approximately 3x coverage of the region of interest.

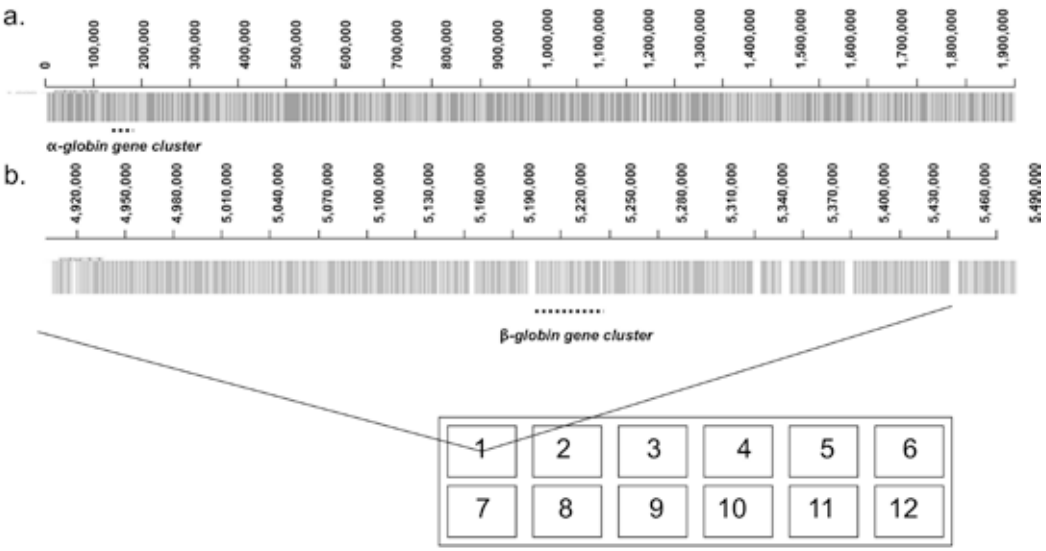

FIG. 7.1
Nimblegen custom fine-tiling array: schematic overview of the coverage of the probes. The vertical grey lines indicate where probes are located, uncovered areas are left in white. The array covers the telomeric 2 Mb of chromosome 16p including the α-globin gene cluster (a) and a 600 kb region including the ß-globin gene cluster on chromosome 11p (b). The stippled bars below the figures represent the location of the globin gene clusters. Positions are according to the UCSC Genome Browser (March 2006, hg18).

The design had to accommodate certain features of the sequences in the α- and ß-globin gene clusters. For example both clusters contain non-unique sequences, such as the duplicated α- and γ-globin genes and the Alu- and LINE-repeat regions. The largest repeat unit in our region of interest is a ~7 kb LINE-repeat in the ß-globin gene cluster. In order to increase specificity and to prevent false positive results, all the probes on the array were selected to be unique, which resulted in non-unique sequences of the globin gene clusters not being covered by the probes. Thus, in cases where the breakpoint is located within a repetitive sequence, the determination of the breakpoint position by the array may be inaccurate by up to a maximum of 7 kb from the true breakpoint.

The experimental conditions were based on the NimbleGen Arrays User's Guide: CGH Analysis v4.0 and the data analysis was performed using the NimbleScan v2.5 and SignalMap v1.9 software (NimbleGen). Details are described in a recent publication by Phylipsen et al, 2011 (10). Application of the customized fine-tiling aCGH technology has demonstrated the capability to detect small and large rearrangements (from ~4 kb up to 2 Mb) in the α- and ß-globin gene clusters with high resolution, as illustrated in Figure 7.2. Based on information provided by the array analysis, it has been possible to design primers to amplify relatively short products including the breakpoint sequence which can then be characterized easily by direct sequencing.

