Instructions for Use
SALSA® MLPA® Probemix P140 HBA

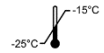
See also the MLPA General Protocol, the product descriptions of the SALSA® MLPA® Reagent Kit, SALSA® Binning DNA SD031, and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P140 HBA product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P140 HBA**
Version	C1
Catalogue numbers	P140-025R (25 reactions) P140-050R (50 reactions) P140-100R (100 reactions)
Basic UDI-DI	872021148P1405M
Ingredients	Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA

Additional Test Components	**Catalogue Numbers**
SALSA® MLPA® Reagent Kit	EK1-FAM EK1-CY5 EK5-FAM EK5-CY5 EK20-FAM
SALSA® Binning DNA SD031	SD031

Storage and Shelf Life

Recommended conditions		

A shelf life of until the expiry date is guaranteed, also after opening when stored in the original packaging under recommended conditions. For the exact expiry date, see the label on the vial. This product should not be exposed to more than 25 freeze-thaw cycles. Do not use the product if the packaging is damaged or opened. Leave chemicals in original containers. Waste material must be disposed of in accordance with the national and local regulations.

Regulatory Status	
IVD	EUROPE CE 2797 COLOMBIA ISRAEL
RUO	ALL OTHER COUNTRIES

Label Symbols			
IVD	In Vitro Diagnostic	RUO	Research Use Only

More Information: www.mrcholland.com	
	MRC Holland BV; Willem Schoutenstraat 1 1057 DL, Amsterdam, the Netherlands
E-mail	info@mrcholland.com (information & technical questions); order@mrcholland.com (orders)
Phone	+31 888 657 200

Any serious incident that has occurred in relation to this product should be reported to MRC Holland and the competent authority of the Member State or country in which the user and/or the patient is located.

Changes in this Product Version

As compared to version B4, 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.

1. Intended Purpose

The SALSA MLPA Probemix P140 HBA is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] for the detection of deletions 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. Detection of duplications of the *HBA* gene cluster and its regulatory region with P140 HBA is relevant only for disease severity assessment when co-inherited with other haemoglobinopathies.

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 on page 1 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 and Coffalyser.Net analysis software.

2. Sample Requirements

Specimen	50-250 ng purified human genomic DNA, free from heparin, **treated with RNase (see Appendix)**, dissolved in 5 µl $TE_{0.1}$ buffer (10 mM Tris-HCl pH 8.0 + 0.1 mM EDTA), pH 8.0-8.5
Collection Method	Standard methods
Extraction Method	Methods tested by MRC Holland: • QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual) • Promega Wizard Genomic DNA Purification Kit (manual) • Salting out (manual)

Sample Types		
Test Sample	• Provided by user	
Reference Samples (Required)	• Provided by user • Extraction method, tissue type, DNA concentration and treatment as similar as possible in all test and reference samples. • Have a normal copy number and ≤0.10 standard deviation for all probes except for the mutation-specific probe. • At least three* independent reference samples required in each experiment for proper data normalisation. Derived from unrelated individuals from families without a history of alpha-thalassaemia	
No-DNA Control (Preferably)	• Provided by user • $TE_{0.1}$ buffer instead of DNA • To check for DNA contamination	
Binning DNA (Initial Experiment)	• SALSA Binning DNA SD031, provided by MRC Holland • Recommended in initial experiment to determine suitable bin set • Should never be used as a reference sample	
Positive Control Samples (Preferably)	• Provided by user, or	
	Available from third parties	See the table of positive samples on the probemix product page on our website
Validation Samples (Required)	• This probemix contains target probes that bind to sequences with natural variation. The validation experiments of this probemix should result in a standard deviation ≤0.10 for all reference probes • In the validation experiments of this probemix, the peak of the mutation-specific probe is expected to be absent in the majority of samples from healthy individuals	

*When testing >21 samples, include one extra reference for each 7 test samples.

3. Test Procedure

See the MLPA General Protocol.

4. Quality Control, Data Analysis, and Troubleshooting

Quality Control Fragments in the Probemix	
Length (nt)	**Function**
64-70-76-82	DNA quantity control fragments
88-96	DNA denaturation control fragments
92	Benchmark fragment
100	Chromosome X presence control fragment
105	Chromosome Y presence control fragment

Coffalyser.Net should be used for data analysis in combination with the appropriate product and lot-specific Coffalyser sheet. See the Coffalyser.Net Reference Manual for details on data analysis and quality control.

