

Nine unknown rearrangements in 16p13.3 and 11p15.4 causing α - and β -thalassaemia characterised by high resolution multiplex ligation-dependent probe amplification

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Background: Approximately 80% of the α - and 10% of the β -thalassaemias are caused by genomic deletions involving the α - and β -globin gene clusters on chromosomes 16p13.3 and 11p15.5, respectively. Gap-PCR, Southern blot analysis, and fluorescent in situ hybridisation are commonly used to identify these deletions; however, many deletions go undetected using conventional techniques.

Methods: Patient samples for which no abnormalities had been found using conventional DNA techniques were analysed by a three colour multiplex ligation-dependent probe amplification assay. Two sets of 35 and 50 probes, covering a region of 700 kb of the α - and 500 kb of the β -globin gene cluster, respectively, were designed to detect rearrangements in the α - and β -globin gene clusters.

Results: In 19 out of 38 patient samples, we found 11 different α -thalassaemia deletions, six of which were not previously described. Two novel deletions leaving the α -globin gene cluster intact were found to cause a complete downregulation of the downstream α -genes. Similarly, 31 out of 51 patient samples were found to carry 10 different deletions involving the β -globin gene cluster, three of which were not previously described. One involves the deletion of the locus control region leaving the β -globin gene cluster intact.

Conclusions: These deletions, which are not easily detected by conventional techniques, may have clinical implications during pregnancy ranging from mild to life threatening microcytic haemolytic anaemia in neonates. The approach as described here is a rapid and sensitive method for high resolution analysis of the globin gene clusters and for any region of the genome.

Thalassaemias are inherited haemoglobin disorders characterised by a quantitative reduction of the α - or β -globin chains.^{1–3} Genomic deletions involving the α -globin gene cluster on chromosome 16p13.3 are the most common molecular cause of α -thalassaemia (~80–90% of cases). Rearrangements in the β -globin gene cluster on 11p15.4 account for ~10% of all β -thalassaemia mutations and hereditary persistence of fetal haemoglobin (HPFH) syndromes. Besides the most common deletions, a large variety of less frequently occurring thalassaemia deletions have been found in different populations. At least 60 different deletions involving the β - and more than 50 involving the α -globin gene cluster have been described to date^{4–5} (<http://globin.cse.psu.edu/hbvar/menu.html>).

The molecular tests commonly used to identify these deletions are gap-PCR, Southern blot analysis, and fluorescent in situ hybridisation (FISH) analysis.^{6–10} Gap-PCR can only be applied to known deletions, Southern blot analysis is time consuming and technically demanding and success is very much dependent upon the hybridisation probes available, and FISH analysis involves laborious cell culturing to generate metaphase chromosome spreads and has a low resolution (>20 kb).

Recently, a simple technique suitable for rapid quantitative analysis, multiplex ligation-dependent probe amplification (MLPA), has been described.¹¹ This technique is based on the ligation and PCR amplification of two adjacently hybridising oligonucleotides. Each oligonucleotide pair is designed to give a product of a unique length, and by using common ends all probes can be amplified with one primer pair. Using a

fluorescent label allows probe separation on a capillary sequencing system. This method has been applied successfully in a number of genes in which deletions and duplications are common.^{12–14} In the original description, the probes were generated by cloning into specially developed M13 vectors. Recently, we have simplified this method by using chemically synthesised oligonucleotides. Discrimination of probes based on chemically synthesised oligonucleotides (~40–60 nt) was doubled using two universal primer sets each labelled with a different fluorophore, allowing up to 40 probes to be used in a single reaction.¹⁵

To simplify the detection of α - and β -thalassaemia deletions and increase the resolutions, we designed two probe sets for each cluster. For the α -cluster, two probe sets of 35 probe pairs in total were designed with an average distance of ~20 kb, covering a genomic region of ~700 kb. For the β -cluster, a total of three probe sets consisting of 50 probe pairs were designed covering a region of ~500 kb and an average distance of ~10 kb. Control DNA of known α - and β -thalassaemia deletion carriers was used and the deletion characterised by an independent method. Two groups of patient samples suspected of having a (large) deletion in either the α - or β -globin gene cluster, based on haematological findings, were analysed in this assay.

Abbreviations: ATR-16, α -thalassaemia mental retardation syndrome; FISH, fluorescent in situ hybridisation; HbH, haemoglobin H; HPFH, hereditary persistence of fetal haemoglobin; MAPH, multiplex amplifiable probe hybridisation; MLPA, multiplex ligation-dependent probe amplification