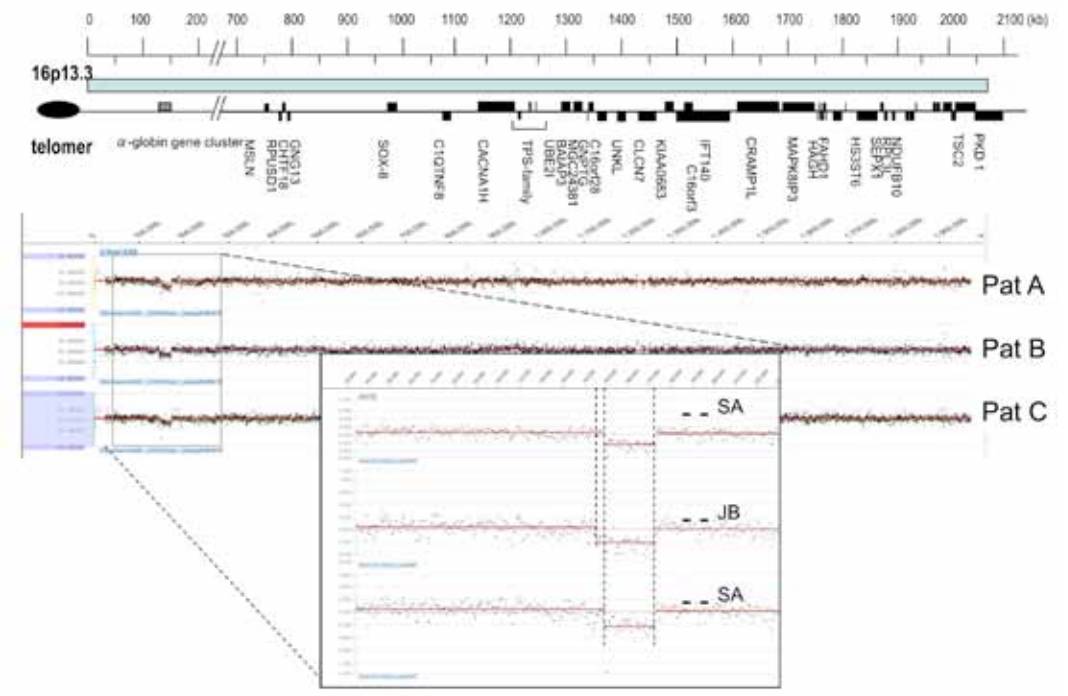

FIG. 7.2
Detection of two α^0-thalassaemia deletion mutations with the Nimblegen custom fine-tiling array: the aCGH results for 3 patients are shown below the schematic presentation of the 2Mb region of 16p containing the α- globin gene clusters covered by overlapping 'tiled' oligonucleotide probes. Patient A and B are carriers of the South African deletion (--^{SA}) and patient C is a carrier of the --^{JB} deletion.

Concluding remarks: Currently, the high cost of aCGH technology limits its wider use in diagnostic applications, estimated at about 9x the cost of MLPA per sample (comparing reagents only, and excluding purchase of the required instruments. However, fine-tiling aCGH technology is a valuable tool to support high resolution breakpoint characterization in α- and ß-globin gene cluster rearrangements. In addition, the information provided can be used to design simple PCR-based tests to detect the variant alleles, useful to laboratories where specific deletions may reach a high frequency in the local population since gap-PCR protocols are easier and cheaper than MLPA and Southern blotting. Since it is not feasible that all laboratories can set-up and apply aCGH, an alternative strategy for characterizing any rare new deletions might be to screen samples using MLPA and then refer any undefined samples to a centralized collaborating laboratory which has fine-tiling aCGH assays available, facilitating the design of gap-PCR assays if required.

