Product Description
SALSA® MLPA® Probemix P140-C1 HBA

To be used with the MLPA General Protocol.

Version C1
For complete product history see page 20.

Catalogue numbers:
- **P140-025R:** SALSA MLPA probemix P140 HBA, 25 reactions.
- **P140-050R:** SALSA MLPA probemix P140 HBA, 50 reactions.
- **P140-100R:** SALSA MLPA probemix P140 HBA, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit and Coffalyser.Net data analysis software. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman/SCIEX capillary sequencers, respectively (see www.mrcholland.com).

Certificate of Analysis
Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mrcholland.com.

Precautions and warnings
For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mrcholland.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

This product requires RNAse sample treatment. For more information, see page 4.

Intended purpose
The SALSA MLPA Probemix P140 HBA is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in the alpha-globin (*HBA*) gene cluster and its regulatory region in genomic DNA isolated from human peripheral whole blood specimens. P140 HBA can be used to confirm parental aberrations in prenatal samples, in DNA isolated from (un)cultured amniotic fluid obtained in week 16 of pregnancy or later and free from blood contamination, or (un)cultured chorionic villi free from maternal contamination. In addition, this probemix can be used to detect the presence of the Hb Constant Spring mutation in the *HBA2* gene. P140 HBA is intended to confirm a potential cause for and clinical diagnosis of alpha-thalassaemia, for molecular genetic testing of at-risk family members and for carrier screening in at-risk populations.

Copy number variations (CNVs) detected with P140 HBA should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Although most defects in the alpha-globin gene cluster are copy number changes, about 15% of the defects are due to point mutations in the *HBA1* and *HBA2* genes, most of which will not be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

This device requires in depth knowledge of the complicated human alpha-globin gene cluster and assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, parental evaluation, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1] Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).
[2] To be used in combination with a SALSA MLPA Reagent Kit, SALSA Binning DNA SD031 and Coffalyser.Net analysis software.

Clinical background

Alpha-thalassaemia is the most common inherited haemoglobin disorder in the world. It is characterised by a reduced production of the alpha-globin chain, resulting in a decrease in the total amount of haemoglobin. In normal adult life, about 97% of the total haemoglobin level comprises haemoglobin A (HbA), which is composed of two alpha- and two beta-globin chains. The remaining 3% of adult haemoglobin consists of HbA_2 and HbF (foetal haemoglobin), consisting of two alpha chains combined with two delta-globin chains or two gamma-globin chains, respectively.

The alpha-globin chains are encoded by the haemoglobin alpha 1 (*HBA1*) and alpha 2 (*HBA2*) genes, located in the alpha-globin gene cluster on chromosome 16p13.3. Defects in the *HBA* genes can lead to two clinically significant forms of alpha-thalassaemia. In the lethal Hb Bart's hydrops foetalis syndrome, the two *HBA1* and two *HBA2* copies are all absent or defect. In HbH disease, only one functional *HBA* copy remains. In addition, two alpha-thalassaemia carrier states can be discriminated: in alpha-thalassaemia trait (heterozygous α^0-thalassaemia or homozygous α^+-thalassaemia), two functional *HBA* copies remain, whereas in "silent" alpha-thalassaemia (heterozygous α^+-thalassaemia), three functional *HBA* copies are present. Next to defects in the *HBA* genes, alpha-thalassaemia can also be caused by deletions in the upstream hypersensitive (HS) sites, which constitute the regulatory elements of the alpha-globin gene cluster.

Alpha-thalassaemia patients can present with a wide variety of clinical symptoms, ranging from very mild anaemia to severe transfusion-dependent haemolytic anaemia. The phenotype depends on the gene that harbours the mutation (*HBA1* or *HBA2*), the type of mutation, and the number of affected alpha-globin genes.

Alpha-thalassaemia is inherited in an autosomal recessive manner, with about 85% of all alpha-thalassaemia phenotypes caused by genomic deletions in the *HBA1* and *HBA2* genes. Most of these deletions can be detected by the MLPA technique, including commonly occurring deletions such as the 3.7 kb deletion ($-\alpha^{3.7}$), the 4.2 kb deletion ($-\alpha^{4.2}$), the South-East Asian deletion ($--^{SEA}$), and the Filipinian deletion ($--^{FIL}$). The remaining 15% of the alpha-thalassaemia cases result from one of at least 70 different point mutations, usually located within the *HBA2* gene (Higgs and Weatherall, 2009; Harteveld and Higgs, 2010). The most common non-deletion mutation, which is frequently seen in Southeast Asia, is Hb Constant Spring, resulting from a mutation in the stop codon of the *HBA2* gene. This mutation leads to the production of an elongated α-globin chain. Hb Constant Spring is produced in very small amounts because its mRNA is unstable. Heterozygotes for elongated globin chain variants such as Hb Constant Spring present with an α^0-thalassemia phenotype. Presence of the Hb Constant Spring mutation can be detected by the P140 probemix.

In addition to many deletion types, several duplications have also been described in the alpha-globin gene cluster. These duplications vary in size, ranging from only a single duplicated *HBA* gene to large segmental duplications of the complete alpha-globin gene cluster, including the regulatory elements. Duplication of one or both *HBA* genes is clinically benign. However, when co-inherited with a beta-thalassaemia mutation, an *HBA* duplication leads to a more severe phenotype in beta-thalassaemia patients because it aggravates the balance between alpha- and beta-globin chains.

More information on alpha-thalassaemia is available on http://www.ncbi.nlm.nih.gov/books/NBK1435/.

Gene structure and transcript variants

The human alpha-globin gene cluster spans about 43 kb and is located on chromosome 16p13.3 at a distance of only 0.2 Mb from the p-telomere. The gene cluster includes seven loci arranged in the order 5'- *HBZ - HBZP1 - HBAP2 - HBAP1 - HBA2 - HBA1 - HBQ1-3*' (see Figure 1). The Genbank chromosomal DNA sequence of this gene cluster is NG_000006.1, which is available at http://www.ncbi.nlm.nih.gov/nuccore/NG_000006.1. For both *HBA1* and *HBA2*, only one transcript variant has been defined: NM_000558.5 for *HBA1* (577 nt, coding sequence 38-466, http://www.ncbi.nlm.nih.gov/gene/3039) and NM_000517.6 for *HBA2* (576 nt, coding

sequence 38-466, http://www.ncbi.nlm.nih.gov/gene/3040). *HBA1* and *HBA2* both contain 3 exons. The two *HBA* genes have identical coding sequences, but differ slightly in the 5' untranslated regions and introns and significantly in the 3' untranslated regions. At least four hypersensitive sites, or regulatory elements, located upstream of the alpha-globin gene cluster have been described (Hughes et al. 2005). Of these, HS-40, which is located 40 kb upstream of *HBZ,* is suggested to be the major regulatory element.