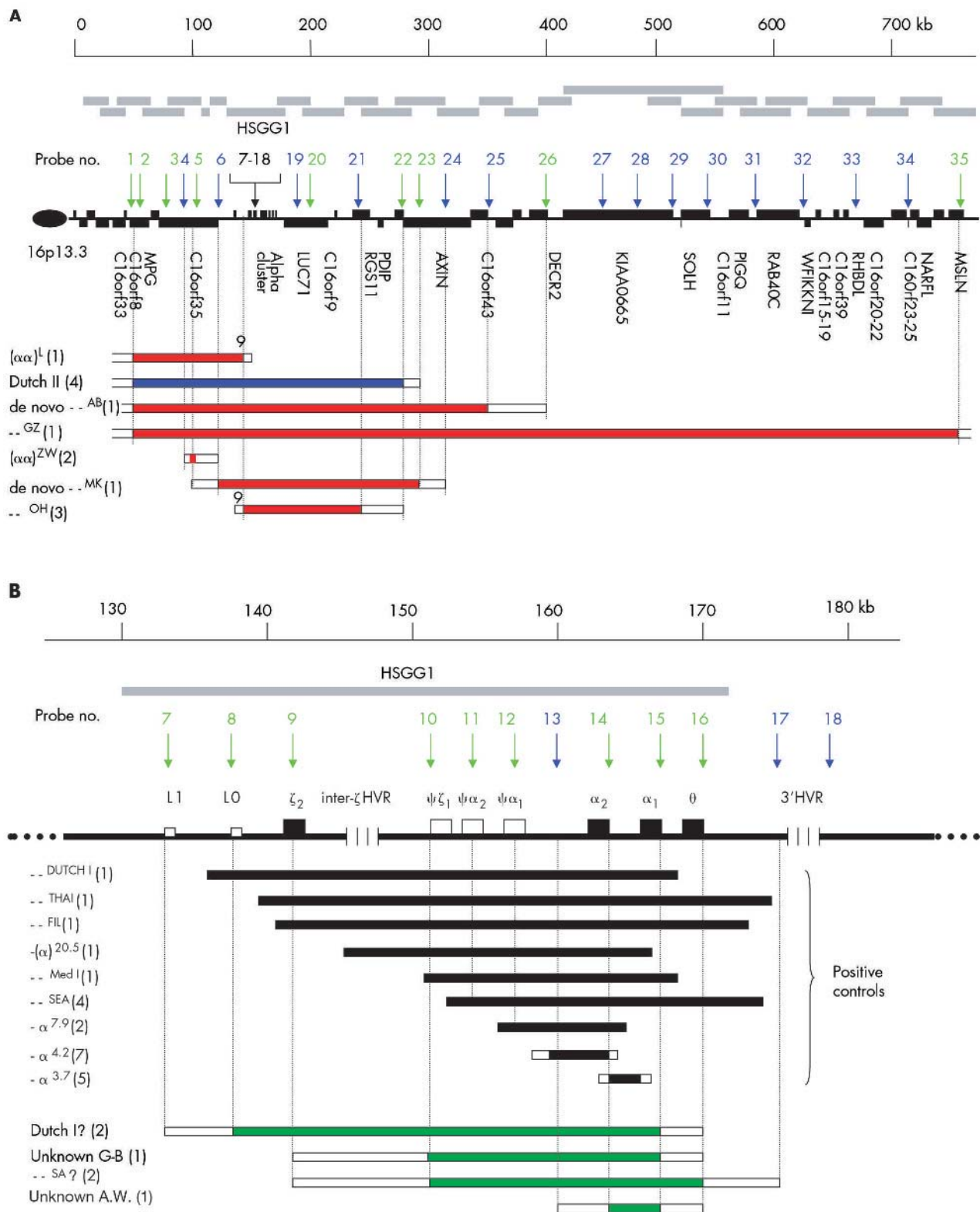


Figure 1 (A) Schematic representation of the short arm of chromosome 16 (16p13.3), showing a 700 kb region containing the α -globin gene cluster. Grey bars above the cluster indicate the minimal tiling path of clones covering this region. Oval shape denotes the telomeric repeat region and solid boxes the genes throughout the regions (adapted from Daniels *et al*²⁵). Vertical arrows show locations of probe pairs; colours correspond to colour label used in MLPA reaction and probe numbers to numbers in table 3. Bars below the figure indicate deletions found by MLPA, vertical lines marking the first and last probe deleted. Open boxes mark the region where deletion breakpoint should be located. Red indicates novel deletions found in this study. Blue indicates deletions previously described, but more accurately mapped by MLPA. The number of unrelated individuals found during analysis is indicated between brackets. (B) Schematic presentation of part of 16p13.3 showing the α -globin gene cluster. Black bars show deletions (all confirmed by gap-PCR or Southern blot analysis) used as positive controls to set up the assay. Green bars show deletions resembling described deletions in length and position of the breakpoints. The identity can only be determined by gap-PCR and direct sequencing.

METHODS

Patients

Patients suspected of having haemoglobinopathies were referred to our laboratory for haematological, biochemical, and DNA analysis.¹⁶ Based on this analysis, they were diagnosed as α - and/or β -thalassaemia carriers. The patients suspected of having α -thalassaemia in whom no abnormalities were found by gap-PCR for the seven most common α -thalassaemia deletions and non-deletion types of α -thalassaemia were excluded by direct sequencing of the α -genes, were selected for MLPA. Some showed either an unbalanced α/β chain synthesis ratio (<0.8) and/or inclusion bodies¹⁷ indicative for a deletion of both α -genes on the same allele.¹⁸ In addition, a few patients presented with haemoglobin H (HbH) disease, but analysis thus far only revealed one mutation, suggesting a deleted allele in trans. Some showed the presence of possible junction fragments by Southern blot, in which the deletion could not be characterised due to lack of probes in the region flanking the potential deletion. In total, 38 possible α -thalassaemia carriers were selected to be screened for rearrangements in 16p13.3. These samples were collected during a period of approximately 5 years.

Patients were selected who presented with a microcytic hypochromic anaemia in the presence of elevated HbA₂ levels, for which standard DNA analysis revealed no abnormalities in the β -globin gene sequence or the 5' and 3'UTR. These samples include patients showing a high HbF expression, indicative for HPFH, ($\delta\beta$)⁰- or γ ($\delta\beta$)⁰-thalassaemia, and patients showing normal HbA₂ and HbF levels with α/β chain synthesis ratios higher than 1.5, indicative for deletions involving the complete cluster and/or the regulatory elements. A total of 51 samples were analysed by MLPA.

As positive controls for MLPA of the α -globin gene cluster, we used seven deletions confirmed previously by gap-PCR (-SEA, - α ^{3.7}, - α ^{4.2}, -Med 1, -FIL, -THAI, and -(α)^{20.5}, indicated as black bars in fig 1B). Two other deletions (33 kb -Dutch I and - α ^{7.9}) were previously characterised by Southern blot analysis and direct sequencing of the amplified break point fragments.^{19, 20} For MLPA of the β -cluster, the Dutch III (ϵ γ δ)⁰-thalassaemia of 112 kb,²¹ the 50 kb Belgian ($\gamma\delta$)⁰-thalassaemia,²² the 25–30 kb Chinese β ⁰-thalassaemia,²³ the 12.6 kb Dutch I β ⁰-thalassaemia,²⁴ and the Indian (-619 bp) β ⁰-thalassaemia deletions,²⁵ all previously characterised by Southern blot analysis, were used as positive controls (fig 2).

Probe design

In total 35 probe pairs were designed to detect rearrangements on 16p13.3, covering approximately 700 kb from the telomere to the MSLN gene (table 3, fig 1). For each probe pair, the common ends correspond to either the MLPA amplification primers (forward tag 5'-GGGTTCCCTAAGGGTTGGA-3'; reverse tag 5'-TCTAGATTGGATCTTGCTGGC-3')¹¹ or the multiplex amplifiable probe hybridisation (MAPH) primers (forward tag 5'-GGCCGCGGAATTCGATT-3'; reverse tag 5'-CACTAGTGAATTCGCGGC-3'),²⁶ which allows simultaneous amplification and detection of the separated fragments in different colours.

Similarly, 34 probe pairs to be analysed in two colours were designed to detect rearrangements on 11p15.4 (table 1, fig 2). A third probe set, consisting of an additional 16 probe pairs, was designed for fine mapping some of the deletions found by MLPA (table 2). In order to detect all 50 probe sets in the same fragment analysis sample run, a third common extension was used for the additional probe set, which allowed the use of a third colour (M13 forward tag 5'-GGCGATTAAGTTGGGTAAC-3'; M13 reverse tag 5'-GTTACACAGGAAACAGC-3').

Unique sequence was identified using the BLAT program (<http://genome.ucsc.edu>),²⁷ and care was taken that no known sequence variants were present in the primer annealing site. Probes within each set were designed to produce PCR products differing by 2 bp in length to allow separation in the size range of 80–125 bp using capillary electrophoresis on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA). Primers have been designed using the RAW program (MRC-Holland, Amsterdam, The Netherlands) such that the melting temperature of the hybridising regions of each probe was at least 65°C, with a GC percentage between 35% and 60%.

The oligonucleotides were from Illumina (San Diego, CA), synthesised in a salt-free environment (50 nmol scale) and used without further purification. The downstream primer of each probe pair was 5' phosphorylated to allow ligation. Separate probe mixes were prepared to allow the detection of deletions in either the α - or β -globin gene clusters, combining two sets of probes with MLPA and MAPH common ends at a final concentration of 4 fmol/ μ l. The α - and β -globin gene MLPA probe mixes are available upon request (<http://www.LGTC.nl>).