For troubleshooting help, see the additional resources offered on our support portal.

5. Interpretation of Results

Determining Typical Values in Normal and Affected Populations

The typical final ratio (FR) values stated in the copy number tables were determined in a validation study with samples containing abnormal copy numbers. The standard deviation of each individual probe over all the reference samples was ≤0.10.

Expected Results of Reference Probes

Final Ratio (FR)	Copy Number	Description
0.80 – 1.20	2	Normal

Typical Results of Probes Targeting Two Copies (*HBA* region)

Final Ratio (FR)	Copy Number	Description
0	0	Homozygous deletion
0.40 – 0.65	1	Heterozygous deletion
0.80 – 1.20	**2**	**Normal**
1.30 – 1.65	3	Heterozygous duplication
1.75 – 2.15	4	Homozygous duplication or Heterozygous triplication
All other values	-	Ambiguous

Typical Results of Probes Targeting Four Copies (combined *HBA1* and *HBA2*[3])

Final Ratio (FR)	Copy Number	Description
0	0	Homozygous deletion of *HBA1* and *HBA2*
~ 0.25	1	Heterozygous deletion of *HBA1* and homozygous deletion of *HBA2* or vice versa
~ 0.50	2	Heterozygous deletion of *HBA1* and *HBA2* **OR** Homozygous deletion of *HBA1* or *HBA2*
~ 0.75	3	Heterozygous deletion of *HBA1* or *HBA2* **OR** Homozygous deletion of *HBA1* or *HBA2* **AND** Heterozygous duplication of *HBA1* or *HBA2*
~ 1	**4**	**Normal**
~ 1.25	5	Heterozygous duplication of *HBA1* or *HBA2* **OR** Homozygous duplication of *HBA1* or *HBA2* **AND** Heterozygous deletion of *HBA1* or *HBA2*
~ 1.50	6	Heterozygous duplication of *HBA1* and *HBA2* **OR** Homozygous duplication of *HBA1* or *HBA2*
~ 1.75	7	Heterozygous duplication of *HBA1* and homozygous duplication of *HBA2* or vice versa
~ 2	8	Homozygous duplication of *HBA1* and *HBA2*

[3] 172, 214 and 220 nt probes detect sequences that are present in both *HBA1* and *HBA2*. See column *Target* in Table named *Content*.

The tables illustrate the relationship between final probe ratio and corresponding copy number. Test results are expected to center around these values. Ambiguous values can indicate a technical problem, but may also reflect a biological cause such as mosaicism or a SNV influencing a single probe. It is important to use Coffalyser.Net to determine the significance of values found.

Possible Results of Mutation Probe

Signal Strength	Mutation Status
≥10% median peak height reference probes	Constant Spring mutation *HBA2*:c.427T>C is detected (expected only in positive samples)
<10% median peak height reference probes	Constant Spring mutation *HBA2*:c.427T>C is not detected (expected in most samples from healthy individuals)

Examples of MLPA results obtained with this probemix can be found on the P140 HBA Product Page on www.mrcholland.com.

6. Performance Characteristics

<table>
<tr><th>Study</th><th>Description</th></tr>
<tr><td>Expected values for copy number in normal and affected populations</td><td>To determine the expected values in normal and affected populations, a study was conducted on over 1500 MLPA reactions using samples with and without abnormal copy numbers. When the standard deviation of each individual probe over all the reference samples is ≤0.10, the ranges stated in the copy number table in the product description can be used. Cut-off values for copy number determination were verified with P140 HBA in 55 samples from healthy individuals with normal copy numbers and 12 samples with known CNVs. The expected FRs for the corresponding copy number were found in 100% (1704/1704) of measurements tested.</td></tr>
<tr><td>Expected values for point mutation detection in normal and affected populations</td><td>The mutation-specific probe will only generate a signal when the Hb Constant Spring mutation (HBA2:c.427T>C) is present. Please note that background signals of the mutation-specific probe can be expected above the threshold in some cases. Users should always compare the relative peak height of the mutation-specific probe in mutation-positive samples to the relative peak height in reference samples. A clear signal (at least 10% of the median peak height of all reference probes in that sample) indicates that the mutation is present. Mutation-specific probes should not be used to determine zygosity. The expected value for mutation-specific probes was verified with P140 HBA using two mutation-positive samples and the expected mutation status was found in both samples tested.</td></tr>
<tr><td>Limit of Detection</td><td>A study using representative probemixes was conducted to evaluate the minimum and maximum amount of DNA acceptable as the assay input. Results support the use of 50-250 ng of human DNA as the recommend input amount. The use of insufficient or too much sample DNA can affect performance. These lower and higher limits of detection were verified using P140 HBA on three samples with known CNVs and mutation status, and on one sample without any mutation. Expected results were obtained in 100% (400/400) of measurements tested using both the lower and upper input amount of DNA.</td></tr>
<tr><td>Interfering substances</td><td>SNVs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl, EDTA, hemoglobin and RNA) can affect the MLPA reaction.