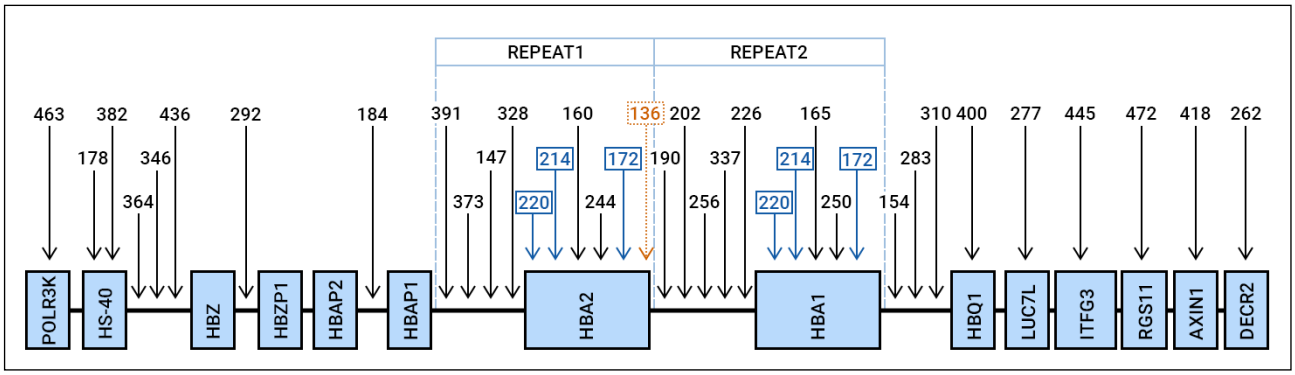

Figure 1. Schematic representation of the target locations of the probes in SALSA MLPA probemix P140-C1 HBA. The numbers above the arrows represent the amplification size (in nt) of the respective probes. The probes that detect sequences in both repeats are blue, framed, with blue arrows. the Hb Constant Spring mutation-specific probe is orange, framed in orange dotted line and similar arrow. This picture is not drawn to scale and is only intended to provide an illustration of the sequential ordering of the probe targets.

Exon numbering

The exon numbering used in this P140-C1 HBA product description is the exon numbering from the NG_000006.1 sequence. As changes to the database can occur after release of this product description, the NG_ sequence and exon numbering may not be up-to-date.

Probemix content

The SALSA MLPA Probemix P140-C1 HBA contains 45 MLPA probes with amplification products between 130 and 481 nucleotides (nt). This includes 34 probes for the alpha-globin gene cluster and its flanking regions, including one probe specific for the Hb Constant Spring mutation in *HBA2* which will generate a clear signal (>10% of the mean peak height of all reference probes in the sample) when the mutation is present (see Figure 1 for the location of the probe targets). In addition, 11 reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

Eight probes are present that detect sequences in the *HBA* genes. This includes two probes for *HBA2* intron 2, two probes for *HBA1* intron 2, three probes (172, 214 and 220 nt) that detect sequences present in both *HBA1* and *HBA2*, and a probe specific for the Hb Constant Spring mutation. Next to these *HBA*-specific probes, the probemix contains 18 probes for sequences elsewhere in the alpha-globin gene cluster and two probes for the HS-40 regulatory element. Finally, one probe detects a sequence telomeric of the HS-40 regulatory element and five probes detect a sequence centromeric of the alpha-globin gene cluster. These flanking probes are included to delineate the extent of larger deletions/duplications in the alpha-globin gene cluster.

This probemix contains five probe pairs targeting locations with a very small sequence difference between *HBA1* and *HBA2*: one probe detecting the *HBA1* sequence and the other probe detecting the *HBA2* sequence. Due to the close proximity of these genes, it is possible that in some healthy individuals the *HBA2* sequence at one or more of these five locations is changed by gene conversion into the *HBA1* sequence (or *vice versa*), without any clinical consequences. Probe pairs that can be affected in this way are the 160 & 165 nt intron 2 probes, the 244 & 250 nt intron 2 probes, the 391 & 190 nt probes, the 328 & 226 nt probes, and the 373 & 202 nt probes.

This probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below).

More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MLPA technique
The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mrcholland.com).

MLPA technique validation
Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation ≤0.10 for all probes over the experiment.

Required specimens
Extracted DNA from peripheral whole blood specimens, free from impurities known to affect MLPA reactions, (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later and free from blood contamination, or (un)cultured chorionic villi free from maternal contamination. DNA samples should be free from RNA (see box below). For more general information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

RNAse sample treatment (essential for HBA and HBB MLPA probemixes)
Since *HBA* is heavily expressed in red blood cells, an RNAse treatment of samples is essential for (whole) blood derived samples. Even though white blood cells are less affected by RNA, they should still be treated with RNAse. Without RNAse treatment, the probes targeting HBA1 and HBA2 exons (i.e., the 172, 214 and 220 nt probes, and the 136 nt probe in the presence of the Hb Constant Spring mutation) can bind to HBA mRNA, thereby reducing the effective concentration of probes. Please note that some automatic DNA purification methods (e.g. Roche Magnapure) do not include an RNAse treatment. The following method can be used to treat RNA containing DNA samples:

Mix 4 µl sample and 1 µl 0.5 mg/ml RNAse A. Incubate 30 minutes at 37 °C. Continue with the 5 minutes 98 °C DNA denaturation step of the MLPA General Protocol.

RNAse A is extremely stable; it can be diluted in TE and stored at -20 °C. We recommend RNAse A from Promega (A7973; 4 mg/ml solution), diluted 8 fold in TE (1 ml of 4 mg/ml RNAse is sufficient for ~8000 samples). Do not use more than the recommended amount.

Reference samples
A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from different unrelated individuals who are from families without a history of alpha-thalassaemia. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).
Please note that approximately 10% of the samples from healthy Caucasian individuals show reproducible copy number changes for one or more of the *HBA* region probes. This percentage may be higher in other

populations. In many cases, these copy number changes are associated with certain polymorphisms (Table 3).

Positive control DNA samples

MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/) have diverse collections of biological resources which may be used as positive control DNA samples in your MLPA experiments. Sample ID numbers listed in the table below have been tested with this P140-C1 probemix at MRC Holland and can be used as positive control samples. The quality of cell lines can change; therefore samples should be validated before use.

Coriell sample ID	Expected copy number alteration
NA10798	Heterozygous deletion of probe 436 nt to 400 nt (=--FIL deletion)
NA10799	Heterozygous deletion of probe 184 nt to 400 nt (=--SEA deletion)
NA10797	Compound heterozygous --SEA and --FIL deletion
NA19122	Homozygous alpha 3.7 kb deletion type D
NA19176	Heterozygous alpha 3.7 kb deletion type D
HG02188	Positive for the Hb Constant Spring mutation (signal for 136 nt probe)
NA09687	Heterozygous deletion of all target probes
NA02325	Heterozygous duplication of all target probes

SALSA Binning DNA SD031

The SD031 Binning DNA provided with this probemix can be used for binning of all probes including the Hb Constant Spring mutation (*HBA2*: 427T>C, p.*143Glnext*31) specific probe (S0585-SP0043-L09493). SD031 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probe. Inclusion of one reaction with 5 µl SD031 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation signal. It is not needed to perform an RNAse treatment on SD031 Binning DNA. For further details, please consult the SD031 Binning DNA product description, available online: www.mrcholland.com.

Performance characteristics

The diagnostic sensitivity of the P140 probemix is estimated to be close to 85%, since ~85% of all alpha-thalassaemias are caused by deletions (http://www.ncbi.nlm.nih.gov/books/NBK1435/), most of which can be detected by the MLPA assay. The diagnostic specificity is considered to be ~100%, because deletion of one or both *HBA* genes will always result in an alpha-thalassaemia phenotype. Therefore, deletions will not be expected in the population without alpha-thalassemia. In addition, this probemix can also detect the Hb Constant Spring mutation, the occurrence of which depends on the population investigated. The analytical sensitivity and specificity for the detection of the Hb Constant Spring mutation and deletions/duplications in the alpha-globin gene cluster (based on a 2004-2022 literature review) is very high and can be considered >99%.

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Data analysis

Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely

downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Note: Slope correction in samples with large deletions
The slope correction algorithm in Coffalyser.Net has been optimised to give the best possible results in as many situations as possible. However, the slope correction algorithm may confuse a large deletion for sloping, which can lead to over- or under-correction. Since large deletions are often detected when using the P140 probemix, this issue may occur relatively frequent. Incorrectly applied slope correction can cause an FSLP warning in Coffalyser.Net or ambiguous results for multiple probes. If you suspect that slope correction was incorrectly applied we recommend to contact info@mrcholland.com for assistance.