MLPA reaction

Reagents for MLPA and subsequent PCR amplification were purchased from MRC-Holland. All primers used for PCR amplification were purchased from Sigma-Genosys (Cambridge, UK). The MLPA reactions were performed as described by Schouten *et al*¹¹ and White *et al*.¹⁵ In brief, approximately 200 ng of genomic DNA in a final volume of 5 μ l was heated for 5 min at 98°C. After cooling to room temperature, 1.5 μ l of the probe mix and 1.5 μ l SALSA hybridisation buffer (MRC-Holland) were added to each sample, followed by heat denaturation (2 min at 95°C) and hybridisation (16 h at 60°C). Ligation was performed by adding 32 μ l of ligation mix at 54°C for 10 min and the reaction was stopped by incubating for 5 min at 95°C. PCR amplification was carried out for 33 cycles in a final volume of 25 μ l, adding both the MAPH-F and -R and the MLPA-F and -R primer sets to a final concentration of 100 and 200 nM, respectively, with MAPH-F being labelled with HEX and MLPA-F being labelled with FAM. The third common primer set used for the β -globin gene cluster were M13-F and M13-R; the primers were labelled with ROX and added to a final concentration of 100 nM. A size standard (0.05 μ l ROX 500; Applied Biosystems, <http://www.appliedbiosystems.com>) was added to each well and products were separated by capillary electrophoresis on the ABI 3700 sequencer (fig 3).

Data analysis

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.¹⁵ In brief, two probes for unlinked loci were included per probe set as a reference in each sample. The height of each α - (or β -) 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 normalise 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 normalising 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.

All samples were tested at least twice. Detection of deletions is simplified by the fact that a series of flanking probes all generate a decreased signal. In cases of unlinked or

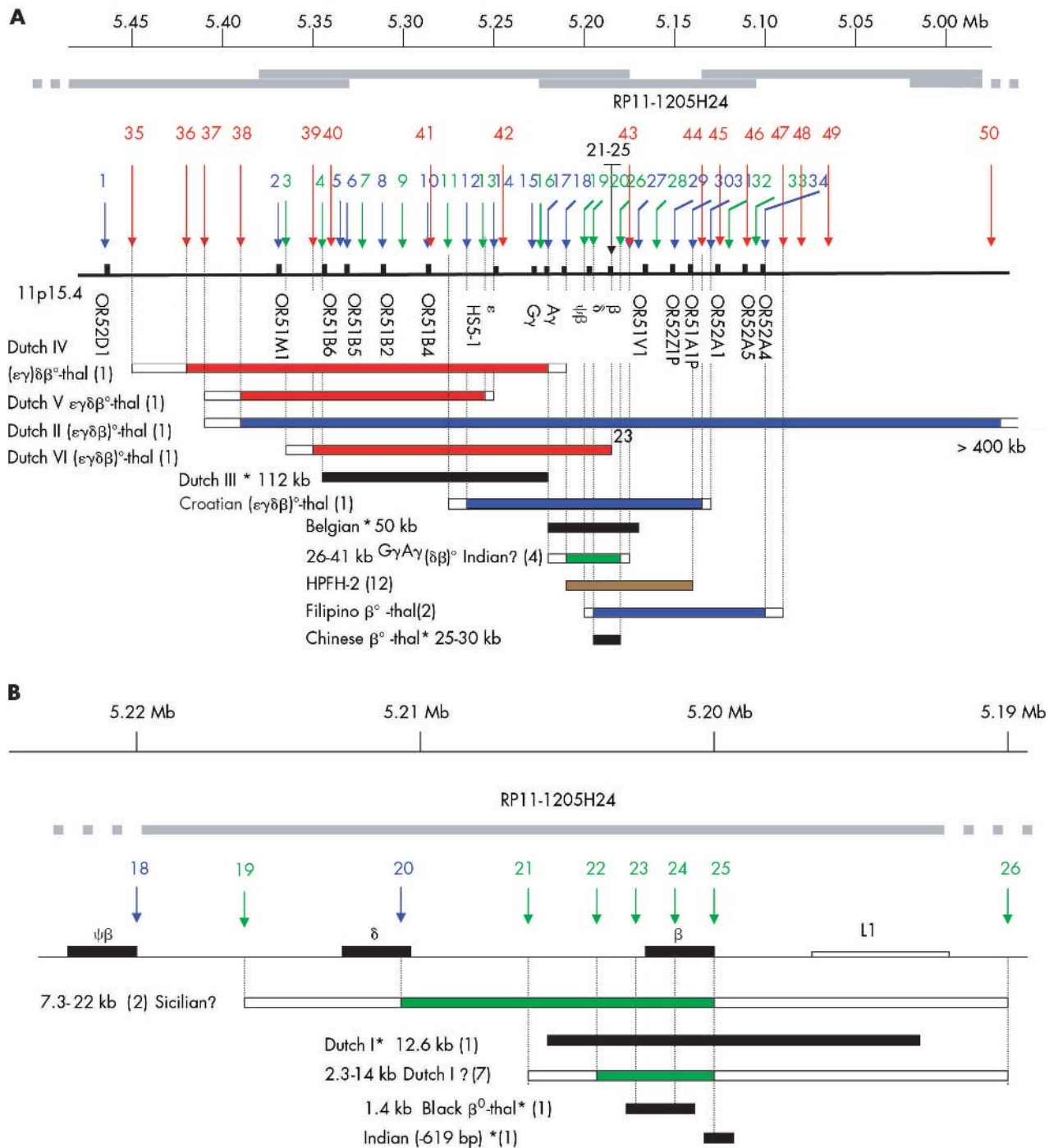


Figure 2 (A) Schematic representation of the short arm of chromosome 11 (11p15.4), showing a 500 kb region containing the β -globin gene cluster. The genes throughout the regions are indicated as solid boxes. The minimal tiling path of clones covering this region is indicated by grey bars above the cluster. Deletions found by MLPA are shown as bars below the figure; the colours of the bars are as indicated in fig 1. The brown bars represent deletions found during this analysis and confirmed by gap-PCR. (B) Schematic presentation of the region surrounding the β -globin gene and deletions found during this analysis or used as positive controls.

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.