7.2 HIGH RESOLUTION MELTING CURVE ANALYSIS (HRMA)

High-resolution melting of DNA is a relatively new method of molecular analysis introduced in 2002 by a collaboration between academia and industry (University of Utah and Idaho Technology, UT, USA) [11]. It is analogous to the principle of DGGE, whereby the melting profile of a PCR product depends on its GC-content, its length and the sequence composition. If two, otherwise identical, double-stranded DNA fragments vary by a single nucleotide, then it is highly likely that the melting profiles of each fragment will be distinct, additionally exacerbated by the creation of heteroduplexes in heterozygous samples. The melting profile is monitored through the use of saturating dyes which produce fluorescence when intercalated within double-stranded PCR products. Under conditions of increasing temperature, the double-stranded DNA will denature and become single-stranded, releasing the fluorescent dye, causing a drop in the intensity of fluorescence. A new generation of saturation dyes, specifically developed for high-resolution melting such as LCGreen® [12], increases the specificity of this technology by ensuring higher levels of dye-saturation along the length of the double-stranded DNA molecules, facilitating both higher levels of fluorescent intensity as well as a higher degree of resolution as each melting-domain along the double-stranded molecule denatures or "dissociates". HRMA is a closed-tube scanning method which does not require any post-PCR processing (other than the melting curve analysis). However, it does require the use of specialized equipment for the sensitive detection of melting profiles, including the LightScanner® (Idaho Technology), or modified real-time thermal cyclers, such as the LC480 (Roche) and the Rotor-Gene 6000 (Corbett) [13-15].

For even greater sensitivity when scanning regions are known to have nucleotide variations, allele-specific probes can be incorporated into the PCR reaction. The probes, designed to cover the site of base variation(s) or mutation(s), are unlabelled and are additionally modified at the 3' end with phosphate to preclude their extension during the PCR step [16]. As the probes are designed to hybridize to one of the two PCR strands, an asymmetric PCR protocol is applied, involving the use in excess (usually a ratio of 5:1) of the primer which favours the generation of the strand complimentary to the probe. During the PCR reaction, both probe and amplicon duplexes will be formed and saturated with the dye. The composite melting curve of the full-length PCR product and the probe-amplicon supports clear distinction between heterozygous, homozygous or wild type sequences samples.

High resolution melting analysis is reported to have a sensitivity and specificity of 100% for PCR products smaller than 400 bp in length, and thus should be well suited to the relatively small globin genes. However, the high number of mutations within the HBB gene located within a relatively small region, along with high homology between the HBB and its neighbouring HBD gene, complicates the design of convenient primers that give amplicons with robust melting curves (J. Traeger-Synodinos, unpublished observations). Previous studies have successfully analyzed heterozygotes and homozygotes for several common ß-gene variants [17] and even the analysis of α-thalassaemia deletions in combination with gap-PCR has been reported [15, 18]. As a relatively new technology, HRMA has not yet been widely incorporated within laboratories performing molecular diagnostics

for haemoglobinopathies. However, this may change, since HRMA is potentially a high-throughput, sensitive, rapid method with low running costs, which allows a high degree of automation and has already been applied to detect mutations in a wide spectrum of genes,. Limitations to the wider use of HRMA in routine clinical diagnostics include the relatively high cost of the instrument, and also that HRMA protocols are technically demanding, requiring detailed optimization and stringent application of protocols.

7.3 NEXT GENERATION SEQUENCING (NGS)

First generation DNA sequencing was first described in 1977, and in a somewhat modified and improved form, still constitutes one of the most fundamental methods for human genome analysis. However, it is limited to producing a maximum of about 1000bp per sequencing reaction, which in addition has to be targeted to a specific region of the genome each time. In 2003 the human genome sequence was published, following 13 years of work based on Sanger sequencing, involving around 3000 scientists from 6 countries and costing nearly US$3 billion. Subsequently, within a few years, nucleic acid sequencing technologies advanced so rapidly such that by 2009 a human genome could be sequenced within weeks at a cost of up to US$50,000 [19].