Interpretation of results
The expected results for most *HBA* region-specific MLPA probes are allele copy numbers of 2 (normal), 0 (homozygous deletion), 1 (heterozygous deletion), 3 (heterozygous duplication), and 4 (heterozygous triplication/homozygous duplication).

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the FR of the probes (except for the 136, 172, 214 and 220 nt probes) can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 2.15
Ambiguous copy number	All other values

Three probes (172, 214 and 220 nt) detect a sequence that is present in both *HBA1* and *HBA2*. In case of normal copy numbers these probes detect 4 copies / cell. The expected results for these three probes are allele copy numbers of 0 (homozygous deletion of both *HBA1* and *HBA2*), 1, 2, 3, 4 (normal), 5, 6, 7 or 8, corresponding to the final ratios listed in the table below. The probe ratios of these probes should be interpreted together with the results of the surrounding probes to determine the copy number.

Copy number status the 172, 214 and 220 nt probes	Final ratio (FR)
Homozygous deletion (0 copies)	FR = 0
Heterozygous deletion of *HBA1* and homozygous deletion of *HBA2* or *vice versa* (1 copy)	FR ≈ 0.25
Heterozygous deletion of *HBA1* and *HBA2*, or homozygous deletion of *HBA1* or *HBA2* (2 copies)	FR ≈ 0.50
Heterozygous deletion of *HBA1* or *HBA2* (3 copies)	FR ≈ 0.75
4 copies **(= normal)**	FR ≈ 1.00
Heterozygous duplication of *HBA1* or *HBA2* (5 copies)	FR ≈ 1.25
Heterozygous duplication of *HBA1* and *HBA2*, or homozygous duplication of *HBA1* or *HBA2* (6 copies)	FR ≈ 1.50
Heterozygous duplication of *HBA1* and homozygous duplication of *HBA2* or *vice versa* (7 copies)	FR ≈ 1.75
Homozygous duplication of *HBA1* and *HBA2* (8 copies)	FR ≈ 2.00

The presence of a clear signal for the 136 nt probe (at least 10% of the mean peak height of all reference probes in the sample), indicates the presence of the Hb Constant Spring mutation. The percentage obtained for the mutation-specific probe can vary between samples and does not determine whether the mutation is present in heterozygous or homozygous state. In the majority of the samples, this probe will generate a signal below 10% of the mean peak height of all reference probes, which indicates absence of the mutation.

Note: The term "dosage quotient", used in older product description versions, has been replaced by "final ratio" to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the alpha-globin gene cluster. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

P140 specific notes:

- So-called 'silent carriers' of α-thalassaemia have a defect or deletion of one *HBA* gene copy, also referred to as heterozygous for α^{+}-thalassaemia. Alpha-thalassaemia trait occurs when two *HBA* gene copies are defective or deleted. This situation can result either from two defective or deleted copies on the same allele (*in cis*, heterozygous α^{0}-thalassaemia), or from one defective or deleted *HBA* copy on each allele (*in trans*, homozygous α^{+}-thalassaemia). Deletion or dysfunction of three *HBA* gene copies results in HbH disease. All four *HBA* gene copies are deleted or inactivated in Hb Bart's hydrops foetalis syndrome.
- The 178 nt and 382 nt probes target sequences within the hypersensitive site (HS)-40, which is one of the upstream regulatory elements. Deletion of these elements lead to an α^{0}-thalassaemia phenotype, even though the *HBA* genes are present and intact. Several studies indicate that HS-40 is the most conserved element and is considered as the major regulatory element (Zhang et al. 2002; Viprakasit et al. 2006).

However, it has also been shown that homozygous deletion of HS-40 does not lead to the lethal Hb Bart's hydrops foetalis syndrome (Sollaino et al. 2010).

- Multiple CpG islands are located within the alpha-globin gene cluster. Therefore, a low signal for probes targeting this cluster can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich region.
- Various - $\alpha^{3.7}$ and - $\alpha^{4.2}$ deletions with different breakpoints exist. The same holds true for the complementary α-triplications. In certain -$\alpha^{3.7}$ deletion cases, it will not be possible to establish which parts of the *HBA1* and *HBA2* genes have been deleted. Homozygous -$\alpha^{3.7}$ deletion samples are frequently a combination of two different -$\alpha^{3.7}$ deletions. To the best of our knowledge, distinguishing the various -$\alpha^{3.7}$ and -$\alpha^{4.2}$ deletions has no clinical significance. The different deletions and duplications are indicated as type A-F for clarity (Table 4A and Table 5). Please note that these indications are not part of the official nomenclature.
- Presence of three *HBA* genes on one allele (α-triplication) is relatively common. The α-triplication can be detected by the P140 probemix, but should be considered as a polymorphism as it is not associated with an α-thalassaemia phenotype (Goossens et al. 1980). However, co-inheritance of multiple alpha-globin genes and beta-thalassaemia may lead to relatively severe (transfusion-dependent) beta-thalassaemia intermedia (Camaschella et al. 1997, Harteveld et al. 2008).
- Due to the very high homology and close proximity of the *HBA1* and *HBA2* genes, sequence exchange (gene conversion) occur frequently. In some individuals, the *HBA2*-typical sequence in the *HBA2* gene has been replaced by the *HBA1*-typical sequence or *vice versa*, without any clinical consequences.
- Five probe pairs target locations with very small sequence differences between *HBA1* and *HBA2*: the 160 & 165 nt intron 2 probes, the 244 & 250 nt intron 2 probes, the 391 & 190 nt probes, the 328 & 226 nt probes, and the 373 & 202 nt probes. When one of the probes in such a probe pair has a ~50% reduced probe signal (ratio 0.5), while the other has a ~50% increased probe signal (ratio 1.5), it is possible that this is a benign polymorphism due to a sequence exchange between *HBA1* and *HBA2* rather than a true deletion and duplication (Table 3).
- The '**African polymorphism**' is a gene conversion between *HBA2* and *HBA1*, causing the intron 2 typical for *HBA1* to be located also in *HBA2* or vice versa. The *HBA* genes have identical coding sequences, but differ at two sites in intron 2: a point mutation T>G (*HBA2*: T; *HBA1*: G) and a 8 nt insertion (where a single G (*HBA2*) is replaced by CTCGGCCC (*HBA1*)). Probe pairs at 160 & 165 nt and at 244 & 250 nt, respectively, detect these intron 2 differences. In the case of African polymorphism 1, the *HBA2* target sites (160 & 244 nt) have disappeared and *HBA2* intron 2 seems deleted. In fact, however, the *HBA2* gene is intact and contains the *HBA1* intronic sequence, as can be seen by an increased probe signal for the 165 & 250 nt *HBA1* intron 2 probes (*vice versa* for the African polymorphism 2).
- SNP rs370305736 is also frequently found in African populations, with an estimated average carrier frequency of approximately 10%, although this frequency can vary between specific subpopulations. rs370305736 is located in the target sequence of the 220 nt probe and can affect this probe's signal.
- The 292 nt probe has been reported to be deleted/duplicated in several individual from Southeast Asia. This is probably a benign polymorphism, referred to as the '**Asian polymorphism**' (Table 3). The exact deletion boundaries are currently unknown, but seem to cover at least position 20361-23609 of the Genbank NG_000006.1 reference sequence.
- A combination of polymorphism 3B, polymorphism 4B (see table below) and SNP rs555255920 affecting the 256 nt probe has frequently been reported by users of the P140 probemix.
- Expected probe final ratios for polymorphisms and common deletions in the *HBA* region are summarised in Tables 3, 4A and 4B. In addition, Table 5 provides a set of selected examples of deletions in the alpha-globin gene cluster. In order to simplify the explanation of the observed polymorphisms in this product description, we used the indications from the table below. However, when reporting clinical results, we strongly recommend using only the official HGVS nomenclature.