RESULTS
Design of the MLPA assay for α -thalassaemia rearrangements

Fragment analysis in the size range of 80–125 bp allows the simultaneous amplification of approximately 20 probes

differing by 2 bp in length. To maximise the number of loci that can be analysed in a single MLPA assay, we used a second primer set with common ends, to allow co-amplification of the two primer sets under the same PCR conditions. Probes were designed for each gene and pseudo-gene in the α -globin gene cluster, in the unique sequences L₀ and L₁, at the HS-40, the MPG gene, and more proximal at conserved sequences, respectively, 20 and 9 kb from the MPG gene (fig 1). More distally, two probes were designed flanking the

Table 1 Names and sequences of probes used for MLPA for the β -globin gene cluster and flanking regions

No.	Name	Standard deviation (range) [†]	Upstream hybridising sequence	Downstream hybridising sequence	Position on chr. 11p15.4 [‡]
1b	OR52D1*	0.04 (0.93-1.09)	GGTGGATGCTGGCCAGATGTTT	TGTGTCATTCATCTATGCTCTGGAGTCC	5466733-5466857
2b	OR51M1*	0.03 (0.95-1.05)	CTGTAATCACTCTATGTCGCAATGCTACCTT	TTTGGCTCCCACTGCTAAACC	5368049-5368106
3b	Probe 79	0.04 (0.93-1.09)	CATGGGTCTCTGCTGGTTTAGATCTGTATGCCCCGTTTGGAGCTC	ATCCCCACAACTTAATCAACCAATGCC	5360654-5360732
4b	Probe 45	0.04 (0.92-1.05)	CCTTGTACAGGGTCCCCCA	CCCTCTCTCTGAGCATGTG	5342559-5342603
5b	OR51B6*	0.06 (0.85-1.07)	CTTCCACTTCAGCTTACTGGCT	TCCAGGCATGGAGAAGGCACATC	5329333-5329380
6b	OR51B5*	0.02 (0.95-1.03)	CTGATCATCGTTTTGAAAGCAGGTTCCACATAITGT	TCACCTCAITATGAGCTATGCCTATTTTCTGTT	5320488-5320559
7b	OR51B5-OR51B2	0.06 (0.85-1.07)	CATGCTCCAGGGCCATTGGTGTATATAACACCTTAGTAAAGTAA	GCAATGTTAGTGTGGAGTGGGAGTGAACCCG	5311149-5311229
8b	OR51B2*	0.05 (0.91-1.12)	CTGGCTGTGCTGACATAACTTCAATAGACTTTACCCGTAAIT	TGATCTTTTAACTTCTCTAGACTGTCTG	5301474-5301551
9b	OR51B3P	0.07 (0.83-1.12)	GGGCTGCTCTTTCAGGTT	TACATATCCACTCCCAITATCCACTGTC	5293186-5293243
10b	OR51B4*	0.03 (0.93-1.05)	CCACTGAGGTACAAGTCACTTACCAAT	TCCCGAGTGAACATAGACTGGG	5279322-5279377
11b	OR51B4-HS4	0.05 (0.91-1.12)	GGGTGAGAAAGCTTAGATCCATGAAGTATTACAGCAITTTGGTAGT	CTTT TTGCACTCAGGTTATTTTACTGTC	5272871-5272947
12b	HS-4*	0.05 (0.94-1.08)	CCGCAATGCTTCTGCCCAT	TCAGGGCTCCAGCATGTAGAAATC	5263645-5263688
13b	HS-1	0.05 (0.83-1.07)	CACCTGCTTAGCTAGGCCCT	CCCTCATCAGCTCAGCATAGTCC	5253777-5253823
14b	Hb ϵ *	0.05 (0.90-1.09)	GCTCTAGGCTGGCAATGCTGGTCAIT	TTACTGCTAGGAGAGGCTGCCG	5247663-5247714
15b	Hb γ C*	0.04 (0.88-1.04)	CGCTAAACACTTTGACGATATAAGCT	TACACAGGATTAAGTCTGAAAGGATCC	5233901-5233960
16b	Hb γ -Hb γ A	0.03 (0.93-1.09)	CCTTTAGCCATCTGATCAATGACGATATAA	GCITTACACAGGATCATGAAGGATGAAAG	5228976-5229038
17b	Hb γ A*	0.05 (0.89-1.10)	CCAAAGGGTTTATTTAGGGAAAGTGTGTGTGTAT	TTCTGATGCTGTTTGTGTTT	5225968-5226029
18b	Hb ν β *	0.05 (0.84-1.09)	CATCTCTTATGATGGGGAGGT	TGGGAGAAGACAGTATCCCTGC	5219835-5219934
19b	Hb ν β -Hb δ	0.03 (0.95-1.07)	CCATACCATGTGGCTACTCT	CCITACATATCCCATTTCCACCTAC	5215712-5215764
20b	Hb δ *	0.05 (0.91-1.14)	GGCTAACTGCTGGCTCAAGTACCATGAGATCTGGACTGTT	TCCTGATAACATAAAGAACCTTATTTCC	5210725-5210800
21b	Hb β (1b)	0.03 (0.95-1.08)	GTGCTAGTATTCATGGAGGAAGTAAITATCTCC	TCTAAGTATACCCAGACTAGGCCATCTG	5207650-5207716
22b	Hb β (2)	0.03 (0.90-1.05)	CTGAGCCAAATGAAAGACCTTCCCTCTACCCCTA	CT TTTAAGTACAGAGGCTTTTGTCCCC	5205743-5205811
23b	Hb β (3)	0.06 (0.91-1.14)	CAGAAAGCCAAAGCAGGTCGGCTGTCAAT	CACITTAGCTCACTCCCTGTGGAGC	5204970-5205024
24b	Hb β (4)	0.05 (0.94-1.11)	GGGTACAGTTTAAATGGAAACACAGCGAA	TGATGATCAGTGTGGAAAGTCTCAGG	5204244-5204300
25b	Hb β (5)	0.03 (0.94-1.06)	GCTGCTTCTGCTGCTCAATTTCTATAAAGGTTCTT	TGTTCCCTAAGTCAACTACTAACTGGGGG	5203333-5203403
26b	Hb β (6)	0.03 (0.96-1.06)	GTATCTTATAAACTGATCTCACACATCC	GTAGGCCAATTAAGTCTTTCTTTTG	5193754-5193813
27b	OR51V1*	0.08 (0.76-1.11)	GCCTCAATGTGGCCCGT	TTTGGCAAGCACCTTCCCCCG	5177669-5177710
28b	OR52Z1P-OR51V1	0.05 (0.89-1.10)	CTAAAGTATTCACAACTCCACTGGAAAGAAGTGGCTAT	TGCTCAAGTATTTCTCAATGTGGCAGGGG	5166593-5166665
29b	OR52Z1P*	0.04 (0.92-1.10)	GCCTGGAAATAAAGTACATAAGGCTCTTCT	TTGCAATCACACTATTTCCAGGATGTG	5156427-5156490
30b	OR51A1P*	0.05 (0.91-1.11)	GCCTCCCACTAGTTTAACTGTCTAGGTACTGAT	TCTCTTCTCTCTCAACCAACACTTTAGAAC	5145679-5145748
31b	OR52A1*	0.06 (0.91-1.13)	CATTTACTGCTGCTCCCTCAATTTCTCAATCCACT	TGCTATGGTCAAAAGCACACAGATCCG	5129268-5129333
32b	OR52A5-OR52A1	0.03 (0.89-1.10)	GGAGTACTGACAAAGTGGACTGCTGGCTTTTATATGTAAAAAGAA	CAGGTCTCTGAAATAGTTCAGACCC	5117804-5117878
33b	OR52A5-OR52A4	0.04 (0.94-1.07)	CAATATGATTAAGTAAAGTACTTTTGGAGATGAGAT	GTTAAGGCGAGTAGGACCTGTC	5107566-5107630
34b	OR52A4*	0.02 (0.95-1.04)	GGCGTCTATAGTATCACTGACAGCTAGTCACTTATAT	TGTAGTTGGAGTGAATTCGGCCCTGCCATCTG	5098908-5098975

*With MLPA-F and -R common ends (probes labelled in blue in figures); [†]calculated on 19 normal individuals; [‡]UCSC Genome Browser (May 2004).