Next Generation Sequencing (NGS), also known as Massively Parallel Sequencing, is about a million times more efficient than Sanger sequencing and allow simultaneous sequencing of huge amounts of DNA in a single analytical procedure. For example, an entire genome can now be sequenced within a few days for approximately US$4000 at 30–40-times coverage. Unlike Sanger sequencing, NGS methods do not require previous knowledge of the sequence of a target region(s), thus precluding the need of target-specific DNA primers. The basic steps of most of the NGS methods involve 1) the creation of a DNA fragment library, involving randomized fragmentation of the DNA sample and ligation of adaptor DNA sequences to each and every fragment, followed by 2) amplification of all fragments simultaneously and finally 3) a 'sequencing by synthesis' step, based either on repeated cycles of polymerase-based nucleotide extension or by oligonucleotide ligation. The amplification and sequencing steps are achieved either in the format of "microreactors" and/or by attaching DNA molecules to be sequenced to solid surfaces or beads, allowing millions of sequencing reactions to be performed in parallel at the same time. NGS can be applied to analyse the entire genome of an individual. Alternatively it can be more targeted by pre-selecting for only the protein-coding regions of the genome (otherwise known as exome sequencing) or even only for a more limited selection of candidate genes likely underlying a clinical phenotype. The latter two approaches involve the capture of target sequences in the randomly fragmented DNA sample by molecular probes or 'baits' in microarray formats, or bead capture in solution or microfluidics and PCR primers [20].

Several NGS systems are commercially available, each with relative advantages and disadvantages, although it is beyond the scope of this mini-overview to discuss details here. In addition the

field is continuously advancing, so information is likely rapidly outdated. Current systems include the Genome Analyzer and Hi-Seq™ (Illumina), 454-FLX (Roche), SOLiD™ (Applied Biosystems), the Polonator (Dover/Harvard) and Heliscope™ (Helicos), Ion Torrent (Ion Torrent Inc) and PacBio RS (Pacific Biosciences) [Reviewed in references 21 and 22].

a. There are many applications for NGS, for research and/or clinical purposes. They include: Identification of the molecular basis of rare, and so far undefined, Mendelian diseases for which linkage analysis and candidate gene screening have failed to deliver results [23, 24]. This application potentially decreases the number of patients who remain without a definitive diagnosis for hereditary diseases.
b. Targeted genetic testing for diseases caused by many potential candidate genes. For example, Bardet-Biedl syndrome for which mutations in 14 different genes have been implicated, or hypogonadotrophic hypogonadism (10 potential known genes) or retinitis pigmentosa (35 potential known genes) [25, 26].
c. The investigation of cancer genetics by comparing the "cancer" genome to the germ-line genome in cancer patients. This application hopes to identify mutations potentially important for initiation versus progression of the cancer. Other applications relevant for cancer genetics include RNA sequencing of disease tissues and also next-generation cytogenetics to identify complex chromosome rearrangements in cancer. Overall NGS applications are expected to lead to a better understanding of cancer pathogenesis and new ways of targeting treatment [27, 28, 29].
d. To contribute to the study of complex diseases and traits [30].
e. To contribute to the study of population genetics [31].
f. To facilitate population wide (neonatal) screening to support prevention of genetic diseases [32] and also for noninvasive prenatal diagnosis [33] (see also section 7.4).

The fact that NGS produces an enormous amount of sequence data per analysis (megabases or even gigabases), means that the NGS methodology at the bench has to be supported by comprehensive data storage and appropriate bioinformatic analysis. The clinically useful interpretation of all sequence variants potentially identified in a single individual genome (which may number many thousands of nucleotide variants in the protein-coding sequences alone) requires prior knowledge of the population-specific variants in both normal and disease states, along with comprehensive databases and bioinformatic tools to differentiate those that are in fact disease-causing [21, 22].

It is anticipated that costs of NGS will continue to decrease and the eagerly anticipated US$1000 genome will soon become a reality. However, several issues remain before NGS becomes acceptable for routine clinical application. NGS still requires improvements with respect to sequence quality, bioinformatics data, as well as data and sample management, and issues of quality control and standardization of protocols need to be addressed. Additionally ethical aspects need to be considered by patients, professional and the public in the light of the huge amount of information potentially generated for each genome analysed, at least until the prognostic significance of individual genotypes and genotype-interactions are fully understood [21, 22].

Assuming that costs do fall, with respect to the haemoglobinopathies, NGS may potentially have

a role by facilitating the simultaneous sequencing of all globin genes in a single test, along with any other genes which have been (will have been) confirmed to modify the clinical expression of patients with a haemoglobin disorder. With the rapid progress in NGS technologies perhaps NGS-based genetic testing may become a reality for some laboratories within the next few years [19].

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