Polymorphisms	HGVS nomenclature using NG_000006.1
Polym 1A	g.30717T>C
Polym 1B	g.34974C>T
Polym 2A	g.31233T>C
Polym 2B	g.35489C>T
Polym 3A	g.33103G>A
Polym 3B	g.36907A>G
Polym 4A	g.34247T>G
Polym 4B	g.38051G>T
Polym 5A	g.34311delGinsCTCGGCCC
Polym 5B	g.38115_38122delCTCGGCCCinsG
African polym 1 *	g.[34247T>G; 34311delGinsCTCGGCCC]
African polym 2 *	g.[38051G>T; 38115_38122delCTCGGCCCinsG]

* As SALSA MLPA Probemix P140 HBA can only detect a limited number of specific sequences, we describe the African polymorphisms as two separate small changes, even though they are expected to be due to gene conversion events spanning a larger region.

Limitations of the procedure

- In most populations, approximately 15% of the genetic defects in the alpha-globin gene cluster are small (point) mutations, which will not be detected by using SALSA MLPA probemix P140 HBA, with exception of the Hb Constant Spring mutation.
- The combination of a deletion on one chromosome and a similarly sized duplication on the other chromosome may result in a false negative MLPA result as there is no net change in copy number (see example 14 in Table 5).
- Not all deletions in the alpha-globin gene cluster can be discriminated from each other using this probemix. The --^{FIL} and --^{THAI} deletions will display identical final ratios (Table 4B). The same accounts for the --^{MED2} and --^{Dutch1} deletions (Table 4B).
- In some cases, two different types of deletions are detected by the P140 probemix in a single patient. It is not possible to determine if two (not-overlapping) deletions are *in cis* or *in trans*, but testing of family members can provide more information.
- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.

Confirmation of results

Copy number changes detected by only a single probe always require confirmation by another method. An apparent deletion detected by a single probe can be due to e.g. a mutation/polymorphism that prevents ligation or destabilises the binding of probe oligonucleotides to the DNA sample. Sequence analysis can establish whether mutations or polymorphisms are present in the probe target sequence. The finding of a heterozygous mutation or polymorphism indicates that two different alleles of the sequence are present in the sample DNA and that a false positive MLPA result was obtained.

Copy number changes detected by more than one consecutive probe should be confirmed by another independent technique such as long range PCR, qPCR, array CGH or Southern blotting, whenever possible. Deletions/duplications of more than 50 kb in length can often be confirmed by FISH.

Haemoglobin mutation databases

http://globin.cse.psu.edu/globin/hbvar/menu.html, https://databases.lovd.nl/shared/genes/HBA1, and https://databases.lovd.nl/shared/genes/HBA2. We strongly encourage users to deposit positive results in the

Database of Human Haemoglobin Variants and Thalassaemias and/or the Leiden Open Variation Database (LOVD). Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results (e.g., a duplication of *HBA1* exons 1 and 3 but not intron 2) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P140-C1 HBA

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*HBA* cluster
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 00797-L13645	5q	
136 § Ж	**HBA2 probe** S0585-SP0043-L09493		**Hb Constant Spring mutation**
142	Reference probe 07641-L07326	8p	
147	**HBA region probe** 18098-L22522		0.9 kb before HBA2
154	**HBA region probe** 08499-L23594		0.1 kb after HBA1
160 +	**HBA2 probe** 08498-L08422		HBA2 intron 2
165 +	**HBA1 probe** 08498-L21607		HBA1 intron 2
172 »	**HBA1 & HBA2 probe** 15857-L21812		**HBA1 + HBA2 exon 3**
178	**HS-40 probe** 04799-L04797		**HS-40**
184	**HBA region probe** 04637-L04018		Between HBAP2 & HBAP1
190 +	**HBA region probe** 18096-L22520		2.5 kb before HBA1
196 ±	Reference probe 05846-L11214	10q	
202 +	**HBA region probe** 18880-L24428		2.0 kb before HBA1
208	Reference probe 11331-L12056	12p	
214 » Δ	**HBA1 & HBA2 probe** 18881-L06288		**HBA1 + HBA2 exon 1**
220 » ±	**HBA1 & HBA2 probe** 18099-L22524		**HBA1 + HBA2 exon 1**
226 +	**HBA region probe** 18093-L22517		0.6 kb before HBA1
238	Reference probe 11435-L12163	1q	
244 +	**HBA2 probe** 04633-L23748		HBA2 intron 2
250 +	**HBA1 probe** 04633-L23600		HBA1 intron 2
256 ±	**HBA region probe** 08494-L08417		1.5 kb before HBA1
262 ¬	**DECR2 probe** 17613-L23601		Centromeric of HBA region
269	Reference probe 03075-L19996	5p	
277 ¬	**LUC7L probe** 15859-L21960		Centromeric of HBA region
283	**HBA region probe** 04638-L23602		0.4 kb after HBA1
292 ~	**HBA region probe** 04624-L04004		Between HBZ & HBZP1
300	Reference probe 03250-L02687	13q	
310	**HBA region probe** 04639-L04020		2.3 kb after HBA1
328 +	**HBA region probe** 18092-L22516		0.6 kb before HBA2
337	**HBA region probe** 14855-L23604		0.9 kb before HBA1
346	**HBA region probe** 04622-L04001		3.5 kb before HBZ
355	Reference probe 00547-L00116	11q	
364	**HBA region probe** 04926-L23886		9.2 kb before HBZ
373 +	**HBA region probe** 18090-L08415		2.5 kb before HBA2
382	**HS-40 probe** 04800-L04175		**HS-40**
391 +	**HBA region probe** 18097-L22521		3.0 kb before HBA2
400	**HBQ1 probe** 19233-L25313		HBQ1 exon 3
409	Reference probe 03272-L02709	3q	
418 ¬	**AXIN1 probe** 17212-L13393		Centromeric of HBA region
436 Ж	**HBA region probe** 17214-SP0457-L20489		0.2 kb before HBZ
445 ¬	**ITFG3 probe** 17227-L20554		Centromeric of HBA region
454	Reference probe 07607-L07292	15q	
463 ¬ £	**POLR3K probe** 19236-L25316		Telomeric of HBA region
472 ¬	**RGS11 probe** 18102-L20488		Centromeric of HBA region
481	Reference probe 15318-L17117	2q	

[a] See section Exon numbering on page 3 for more information.

For explanation of symbols, see page 13 (below Table 2).