A study using P140 HBA was performed to assess the potential for interference of endogenous and exogenous substances on genomic DNA on samples with known CNVs and mutation status. For most probes, expected FRs (FRs within the expected cut-off category) and mutation status were obtained even in the presence of potential interferents at concentrations shown in the table below.
<table>
<tr><th>Interferent</th><th>Source</th><th>Testing Concentration</th><th>Results*</th></tr>
<tr><td>EDTA</td><td>Exogenous – specimen collection tubes</td><td>1.5 mM</td><td>Copy number: Expected FR for 288/288 measurements
Mutation status: Expected for 12/12 measurements</td></tr>
<tr><td>NaCl</td><td>Exogenous – DNA extraction</td><td>40 mM</td><td>Copy number: Expected FR for 125/288 measurements
Mutation status: Expected for 12/12 measurements</td></tr>
<tr><td>Fe^{3+} ($FeCl_3$)</td><td>Exogenous – DNA extraction</td><td>1 µM</td><td>Copy number: Expected FR for 288/288 measurements
Mutation status: Expected for 12/12 measurements</td></tr>
<tr><td>Heparin</td><td>Exogenous – specimen collection tubes</td><td>0.02 U/mL</td><td>Copy number: Expected FR for 288/288 measurements
Mutation status: Expected for 12/12 measurements</td></tr>
<tr><td>Hemoglobin</td><td>Endogenous – blood sample</td><td>0.02 µg/µl</td><td>Copy number: Expected FR for 62/288 measurements
Mutation status: Expected for 12/12 measurements</td></tr>
<tr><td>RNA</td><td>Endogenous – blood sample</td><td>0.8 ng/µl</td><td>Copy number: Expected FR for 288/288 measurements
Mutation status: Expected for 12/12 measurements</td></tr>
</table>
* Results are summarised for all probes across all three samples tested.

EDTA, $FeCl_3$, heparin and RNA did not interfere with copy number and mutation status determination. Hemoglobin and NaCl had a significant effect on copy number determination in all samples tested, which was consistent between copy number two and copy number four probes. Importantly, Coffalyser.Net issues warnings for the samples in which the interferents showed an effect, as well as lowered quality scores, this also leading to the samples needing a re-test according to the MLPA General Protocol.

To minimise variability across samples, 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.</td></tr>
<tr><td>Cross-reactions</td><td>Cross-reactivity is the potential for probes to bind to homologous regions (e.g. pseudogenes) or other cross-reactive sequences. Quality tests were carried out to determine whether probes are specific to their target sequence and all probes met the quality criteria for specificity.</td></tr>
</table>

Study	Description
Accuracy	Results of accuracy are derived from trueness and precision studies. For trueness, thirteen previously genotyped samples were tested using P140 HBA and found to have the expected results in 100% (425/425) of measurements tested. Assay precision was tested by repeatedly testing samples with known copy number and mutation status over multiple days, and by multiple operators. Results showed a correct call in 1500/1500 data points, leading to a precision of 100%.
Clinical validity*	Approximately 85% of alpha-thalassaemia is explained by deletions in the *HBA* gene involving part or the complete gene cluster, while the remaining ~15% is explained by SNVs, including the Hb Constant Spring mutation (*HBA2*:c.427T>C), and <1% is explained by deletions in the *HBA* regulatory regions (Gene Reviews). *Based on a 2005-2026 literature review

Summary of Safety and Performance (SSP)
The SSP is available in the European database on medical devices (Eudamed), https://ec.europa.eu/tools/eudamed, or upon request.