Table 2 Names and sequences of third probe set for the β -globin gene cluster designed to map the large deletions

No.	Name	Standard deviation (range)†	Upstream hybridising sequence	Downstream hybridising sequence	Position on chr. 11p15.4‡
35b	OR51A10P*	0.05 (0.91-1.08)	CCCTCAACATCTTCAACCAAAATATCTGCAGAACC	TTCTGAGGATCCACCCAGCTAGTATCATGCCTCT	5448616-5448685
36b	OR5111*	0.04 (0.93-1.09)	CATGTCCAACTCTCTCCAA	AGGAGCTTTGGGGCTTCAGCCCTC	5418327-5418374
37b	OR51K1P*	0.05 (0.88-1.05)	GGAAGAAGAAATCCATTACTTCTGCCCCA	AAGTACCATATCTGGTTAGAGAAGAAAGTCATGTA	5408181-5408246
38b	OR51Q1-OR51J1*	0.06 (0.91-1.11)	CAAGAGATAATGGTTGAATCCCTAAACTG	GTAACCCGCATCAATCCATATCAAGAAG	5387552-5387616
39b	79-45*	0.06 (0.88-1.09)	CAGTCCAGTAATCTCAACAAAGGCCACCA	CACAAAGGGGAAGGATAGTCTCTC	5350883-5350888
40b	45-OR51B6*	0.05 (0.93-1.09)	GTAGGGTCAAGGGTATAGGGTACCAAAAT	TTTCTAAAGGATCAAAATGAAATGCCCTGCTCTGA	5333585-5333648
41b	OR51B3P-OR51B4*	0.09 (0.84-1.09)	GIITTAGATAAGATTTAGGGTAGGTTGTATCT	GCACGATCTAGACTCTAAATAGAAACTCAGCA	5284744-5284815
42b	Hb ϵ -Hb γ G*	0.06 (0.86-1.14)	GGAGAGACAAATAATTGTTAGTTCAGGCTATCAACA	TACTGGTCTCTGAGCCCTATAACCTTCAA	5244693-5244766
43b	Hb β (16)-OR51V1*	0.06 (0.93-1.16)	GATCCCACTCTTATAGCAATGGG	AGGGCTAAGGCAAGTCATGTCAIG	5184855-5184904
44b	OR51A1P-OR52A1*	0.05 (0.86-1.06)	GGGAAACCCTCCAGGGCAIT	CATGTATGGGCAAAATCTGTCTATGAGACTTC	5135864-5135917
45b	OR52A1-75*	0.11 (0.89-1.19)	GCAGGTAAGTACAGCACCTCCCCACA	CCAGAGACTCAACATCATGGTTACAAGTTAC	5124990-5125047
46b	OR52A5*	0.09 (0.86-1.21)	GCCGACATTCATGGCTCAGTCTT	CATGCCCTCTGCGTTTACTAATTTGGG	51110395-51110446
47b	OR52A4-OR52J1P*	0.09 (0.79-1.15)	CCACTATATACTGGAACCTCGCTTGCAATGTCAGTGGCAAGG	TATGGCAGAAGAAAGCTCAATAATCTTCCAAITA	5088458-5088533
48b	OR52J1P-OR52E3P*	0.07 (0.90-1.14)	GAITTAGTGTCTTATTCCTCCGAACTATCTGTAGTG	TCTCTCTGCTGCTTCTAAACACATTCAGAITA	5078734-5078811
49b	OR52E3P-OR52S1P*	0.09 (0.86-1.17)	GITGCCGTGTGTTCCTCAATAGGCAAGGCTTATGAGGGCACCA	CTCCTTGCCCTGAAAAGAGCTTTCATAGAAAATCCCC	5066851-5066932
50b	OR51L1*	0.05 (0.94-1.13)	GTGTCAAGCAAAAAGCAGATCTGTAGGAATCTCCACAAGTT	TGTCCTAAGGAGGGGTTTTAAGTAAACCTGTCTCCTC	4977672-4977751

*With MLPA-2-F and -R common ends (fragments labelled in red); †calculated on 16 normal individuals; ‡UCSC Genome Browser (May 2004).

3'HVR, known to be involved in many rearrangements of the α -cluster, and 15 probes at approximate intervals of 13-50 kb with the most proximal probe localised in the MSLN gene, known to be deleted in the α -thalassaemia mental retardation syndrome (ATR-16).^{28, 29} The 35 probe pairs shown in table 3 can detect all the deletions described to date.

Of the 35 probes tested in triplicate on 14 healthy individuals, two gave a standard deviation of greater than 12% (table 3, probes 17a and 21a). These probes were considered to be unreliable and were excluded from further calculations. To investigate the efficacy of the assay, DNA samples of nine carriers with known deletions were used as positive controls. All could be detected unequivocally and their extent could be confirmed (black bars in fig 1B).

To demonstrate that duplications are also reliably detected, we tested a homozygote and heterozygote carrier for the common $-\alpha^{3,7}$ deletion, which results in the loss of the α_2 -specific 3'UTR and a heterozygote for the so called α_2 -triplication, which is characterised by a duplication of the α_2 -specific 3'UTR. The results are summarised in fig 3.

MLPA for β -thalassaemia rearrangements and HPFH

Similar to the α -cluster, 34 probes were designed for loci in the β -globin gene cluster and flanking regions. The region spans from the olfactory receptor gene OR52D1 to OR52A4 and covers an area of approximately 370 kb (table 1, fig 2). Most large deletions reported so far are located in this region and all should be detectable. In order to detect small deletions removing part or all of the β -gene,³⁰ a subset of closely spaced probes (fig 2B) surrounding the β -globin gene were selected. A third probe set was designed with different common ends (M13-F and -R) to allow amplification and detection with a third colour. Loci were selected in between some widely spaced probes and towards the centromere. Standard deviations for these probe sets were calculated in 19 healthy individuals; none showed standard deviations greater than 12%.

Positive controls (marked as black bars in fig 2) were used to test the capacity of the MLPA assay to detect the deletions found by other methods in these patients. Probes covering deleted loci showed half the intensity of the surrounding probes, matching the positions and extensions of all the six known deletions.