Table 2. *HBA* region probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene / exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
				From p-telomere to POLR3K probe	37.2 kb
463 ¬ £	19236-L25316	*POLR3K*		ATATGCTCTGTG-TAAAGTCCTATT	66.4 kb
			AY327548.1		
178	04799-L04797	HS-40	61-62	CTGCCCAAGCCA-AGGGTGGAGGCA	0.2 kb
382	04800-L04175	HS-40	220-221	GGTACTGCTGAT-TACAACCTCTGG	30.0 kb
			NG_000006.1		
364	04926-L23886	9.2 kb before *HBZ*	4528-4529	GGCTGGGGCTCA-AACCAAGGCCCA	5.7 kb
346	04622-L04001	3.5 kb before *HBZ*	10229-10230	CGCAGTGCTAGA-AGGGAGTTCCTG	3.3 kb
436 Ж	17214-SP0457-L20489	0.2 kb before *HBZ*	13499-13498 & 13536-13535 **R**	TTCCTCTCCTGT-37 nt spanning oligo-AGTCTAGGAGAG	6.9 kb
292 ~	04624-L04004	Between *HBZ* & *HBZP1*	20361-20362	GTGGAGTAGGCT-TTGTGGGGAACT	7.8 kb
184	04637-L04018	Between *HBAP2* & *HBAP1*	28168-28169	AGTGGCCACAAT-TTGGCAGACAGA	2.5 kb
391 +	18097-L22521	3.0 kb before *HBA2*	30717-30716 **R**	AGGAAGGGGTGA-GAATGAGAGAAA	0.5 kb
373 +	18090-L08415	2.5 kb before *HBA2*	31233-31232 **R**	ATGTCCAGAAGA-AAAGCGGTGACA	1.6 kb
147	18098-L22522	0.9 kb before *HBA2*	32850-32849 **R**	GATAAACAAACT-TGGCTCTGGGTA	0.2 kb
328 +	18092-L22516	0.6 kb before *HBA2*	33103-33102 **R**	CCGGGAAGGAAC-AAACACCAGGAC	0.6 kb
220 » ±	18099-L22524	*HBA1* + *HBA2* exon 1	33733-33732 **R**	AAGAGTGCCGGG-CCGCGAGCGCGC	0.1 kb
214 » Δ	18881-L06288	*HBA1* + *HBA2* exon 1	33824-33823 **R**	CGCGCCGACCTT-ACCCCAGGCGGC	0.4 kb
160 +	08498-L08422	*HBA2* intron 2	34247-34248	GCGCCTTCCTCT-CAGGGCAGAGGA	0.1 kb
244 +	04633-L23748	*HBA2* intron 2	34311-34312	GGGCCTGGGCCG-CACTGACCCTCT	0.1 kb
172 »	15857-L21812	*HBA1* + *HBA2* exon 3	34385-34384 **R**	GCAGGGGTGAAC-TCGGCGGGGAGG	0.1 kb
136 § Ж	S0585-SP0043-L09493	Hb Constant Spring mutation	34461-34462 & 34489-34490	CCAAATACCGTC-28 nt spanning oligo-TGCCCGCTGGGC	0.5 kb
190 +	18096-L22520	2.5 kb before *HBA1* (0.4 kb after *HBA2*)	34974-34975	TTCTCTCATTCC-CACCCCTTCCTG	0.5 kb
202 +	18880-L24428	2.0 kb before *HBA1*	35489-35490	GTCACTGCTTTC-CTTCTGGACATG	0.6 kb
256 ±	08494-L08417	1.5 kb before *HBA1*	36050-36051	TTCTCTGCCCAA-GGCAGCTTACCC	0.6 kb
337	14855-L23604	0.9 kb before *HBA1*	36629-36628 **R**	CTGGAGCATTCA-ACCTCCTCTGGG	0.3 kb
226 +	18093-L22517	0.6 kb before *HBA1*	36907-36908	TCCTGGTGTTTA-TTCCTTCCCGGT	0.6 kb
220 » ±	18099-L22524	*HBA1* + *HBA2* exon 1	37537-37536 **R**	AAGAGTGCCGGG-CCGCGAGCGCGC	0.1 kb
214 » Δ	18881-L06288	*HBA1* + *HBA2* exon 1	37628-37627 **R**	CGCGCCGACCTT-ACCCCAGGCGGC	0.4 kb
165 +	08498-L21607	*HBA1* intron 2	38051-38052	GCGCCTTCCTCG-CAGGGCAGAGGA	0.1 kb
250 +	04633-L23600	*HBA1* intron 2	38122-38123	GGCCCTCGGCCC-CACTGACCCTCT	0.1 kb
172 »	15857-L21812	*HBA1* + *HBA2* exon 3	38196-38195 **R**	GCAGGGGTGAAC-TCGGCGGGGAGG	0.3 kb
154	08499-L23594	0.1 kb after *HBA1*	38459-38460	TGGGACACACAT-GGCTAGAACCTC	0.3 kb
283	04638-L23602	0.4 kb after *HBA1*	38785-38786	AAGTCCCACTCC-AGCATGGCTGCA	1.9 kb
310	04639-L04020	2.3 kb after *HBA1*	40637-40638	GTTCACTGCCCT-GAAGAAACACCT	1.4 kb
400	19233-L25313	*HBQ1* exon 3	42066-42067	TGCTCTCTCGAG-GTCAGGACGCGA	25.1 kb
277 ¬	15859-L21960	*LUC7L*		ATGTTCCAATGA-AACCAGTGGCAC	33.5 kb
445 ¬	17227-L20554	*ITFG3*		GCTGTGATACTT-TTGCCTTTGTCA	31.9 kb
472 ¬	18102-L20488	*RGS11*		GAACCTGAGGTT-CCGTGGAATATT	16.3 kb
418 ¬	17212-L13393	*AXIN1*		GATCATCGGCAA-AGTGGAGAAGGT	119.4 kb
262 ¬	17613-L23601	*DECR2*		CAGAATCGACAT-TCTCATTAACTG	

[a] See section Exon numbering on page 3 for more information.
[b] Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

The two sequence blocks between the grey lines are almost identical.

§ Mutation-specific probe. This probe will only generate a clear signal when the Hb Constant Spring mutation (*HBA2*:c.427T>C, p.*143Glnext*31) is present. Coffalyser.Net displays the final ratio of this probe as a percentage, which indicates the height of the probe signal normalized by the average signal of the reference probes. The mutation-specific probe will give a percentage of 0-10% on negative samples and a percentage of >10% on positive samples.
» This probe detects a sequence that is present in both *HBA1* and *HBA2*. Deletion of a single target site results in a 20-25% decrease in signal intensity of this probe. This probe is RNA sensitive. See box on RNAse sample treatment on page 4.
Ж This probe consists of three parts and has two ligation sites. This type of probe is more sensitive to depurination, for instance, when buffer capacity is insufficient.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.
~ This probe has been reported to be deleted/duplicated in several samples from Southeast Asia. This is probably a benign polymorphism.
± SNPs rs370305736 and rs750737757 could influence the 220 nt probe signal. SNPs rs555255920 and rs556530054 influence the 256 nt and 196 nt probe signals, respectively. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
Δ More variable. This probe may be sensitive to certain experimental variations (e.g. extra EDTA in the reaction. Aberrant results should be treated with caution.
+ In the sequence detected by this probe, there is only a small difference between *HBA1* and *HBA2*. Due to the close proximity of these genes, it is possible that in some healthy individuals the *HBA2* sequence is changed by gene conversion into the *HBA1* sequence (or vice versa), without any clinical consequences.
£ Duplications have been described at this location, which is very close to the telomere. SALSA MLPA Probemix P036 Subtelomeres Mix 1 can be used to confirm copy number changes at this position.
R The sequence of these probes is the reverse complement as compared to the NG_000006.1 sequence.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

The tables on the following pages show expected final ratios for a selected subset of copy number changes and polymorphisms. Please note that additional copy number changes, combinations of multiple copy number changes and combinations of one or more polymorphism with copy number changes can be detected by the P140 probemix. Interpretation of those cases should be done carefully considering the location and coverage of the probes, and the borders of these aberrations.

MLPA users are responsible for correct interpretation of their results. No rights can be derived from these tables.

Table 3. Expected probe ratios for predicted polymorphisms in the *HBA* region

Probe number	length (nt)	Polym 1A	Polym 1B	Polym 2A	Polym 3A	Polym 4A	Polym 5A	African polym 1	African polym 2	Asian polym	Asian polym + polym 3B
19236-L25316	463	1	1	1	1	1	1	1	1	1	1
04799-L04797	178	1	1	1	1	1	1	1	1	1	1
04800-L04175	382	1	1	1	1	1	1	1	1	1	1
04926-L23886	364	1	1	1	1	1	1	1	1	1	1
04622-L04001	346	1	1	1	1	1	1	1	1	1	1
17214-L20489	436	1	1	1	1	1	1	1	1	1	1
04624-L04004	292	1	1	1	1	1	1	1	1	**0.5**	**0.5**
04637-L04018	184	1	1	1	1	1	1	1	1	1	1
18097-L22521	391	**0.5**	**1.5**	1	1	1	1	1	1	1	1
18090-L08415	373	1	1	**0.5**	1	1	1	1	1	1	1
18098-L22522	147	1	1	1	1	1	1	1	1	1	1
18092-L22516	328	1	1	1	**0.5**	1	1	1	1	1	**1.5**
18099-L22524	***220*** »	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
18881-L06288	***214*** »	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
08498-L08422	160	1	1	1	1	**0.5**	1	**0.5**	**1.5**	1	1
04633-L23748	244	1	1	1	1	1	**0.5**	**0.5**	**1.5**	1	1
15857-L21812	***172*** »	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
18096-L22520	190	**1.5**	**0.5**	1	1	1	1	1	1	1	1
18880-L24428	202	1	1	**1.5**	1	1	1	1	1	1	1
08494-L08417	256	1	1	1	1	1	1	1	1	1	1
14855-L23604	337	1	1	1	1	1	1	1	1	1	1
18093-L22517	226	1	1	1	**1.5**	1	1	1	1	1	**0.5**
18099-L22524	***220*** »	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
18881-L06288	***214*** »	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
08498-L21607	165	1	1	1	1	**1.5**	1	**1.5**	**0.5**	1	1
04633-L23600	250	1	1	1	1	1	**1.5**	**1.5**	**0.5**	1	1
15857-L21812	***172*** »	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
08499-L08423	154	1	1	1	1	1	1	1	1	1	1
04638-L23602	283	1	1	1	1	1	1	1	1	1	1
04639-L04020	310	1	1	1	1	1	1	1	1	1	1
19233-L25313	400	1	1	1	1	1	1	1	1	1	1
15859-L21960	277	1	1	1	1	1	1	1	1	1	1
17227-L20554	445	1	1	1	1	1	1	1	1	1	1
18102-L20488	472	1	1	1	1	1	1	1	1	1	1
17212-L13393	418	1	1	1	1	1	1	1	1	1	1
17613-L23601	262	1	1	1	1	1	1	1	1	1	1

» This probe detects a sequence that is present in both *HBA1* and *HBA2*. Deletion of a single target site results in a 20-25% decrease in signal intensity of this probe. Although this is a single probe, it is mentioned twice in the Table.

Due to the very high homology and close proximity of the *HBA1* and *HBA2* genes sequence exchange (gene conversion) frequently occur. In some healthy individuals the *HBA2* sequence is therefore changed into the *HBA1* sequence or vice versa, without any clinical consequences. Examples of the resulting benign polymorphisms are indicated in the Table above. Not shown are polymorphisms 2B, 3B, 4B and 5B, which are the complement of 2A, 3A, 4A and 5A, respectively.

The Asian polymorphism is a benign polymorphism that has been reported to be deleted/duplicated in several individuals from Southeast Asia. The exact breakpoint boundaries are currently unknown, but seem to cover at least position 20361-23609 of the Genbank NG_000006.1 reference sequence.

Please note that not all of these predicted polymorphisms have been observed in research samples tested at MRC Holland.

Table 4A. Expected final ratios for common -$\alpha^{3.7}$ and -$\alpha^{4.2}$ *HBA* heterozygous deletion/duplication types

Probe number	length (nt)	-$\alpha^{3.7}$ (A)	-$\alpha^{3.7}$ (B)	-$\alpha^{3.7}$ (C)	-$\alpha^{3.7}$ (D)	-$\alpha^{3.7}$ (E)	-$\alpha^{3.7}$ (F)	-$\alpha^{4.2}$ (A)	-$\alpha^{4.2}$ (B)	-$\alpha^{4.2}$ (C)	$\alpha\alpha\alpha$ anti 3.7 (A)	$\alpha\alpha\alpha$ anti 3.7 (F)
19236-L25316	463	1	1	1	1	1	1	1	1	1	1	1
04799-L04797	178	1	1	1	1	1	1	1	1	1	1	1
04800-L04175	382	1	1	1	1	1	1	1	1	1	1	1
04926-L23886	364	1	1	1	1	1	1	1	1	1	1	1
04622-L04001	346	1	1	1	1	1	1	1	1	1	1	1
17214-L20489	436	1	1	1	1	1	1	1	1	1	1	1
04624-L04004	292	1	1	1	1	1	1	1	1	1	1	1
04637-L04018	184	1	1	1	1	1	1	1	1	1	1	1
18097-L22521	391	1	1	1	1	1	1	1	1	**0.5**	1	1
18090-L08415	373	1	1	1	1	1	1	1	**0.5**	**0.5**	1	1
18098-L22522	147	1	1	1	1	1	1	**0.5**	**0.5**	**0.5**	1	1
18092-L22516	328	1	1	1	1	1	**0.5**	**0.5**	**0.5**	**0.5**	1	**1.5**
18099-L22524	***220*** »	*0.75*	*0.75*	*0.75*	**0.75**	**0.5**	**0.75**	**0.75**	**0.75**	**0.75**	*1.25*	**1.25**
18881-L06288	***214*** »	*0.75*	*0.75*	*0.75*	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	*1.25*	**1.25**
08498-L08422	160	1	1	1	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	**1.5**
04633-L23748	244	1	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	**1.5**
15857-L21812	***172*** »	*0.75*	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**	*1.25*	**1.25**
18096-L22520	190	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	**1.5**	**1.5**
18880-L24428	202	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1	**1.5**	**1.5**
08494-L08417	256	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1	1	**1.5**	**1.5**
14855-L23604	337	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1	1	**1.5**	**1.5**
18093-L22517	226	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1	1	1	**1.5**	1
18099-L22524	***220*** »	**0.75**	**0.75**	**0.75**	0.75	**0.5**	*0.75*	*0.75*	*0.75*	*0.75*	**1.25**	*1.25*
18881-L06288	***214*** »	**0.75**	**0.75**	**0.75**	0.75	*0.75*	*0.75*	*0.75*	*0.75*	*0.75*	**1.25**	*1.25*
08498-L21607	165	**0.5**	**0.5**	1	1	1	1	1	1	1	**1.5**	1
04633-L23600	250	**0.5**	1	1	1	1	1	1	1	1	**1.5**	1
15857-L21812	***172*** »	**0.75**	*0.75*	*0.75*	*0.75*	*0.75*	*0.75*	*0.75*	*0.75*	*0.75*	**1.25**	*1.25*
08499-L08423	154	1	1	1	1	1	1	1	1	1	1	1
04638-L23602	283	1	1	1	1	1	1	1	1	1	1	1
04639-L04020	310	1	1	1	1	1	1	1	1	1	1	1
19233-L25313	400	1	1	1	1	1	1	1	1	1	1	1
15859-L21960	277	1	1	1	1	1	1	1	1	1	1	1
17227-L20554	445	1	1	1	1	1	1	1	1	1	1	1
18102-L20488	472	1	1	1	1	1	1	1	1	1	1	1
17212-L13393	418	1	1	1	1	1	1	1	1	1	1	1
17613-L23601	262	1	1	1	1	1	1	1	1	1	1	1

» This probe detects a sequence that is present in both *HBA1* and *HBA2*. Deletion of a single target site results in a 20-25% decrease in signal intensity of this probe. Although this is a single probe, it is mentioned twice in the Table.

There are many similar -$\alpha^{3.7}$ and -$\alpha^{4.2}$ deletions with different breakpoints. The same holds true for α triplications. To the best of our knowledge, distinguishing the various -$\alpha^{3.7}$ and -$\alpha^{4.2}$ deletions and complementary duplications has no clinical significance. The different deletions and duplications are indicated as type A-F for clarity.. Please note that these types are not part of the official nomenclature.

In example -$\alpha^{3.7\,(E)}$, the deletion breakpoints are likely located within the sequence detected by the 220 nt probe, thereby eliminating two of the four sequence copies detected by this probe. As a result, the final ratio for the 172 and 214 nt probes is 0.75 (4>3 copies), while the 220 nt probe shows a final ratio of 0.5 (4>2 copies).