Patient samples for α -thalassaemia

Our MLPA analysis revealed a large deletion involving the α -globin genes in 19 out of 38 patients. In the remaining 19 patients, 11 different deletions were detected, affecting either the α -globin genes or the regulatory elements known to be involved in globin gene expression. Six showed no resemblance to previously described deletions and were considered to be new ($-\alpha^{GZ}$, $-\alpha^{OH}$, $(\alpha\alpha)^L$, $(\alpha\alpha)^{ZW}$, $-\alpha^{AB}$, $-\alpha^{MK}$). One has been described (Dutch II α^0 -thalassaemia) but the breakpoint position and deletion length could not be determined at the time³¹; FISH analysis performed in John Radcliff Hospital in Oxford revealed an approximate deletion length of 300 kb (Higgs, personal communication). Four deletions show similarity with previously described deletions (fig 1B, last four deletions). One 14 year old Dutch girl showed haematological parameters typical for an α^0 -thalassaemia carrier (MCV 65 fl, MCH 19.5 pg, RBC 5.79×10^{12} , and positive HbH inclusion bodies test). The α -genes were structurally intact and we only detected the deletion of a single probe 5a (fig 1A $(\alpha\alpha)^{ZW}$). The location of this probe coincides with one of the *cis* acting elements that regulate α -gene expression, known as the HS-40.

Patient sample for β -thalassaemia

Analysis of the 51 samples suspected for β -thalassemic rearrangements or HPFH using MLPA revealed 10 different

Table 3 Names and sequences of probes used for MLPA for the α -globin gene cluster and flanking regions

No.	Name	Standard deviation (range) [†]	Upstream hybridising sequence	Downstream hybridising sequence	Position on chr. 16p13.3 [‡]
1a	c16orf33	0.05 (0.93-1.10)	CACAGTCTGGACCTGAAGAAGGCCATC	CAGATAGCTGACGAGCTCAAGCAGGACGCT	45799-45856
2a	c16orf8/RHBD1	0.05 (0.90-1.08)	CACGAGCACCTCGCAGCGCAAGAA	GCCACCTGGCTAAAGCTGACATTCCTC	54937-54990
3a	MPC	0.06 (0.84-1.07)	CAAGAGCTTTGACCAGAGGGAC	CTGGCACAGGATGAAGCTGTATGGCT	75563-75610
4a	c16orf35 (2)*	0.04 (0.97-1.14)	CTAACCATGCACACAGATCAGAAACACC	CTGCTAATGGTGTGATCCCTCCCTGCT	90548-90603
5a	c16orf35 (HS-40)	0.09 (0.86-1.17)	GTAAATGGTACTGTATTACAACTCTGGTGTGCTGCCTCCCTCCCTC	CTGTTTATCTGAGAGGAAAGCCATGCCCAAAAGTG	103695-103774
6a	c16orf35 (3)*	0.11 (0.84-1.18)	GTAAAGCCGGTAGCAGATAGCTGC	CAGCAACAGGGGGACCATGCTGAT	120541-120590
7a	L1	0.07 (0.86-1.12)	CTGGACAATGAAGCACCCAGGGCCAAC	CTCATTTGCTACAGGGACATCT	132952-133003
8a	L0	0.04 (0.93-1.09)	GTACACGGGAGGGCAGTTTCTCTGGTCTGAAAAGAGC	CCCAATGAGCAAAAGGATACACTGGCCCTCCTG	139157-139228
9a	HBZ	0.05 (0.90-1.09)	CAGATCCAGTACATCTCCCTCAGCGCTGGTGGACCTAAC	CCTTGCTTTCTGGAGAAACCCAGGAATCCAG	142344-142417
10a	HBZP2	0.05 (0.89-1.09)	CTTAGGTCA CCTGTATCAGAGGACAGGGAG	GTCAAAGCAGTCACTCTCTGAGGCCA	152736-152795
11a	HBAP1	0.09 (0.92-1.21)	GTGCAATGCAAGCGGCTGGGACAAATTC	CTGACTGGTGGCCGTGGTCTGACCCGAAAA	156636-156679
12a	HBAP1	0.04 (0.92-1.07)	GACTCAGAAATAAGCTCCGCTGGTGTCTCT	CTGAGCAAAAGGCTAACACCAAGCCGCTGGGAGA	158659-158726
13a	1/21- α 2*	0.09 (0.87-1.16)	CATCCATCTGAGGAAACACAG	CTACATCAAACTACTGCCACAGGCTCTCT	160969-161022
14a	HBA2	0.11 (0.84-1.15)	GAAATCCAAAGCGGGAAAGCATTG	CTAAGCTGGTGGAGCTACTCTCTC	165128-165176
15a	HBA1	0.04 (0.88-1.03)	GTGCCAAGAACTGGCTGGCTTCTGCCTG	GGACGCTACTGGTCCAGAGGCTCT	167825-167880
16a	HBQ	0.09 (0.71-1.05)	CTGGACAAGTCTCTGAGCCAC	GTATCTGGCGCTGGTTTCCGGAT	171054-171099
17a	3'HVR (hel)*	0.15 (0.78-1.26)	GATGGCTCTGAGGGTGACGCTGTCTGCTTAAGGC	CCAGGAAACCCAGTGCACAACTCACACTC	175806-175869
18a	3'HVR (cen)*	0.06 (0.89-1.09)	CTCAAGGGTGGCATGTACTC	CCTGCAGAAACAGAGCCGATGAGA	177548-177599
19a	LUC7L (2)*	0.06 (0.88-1.08)	GTCITTCACCTTACCTTGGTCTCATGACAAITGACCTGGC	CTGGCAGACCACTTCGGTGGCAAGTTACCTTGGGG	189203-189080
20a	LUC7L	0.05 (0.91-1.10)	GCTGAATGTGATCGGAACTGACTCGCCAAAGAGCG	GCTGGCAGAAACACAGGAGAAATCAGTCCG	198092-198161
21a	c16orf9*	0.21 (0.81-1.37)	CTTGCAAGGCCACAGATCCCGCTCACAGCTCAC	GACCAGATGCACCAGCAGAGATCCACATCGAGGAC	239560-239629
22a	PDJP	0.08 (0.77-1.11)	GAGCTGGCTGAGGAGTTGGTGTGACGGAGTAC	CCTACGCTCAAGTCTTCCGCAITGGGAACCGC	274507-274572
23a	AXIN1	0.04 (0.92-1.09)	GGATGCACACGAGGAAACCTGAGACATCTCTG	GACGACCGCTACACGCTGTCTGAGGACA	288064-288127
24a	AXIN1 (2)*	0.06 (0.89-1.13)	CTCAGAGCTCTCAGCAGAAAGTCTGAATACATCACTTACC	CAGAACAGGTTGCCGCTACAGTCAATGTT	321023-321094
25a	c16orf43*	0.04 (0.95-1.07)	GGAGAGTCCATTGACAAAGCTTAAATCGAGCTGCCCTTGCAGGGC	CTGTCCCTGAAGTCCCGAGTAATGGGATTT	350871-350946
26a	DEC2	0.09 (0.81-1.16)	CTTGTCTTCAAAGCTTCAAAGCCGATGGACATCGATAC	CAGCGCACCTCAATGTGTCTGTGTCTCTATGA	400278-400355
27a	KIA0665 (1)*	0.06 (0.91-1.08)	GGACTTAACTAAGTACTGGATCCAGTGC	GGCTGGCGTGTATGACTTGAAGACTTC	451410-451467
28a	KIA0665 (3)*	0.08 (0.86-1.13)	GAAAGGATCTTCTCTCTAGCACTGACCTTTGGCCGAAAG	CTGCACAGCATCTCACTGATGAGCGTTTGAAT	481111-481190
29a	KIA0665 (2)*	0.08 (0.85-1.14)	CGCAACTGAAAGGACGACAGCAGGAGCTGAACG	GGCAGATCACTCCCTCAGCATCCAGGGCCCAA	510466-510533
30a	SOLH*	0.08 (0.76-1.09)	GTCAAGAAATGCTAGCTGCGACGATCTGAGGAGCTGGC	GAGTACGCTGGTGTGCTGGCCCTCAACCA	542420-542493
31a	RAB40C*	0.05 (0.92-1.09)	CAGACAGGTTGTGAGCGTACATITGCCCTCTCCAG	CTTCAAAAACAATGAGTCAATCGAAGTACC	583598-583663
32a	WFKKN1*	0.06 (0.85-1.11)	CGAGGAGCTGTCAAAGGATGACAAAGATGGCC	CTCAAGTCTGGCCACCAAGTACCTGGAGG	623788-623849
33a	RHBDL1*	0.07 (0.86-1.10)	GATGAGCCAGGCTAGGTGTCTACAAG	CGGTTTGGCTAGTGGCCACGAGATCTCTG	666751-666810
34a	c16orf25*	0.05 (0.89-1.09)	GTGCTGGTCCACTACTCTCTGTAG	CTCAGAAATGGCTTCAAGCCATCTG	712920-712982
35a	MSLN (ATR-16)	0.07 (0.85-1.14)	CTGAGGACAATCGCAAGTGGAAATGTGACGCTCCCTGGAGAC	CCTGAAGGCTTGTGCTGAAGTCAACAAGGGCCACGA	756403-756478