Table 4B. Expected final ratios for common heterozygous *HBA* deletion types and the Constant Spring (CS) mutation

Probe number	length (nt)	--SEA	--MED1	--MED2 ∞	$(-\alpha)^{20.5}$	--FIL	--THAI	--Dutch1	del HS-40	CS
19236-L25316	463	1	1	1	1	1	1	1	1	1
04799-L04797	178	1	1	1	1	1	1	1	**0.5**	1
04800-L04175	382	1	1	1	1	1	1	1	**0.5**	1
04926-L23886	364	1	1	1	1	1	1	1	1	1
04622-L04001	346	1	1	**0.5**	1	1	1	**0.5**	1	1
17214-L20489	436	1	1	**0.5**	1	**0.5**	**0.5**	**0.5**	1	1
04624-L04004	292	1	1	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
04637-L04018	184	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18097-L22521	391	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18090-L08415	373	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18098-L22522	147	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18092-L22516	328	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18099-L22524	***220*** »	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	*1*	*1*
18881-L06288	***214*** »	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	*1*	*1*
08498-L08422	160	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
04633-L23748	244	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
15857-L21812	***172*** »	***0.5***	***0.5***	***0.5***	***0.75***	***0.5***	***0.5***	***0.5***	*1*	*1*
18096-L22520	190	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18880-L24428	202	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
08494-L08417	256	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
14855-L23604	337	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18093-L22517	226	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	1	1
18099-L22524	***220*** »	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	*1*	*1*
18881-L06288	***214*** »	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	*1*	*1*
08498-L21607	165	**0.5**	**0.5**	**0.5**	1	**0.5**	**0.5**	**0.5**	1	1
04633-L23600	250	**0.5**	**0.5**	**0.5**	1	**0.5**	**0.5**	**0.5**	1	1
15857-L21812	***172*** »	***0.5***	***0.5***	***0.5***	*0.75*	***0.5***	***0.5***	***0.5***	*1*	*1*
08499-L08423	154	**0.5**	**0.5**	**0.5**	1	**0.5**	**0.5**	**0.5**	1	1
04638-L23602	283	**0.5**	**0.5**	**0.5**	1	**0.5**	**0.5**	**0.5**	1	1
04639-L04020	310	**0.5**	**0.5**	1	1	**0.5**	**0.5**	1	1	1
19233-L25313	400	**0.5**	1	1	1	**0.5**	**0.5**	1	1	1
15859-L21960	277	1	1	1	1	1	1	1	1	1
17227-L20554	445	1	1	1	1	1	1	1	1	1
18102-L20488	472	1	1	1	1	1	1	1	1	1
17212-L13393	418	1	1	1	1	1	1	1	1	1
17613-L23601	262	1	1	1	1	1	1	1	1	1
0585-L09493 **CS**	136 ∧	-	-	-	-	-	-	-	-	**>10%**

» This probe detects a sequence that is present in both *HBA1* and *HBA2*. Deletion of a single target site results in a 20-25% decrease in signal intensity of this probe. Although this is a single probe, it is mentioned twice in the table.
∞ The approximate breakpoints of the $-^{Med2}$ deletion have been described by Kutlar et al. (1989).
∧ Coffalyser.Net displays the final ratio of the Hb Constant Spring mutation-specific probe as a percentage. This percentage indicates the height of the probe signal normalized by the average signal of the reference probes. The mutation-specific probe will give a percentage of 0-10% on negative samples and a percentage of >10% on positive samples. The percentage obtained for the mutation-specific probe can vary between samples and does not determine whether the mutation is present in heterozygous or homozygous state.

Interpretation Table 4B is courtesy of dr. C.L. Harteveld and A.C. Schaap, Leiden University Medical Centre, Leiden, The Netherlands. It is intended as an aid only.

Table 5. Selected examples of *HBA* deletions/duplications

Probe number	length (nt)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
19236-L25316	463	1	1	1	1	1	1	1	1	1	1	1	1	1	1
04799-L04797	178	1	1	1	1	1	1	1	1	1	1	1	1	1	1
04800-L04175	382	1	1	1	1	1	1	1	1	1	1	1	1	1	1
04926-L23886	364	1	1	1	1	1	1	1	1	1	1	1	1	1	1
04622-L04001	346	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17214-L20489	436	1	1	1	1	1	1	1	1	1	1	1	1	1	1
04624-L04004	292	1	**0.5**	1	1	1	1	1	1	1	1	1	1	1	1
04637-L04018	184	1	1	1	1	1	1	1	1	1	1	**0.5**	**0.5**	1	1
18097-L22521	391	1	1	1	1	1	1	**0.5**	1	1	1	**0.5**	**0.5**	1	1
18090-L08415	373	1	1	1	**1.5**	1	1	**0**	1	1	1	**0.5**	**0.5**	1	1
18098-L22522	147	1	1	1	1	1	1	**0**	1	1	1	**0.5**	**0.5**	1	1
18092-L22516	328	**1.5**	**1.5**	1	1	1	1	**0.5**	1	1	**0.5**	**0.5**	**0.5**	1	**1.5**
18099-L22524	***220***	***0.75***	***0.75***	***0.75***	***0.75***	*1.25*	***0.5***	***0.5***	***0.25***	***0.25***	***0.5***	***0.25***	***0.75***	***1.5***	***1***
18881-L06288	***214***	***0.75***	***0.75***	***0.75***	***0.75***	*1.25*	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.25***	***0.75***	***1.5***	***1***
08498-L08422	160	**0.5**	**0.5**	**0**	**0**	0.5	**0**	**0**	**0.5**	**1**	**0**	**0**	**1**	**2**	**1**
04633-L23748	244	**0.5**	**0.5**	**0**	**0**	1	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**2**	**1**
15857-L21812	***172***	***0.75***	***0.75***	***0.75***	***0.75***	*1.25*	***0.5***	***0.5***	***0.5***	***0.5***	***0.5***	***0.25***	***0.75***	***1.5***	***1***
18096-L22520	190	**0.5**	**0.5**	**0.5**	**0.5**	**1.5**	**0**	**0.5**	**0**	**0**	**0**	**0**	**1**	**2**	**1**
18880-L24428	202	**0.5**	**0.5**	**0.5**	**0**	**1.5**	**0**	1	**0**	**0**	**0**	**0**	**1**	**2**	**1**
08494-L08417	256	**0.5**	**0.5**	**0.5**	**0.5**	**1.5**	**0**	1	**0**	**0**	**0**	**0**	**1**	**2**	**1**
14855-L23604	337	**0.5**	**0.5**	**0.5**	**0.5**	**1.5**	**0**	1	**0**	**0**	**0**	**0**	**1**	**2**	**1**
18093-L22517	226	**0**	**0**	**0.5**	**0.5**	**1.5**	**0**	**0.5**	**0**	**0**	**0.5**	**0**	**1**	**2**	**0.5**
18099-L22524	***220***	*0.75*	*0.75*	*0.75*	*0.75*	***1.25***	*0.5*	*0.5*	***0.25***	***0.25***	*0.5*	***0.25***	***0.75***	*1.5*	*1*
18881-L06288	***214***	*0.75*	*0.75*	*0.75*	*0.75*	***1.25***	*0.5*	*0.5*	***0.5***	***0.5***	*0.5*	***0.25***	***0.75***	*1.5*	*1*
08498-L21607	165	1	1	**1.5**	**1.5**	**2**	1	1	**0.5**	**0**	1	**0.5**	**0.5**	1	1
04633-L23600	250	1	1	**1.5**	**1.5**	**1.5**	1	1	1	1	1	**0.5**	**0.5**	1	1
15857-L21812	***172***	*0.75*	*0.75*	*0.75*	*0.75*	***1.25***	*0.5*	*0.5*	*0.5*	*0.5*	*0.5*	***0.25***	***0.75***	*1.5*	*1*
08499-L08423	154	1	1	1	1	1	1	1	1	1	1	**0.5**	**0.5**	1	1
04638-L23602	283	1	1	1	1	1	1	1	1	1	1	**0.5**	**0.5**	1	1
04639-L04020	310	1	1	1	1	1	1	1	1	1	1	**0.5**	**0.5**	1	1
19233-L25313	400	1	1	1	1	1	1	1	1	1	1	**0.5**	**0.5**	1	1
15859-L21960	277	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17227-L20554	445	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18102-L20488	472	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17212-L13393	418	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17613-L23601	262	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Deletions are indicated by boxes, polymorphisms are indicated in grey. The results can be explained by the genotypes described below. Please note that other explanations are possible in some cases.