*With MLPA-F and -R common ends (probes labelled in blue in figures); †calculated on 12 normal individuals; ‡UCSC Genome Browser (May 2004).

deletions in 31 out of 51 patient samples. In the remaining 20 samples, a deletion of the probe sets tested could be excluded. In three cases, deletions were detected which do not match those described to date and are considered to be new. All three deletions, found in Dutch carriers, silence the expression of the complete globin gene locus and were named Dutch IV ($\epsilon^G\gamma^A\gamma$) $\delta\beta^0$ -, Dutch V $\epsilon^G\gamma^A\gamma\delta\beta^0$ -, and Dutch VI ($\epsilon^G\gamma^A\gamma\delta\beta$) 0 -thalassaemia. One matched the HPFH-2 deletion and was confirmed by breakpoint PCR.⁸ One sample belonged to a patient described in 1996 by Abels *et al*³² as a carrier of Dutch II ($\epsilon^G\gamma^A\gamma\delta\beta$) 0 -thalassaemia, however the deletion length was not determined at that time. Now the deletion length is estimated to be at least larger than 400 kb and the 5' breakpoint located between position 5408246 and 5387552 (UCSC Genome Browser, May 2004) (fig 2A). Five deletions match the length and breakpoint locations of previously described deletions, two of which, the Croatian ($\epsilon\gamma\delta\beta$) 0 - (at least >108 kb) and the Filipino β^0 -thalassaemia (at least >45 kb), were incompletely mapped. More accurate length estimations were obtained by MLPA, being between 128–143 kb and 109–122 kb, respectively. The other three showed similarity to the Dutch I 12.6 kb β^0 -thalassaemia deletion (in seven independent patients of Dutch origin), the 13.4 kb Sicilian ($\delta\beta$) 0 -thalassaemia deletion, which are also frequently found in the Mediterranean basin,^{33,34} and the 32.6 kb Indian $\epsilon^G\gamma^A\gamma(\delta\beta)$ 0 -thalassaemia,³⁵ found in four independent chromosomes from Surinam-Hindustani subjects (fig 2B).

DISCUSSION

We describe the application of MLPA for high resolution mapping of deletions causing α - and β -thalassaemia. Using synthetic oligonucleotides, 35 loci along a genomic region of 700 kb from the tip of the short arm of chromosome 16, containing the α -globin gene cluster, could be analysed in two colours in a single reaction. More loci could be analysed simultaneously by using a third pair of amplification primers, labelled with a third fluorophore. This increased the number of probes to 50 loci spanning a genomic region of 500 kb on 11p15.4 and used to detect rearrangements causing β -thalassaemia or HPFH. Although slightly better results can be obtained when performing the PCR with the three sets of labelled universal primers separately, the ligation of all 50 probes was done in a single tube reaction. The fragment analysis was performed on a single sample of the three pooled PCR products per patient, which allowed the simultaneous analysis of 86 patient samples along with 10 normal controls in a 96 well format fragment analysis run on the ABI 3700.

The use of chemically synthesised oligonucleotides instead of cloning the half probes into M13 vectors, as originally described for MLPA,¹¹ allows cheap and rapid probe development, which increases the flexibility of MLPA for characterising genomic rearrangements. Only two out of 85 probes (2%) were excluded from further calculations due to standard deviations higher than 12% when tested on a validation set of 12 wildtype controls. The majority showed standard deviations of between 0.05 and 0.08. Although these deviations seem significant, please note that due to the probe density rearrangements are mostly detected using a series of flanking probes (>2).

The ability to detect rearrangements in both regions was tested using positive controls, heterozygous for the seven most common α -thalassaemia deletions confirmed by gap-PCR, and for two less frequent mutations, Dutch I and $-\alpha^{7,9}$, confirmed by Southern blot analysis. By selecting 12 probes closely distributed along the 40 kb α -globin gene cluster, all of the common deletion types (except for the $-\text{THAI}$ and

$-\text{THAI}$) could be distinguished from each other by MLPA. In our eyes, the simplicity, work-load, and cost make MLPA a superior alternative to Southern blot analysis when a single technique is preferred for the detection of deletions causing α -thalassaemia in a research setting. When desired, gap-PCR can be used for independent confirmation. Similarly, six positive controls were selected, based on confirmation by different methods (Southern blotting and/or direct sequencing of breakpoint fragments) and tested for the β -cluster probe set. All the probes expected to be deleted were confirmed in the heterozygotes tested.

In 19 and 20 samples large rearrangements involving the α - and β -globin genes, respectively, could be excluded. Point mutations or micro-deletions affecting expression and located in between the probes, would not be picked up by MLPA. However, since iron levels were not known for some patients and anaemia due to iron deficiency could easily be mistaken for α - or normal HbA₂ β -thalassaemia, we believe that negative samples may fall into this category.

Polymorphisms in the genome, interfering with probe annealing and ligation of the two probe pairs, may cause the loss of probe signal leading to a false positive MLPA result.^{11,14,36} During the screening of patient samples suspected for α -thalassaemia, one case repeatedly showed the deletion of a single probe 5a (in fig 2A), named the ($\alpha\alpha$)^{ZW} deletion found in an adopted child. This probe was selected in a highly conserved region of the HS-40, not containing the polymorphic sites known to be present in human populations.³⁷ Deletion of this regulatory element is expected to cause severe down regulation of α -gene expression of the affected chromosome. Even though nothing can be said about the extent of deletion, the fact that the HbH inclusion bodies test was positive and that no other rearrangements involving the α -genes were found, is strongly in favour of a deletion involving the HS-40. Whether or not this deletion, which is at maximum 30 kb in length, also involves HS-33 as found by Higgs *et al*³⁸ needs further analysis. These types of deletions in human carriers may contribute to understanding the mechanisms involved in regulation of downstream α -gene expression³⁹ and will be studied further.

In conclusion, MLPA is an attractive alternative for FISH analysis for screening large deletions, for example in ATR-16 syndrome.^{9,10} The tiling paths of cloned probes currently available for cytogenetic analysis of 16p13.3 and 11p15.4 are shown in figs 1 and 2. The distribution of synthetic probes coincides with the available cosmids, and allows a higher resolution of mapping than the available BAC or PAC probes. In contrast to *in situ* hybridisation, no laborious cell culture to generate metaphase spreads is necessary. MLPA can be performed directly on (stored) DNA samples.

MLPA uses standard technology only, that is, hybridisation, ligation, PCR, and capillary electrophoresis. Since most diagnostic laboratories have these technologies operational, implementation of MLPA should be straightforward. The robustness, simplicity, and intrinsic redundancy (probe density) of this approach, and the additional specificity offered by the ligation step, make MLPA an attractive technique for the detection and characterisation of copy number variation (deletions/duplications) in any region of the genome, particularly for high resolution analysis, and those regions not amenable to analysis by array comparative genomic hybridisation (array CGH).⁴⁰

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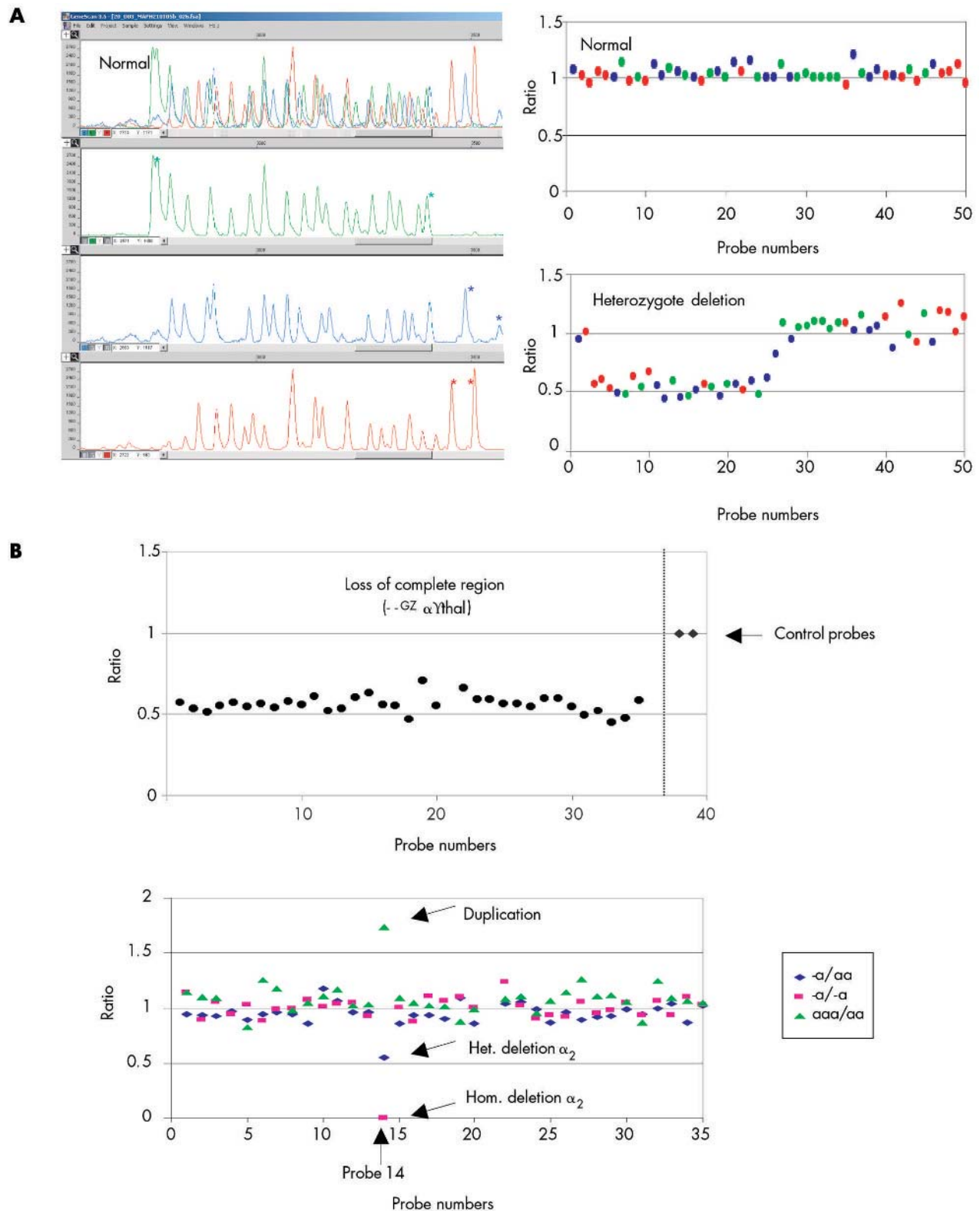


Figure 3 Peak patterns and scatterplots. (A) Peak patterns of an ABI 3700 fragment run of 50 β -specific probes labelled in three colours tested on a wildtype DNA sample. The upper window shows 17 probes in green, 17 probes in blue, and 16 probes in red, all between 80 and 125 bp in length. In the lower three windows, the peaks are split by colour. Within each probe set, two probes for unlinked loci were included as a reference (marked by an asterisk). At the right are scatter plots of a wildtype and a heterozygote for the Dutch IV ($\epsilon\gamma$) $\delta\beta^0$ -thalassaemia deletion. The corresponding probes in different colours are ordered according to their position along the β -globin gene cluster on the x axis, the y axis showing the ratios calculated for each probe. (B) The first scatter plot represents the deletion of all the α -cluster specific probes on one allele as found in the - - GZ α^0 -thalassaemia deletion; the two independent control probes are indicated on the right. The second scatter plot shows heterozygosity for a duplication, a deletion, and homozygosity for a deletion of probe 14, located in the 3'UTR of the α_2 -globin gene, which demonstrates the capacity of the assay to detect copy number changes.

ELECTRONIC-DATABASE INFORMATION



The following URLs have been mentioned in this article: HbVar: A Database of Human Hemoglobin Variants and Thalassemias, <http://globin.cse.psu.edu/hbvar/menu.html>; UCSC Genome Bioinformatics, <http://genome.ucsc.edu>; and Leiden Genome Technology Center (LGTC²), <http://www.LGTC.nl>; Applied Biosystems: <http://www.appliedbiosystems.com>; Microsoft, <http://www.microsoft.com/>.

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