Monoallelic rearrangements + polymorphisms:

1. -$\alpha^{3.7\,(D)}$ + polymorphism 3B (328 nt probe: one extra copy; 226 nt probe: one copy less)
2. -$\alpha^{3.7\,(D)}$ + polymorphism 3B (328 nt probe: one extra copy; 226 nt probe: one copy less) + Asian polymorphism
3. -$\alpha^{3.7\,(D)}$ + African polymorphism 1 (165 & 250 nt probes: one extra copy; 160 & 244 nt probes: one copy less).
4. -$\alpha^{3.7\,(D)}$ + African polymorphism 1 (165 & 250 nt probes: one extra copy; 160 & 244 nt probes: one copy less) + polymorphism 2B (373 nt probe: one extra copy; 202 nt probe: one copy less)
5. $\alpha\alpha\alpha^{\text{anti } 3.7\,(A)}$ + polymorphism 4A (165 nt probe: one extra copy; 160 nt probe: one copy less)

Biallelic rearrangements:

6. -$\alpha^{3.7\,(D)}$ / -$\alpha^{3.7\,(D)}$
7. -$\alpha^{4.2\,(B)}$ / -$\alpha^{4.2\,(C)}$ + polymorphism 3B (328 nt probe: one extra copy; 226 nt probe: one copy less)
8. -$\alpha^{3.7\,(B)}$ / -$\alpha^{3.7\,(E)}$
9. -$\alpha^{3.7\,(B)}$ / -$\alpha^{3.7\,(E)}$ + polymorphism 4B (160 nt probe: one extra copy; 165 nt probe: one copy less)
10. -$\alpha^{3.7\,(D)}$ / -$\alpha^{3.7\,(F)}$
11. -$\alpha^{3.7\,(D)}$ / --^{SEA} deletion
12. $\alpha\alpha\alpha^{\text{anti } 3.7\,(D)}$ / --^{SEA} deletion
13. $\alpha\alpha\alpha^{\text{anti } 3.7\,(D)}$ / $\alpha\alpha\alpha^{\text{anti } 3.7\,(D)}$
14. Theoretical example: this looks like polymorphism 3B (328 nt probe: one extra copy; 226 nt probe: one copy less), but could also be -$\alpha^{3.7\,(D)}$ / $\alpha\alpha\alpha^{\text{anti } 3.7\,(F)}$!

Interpretation aid for Table 5:

Below is an explanation of example 5 in Table 5 describing $\alpha\alpha\alpha^{\text{anti } 3.7\,(A)}$ + polymorphism 4A. This shortened Table shows a selection of the probes. To obtain the final copy number in the patient sample, you need to count the number of copies detected by a particular probe. The final ratio obtained after data analysis is the copy number in the patient sample divided by the copy number in samples of healthy individuals. Additional tables explaining the other examples in Table 5 are available upon request: info@mrcholland.com.

Probe number	length (nt)	Copy number healthy individual	Polym 4A	$\alpha\alpha\alpha^{\text{anti } 3.7(A)}$	Copy number patient	Final ratio
18092-L22516	328	2			2	1
18099-L22524	***220 »***	***4***			***5***	***1.25***
18881-L06288	***214 »***	***4***			***5***	***1.25***
08498-L08422	160	2	-1 copy		1	0.5
04633-L23748	244	2			2	1
15857-L21812	***172 »***	***4***			*5*	***1.25***
18096-L22520	190	2		+1 copy	3	1.5
18880-L24428	202	2		+1 copy	3	1.5
08494-L08417	256	2		+1 copy	3	1.5
14855-L23604	337	2		+1 copy	3	1.5
18093-L22517	226	2		+1 copy	3	1.5
18099-L22524	***220 »***	***4***		*+1 copy*	*5*	***1.25***
18881-L06288	***214 »***	***4***		*+1 copy*	*5*	***1.25***
08498-L21607	165	2	+1 copy	+1 copy	4	2
04633-L23600	250	2		+1 copy	3	1.5
15857-L21812	***172 »***	***4***		*+1 copy*	*5*	***1.25***
08499-L08423	154	2			2	1

» This probe detects a sequence that is present in both *HBA1* and *HBA2*. Although this is a single probe, it is mentioned twice in the table.

Related SALSA MLPA probemixes

P102 HBB Contains probes for the *HBB* gene region

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P140 product history	
Version	*Modification*
C1	Nine probes in the *HBA* region have been removed and 12 new probes have been added. In addition, five new flanking probes centromeric of the *HBA* region have been included and seven reference probes have been replaced.
B4	The 88 and 96 nt DNA denaturation control fragments have been replaced (QDX2).
B3	Small change in the length of one probe.
B2	The 100 and 105 nt X and Y chromosome specific control fragments have been added (QDX1).
B1	Ten probes for the *HBA* region and three reference probes have been added or replaced. In addition, one reference probe was removed.
A1	First release.

Implemented changes in the product description
Version C1-06 – 05 April 2023 (04P) - Figure 1 and its legend was updated and moved. - In section *Interpretation of results*, new table added for interpretation of copy number status the 172, 214 and 220 nt probes. - Information regarding SNP rs370305736 and rs555255920 updated in the remarks under Table 1 and 2, and in P140 specific notes. - In Table 1 and 2, warning for variability added to 214 nt probe. - Minor textual edits, lay-out changes, and updates to Table 1 and 2. - Titles of Table 4A, 4B, and 5 updated. - Selected publications curated. - Morocco removed from the list of countries with IVD status. Version C1-05 – 11 May 2021 (04P) - Product description rewritten and adapted to a new template. - Intended purpose adjusted. - UK has been added to the list of countries in Europe that accept the CE mark. - Updated the NM_ reference sequences to the most recent versions for *HBA1* and *HBA2* in the section 'Gene structure and transcript variants'. - Positive control samples added. - Warning added to Table 1 and 2 for SNPs in the hybridising sequence of the 196 nt (05846-L11214) and 256 nt (08494-L08417) probes. - Warning added to Table 1 and 2 for the occurrence of duplications in the sequence targeted by the 463 nt (19236-L25316) probe. - Added information to the warning on the 136 nt mutation-specific probe (S0585-SP0043-L09493): mutation-negative samples may show a background signal (<10%). Mutation-positive samples will show a clear signal of >10%. - Note added on page 12 regarding Tables 3, 4 and 5: additional CNVs can be detected by the probemix. - Reference added for the --^{Med2} deletion in Table 4B. - New references added and references with publication date of 2016 and earlier removed from the list of selected publications. Version C1-04 — 12 November 2019 (02P) - Product description rewritten and adapted to a new template. - Note on slope correction added. - Warning added to Table 1 and 2 for SNPs in the hybridising sequence of the 220 nt probe (18099-L22524). - Various minor textual or layout changes. - New references added. Version C1-03 – 31 January 2019 (03) - Product is now registered for IVD use in Colombia, Morocco and Israel.

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IVD	EUROPE* CE COLOMBIA ISRAEL
RUO	ALL OTHER COUNTRIES

*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.