Content – Probe Details Sorted by Chromosomal Position

Chr. position	Target	Exon	Distance to next probe	Mutation	Length (nt)	Probe number	Warnings
16p13.3	*POLR3K*		66.4 kb		463	19236-L25316	¬ £
16p13.3	*HBA* region	HS-40	0.2 kb		178	04799-L04797	
16p13.3	*HBA* region	HS-40	30.0 kb		382	04800-L04175	
16p13.3	*HBZ* region	Upstream (9.0 kb before *HBZ*)	5.7 kb		364	04926-L23886	Ø «
16p13.3	*HBZ* region	Upstream (3.3 kb before *HBZ*)	3.3 kb		346	04622-L04001	Ø «
16p13.3	*HBZ*	Exon 1	6.9 kb		436	17214-SP0457-L20489	Ж «
16p13.3	*HBZP1* region	Between *HBZ* & *HBZP1*	7.8 kb		292	04624-L04004	Ø ~ «
16p13.3	*HBM region*	Downstream *HBM*	2.5 kb		184	04637-L04018	Ø «
16p13.3	*HBA2* region	Upstream (3.0 kb before *HBA2*)	0.5 kb		391	18097-L22521	Ø # «
16p13.3	*HBA2* region	Upstream (2.5 kb before *HBA2*)	1.6 kb		373	18090-L08415	Ø # «
16p13.3	*HBA2* region	Upstream (0.9 kb before *HBA2*)	0.2 kb		147	18098-L22522	Ø «
16p13.3	*HBA2* region	Upstream (0.6 kb before *HBA2*)	0.6 kb		328	18092-L22516	Ø # «
16p13.3	*HBA1*& *HBA2*	Exon 1 *HBA2*	0.1 kb		220	18099-L22524	» ± «
16p13.3	*HBA1*& *HBA2*	Exon 1 *HBA2*	0.4 kb		214	18881-L06288	» Δ «
16p13.3	*HBA2*	Intron 2	0.1 kb		160	08498-L08422	Ø # «
16p13.3	*HBA2*	Intron 2	0.1 kb		244	04633-L23748	Ø # «
16p13.3	*HBA1*& *HBA2*	Exon 3 *HBA2*	0.1 kb		172	15857-L21812	» «
16p13.3	*HBA2* CS	Exon 3	0.5 kb	Hb Constant Spring mutation	136	S0585-SP0043-L09493	§ Ж «
16p13.3	*HBA1* region	(0.4 kb after *HBA2* and 2.5 kb before *HBA1*)	0.5 kb		190	18096-L22520	Ø # «
16p13.3	*HBA1* region	(0.9 kb after *HBA2* and 2.0 kb before *HBA1*)	0.6 kb		202	18880-L24428	Ø # «
16p13.3	*HBA1* region	Upstream (1.5 kb before *HBA1*)	0.6 kb		256	08494-L08417	± Ø «
16p13.3	*HBA1* region	Upstream (0.9 kb before *HBA1*)	0.3 kb		337	14855-L23604	Ø «
16p13.3	*HBA1* region	Upstream (0.6 kb before *HBA1*)	0.6 kb		226	18093-L22517	Ø # «
16p13.3	*HBA1*& *HBA2*	Exon 1 *HBA1*	0.1 kb		220	18099-L22524	» ± «
16p13.3	*HBA1*& *HBA2*	Exon 1 *HBA1*	0.4 kb		214	18881-L06288	» Δ «
16p13.3	*HBA1*	Intron 2	0.1 kb		165	08498-L21607	Ø # «
16p13.3	*HBA1*	Intron 2	0.1 kb		250	04633-L23600	Ø # «
16p13.3	*HBA1*& *HBA2*	Exon 3 *HBA1*	0.3 kb		172	15857-L21812	» «
16p13.3	*HBA1*	Downstream (0.1 kb after *HBA1*)	0.3 kb		154	08499-L23594	Ø «
16p13.3	*HBA1* region	Downstream (0.4 kb after *HBA1*)	1.9 kb		283	04638-L23602	Ø «
16p13.3	*HBA1* region	Downstream (2.3 kb after *HBA1*)	1.4 kb		310	04639-L04020	Ø «
16p13.3	*HBQ1*	Exon 3	25.1 kb		400	19233-L25313	«
16p13.3	*LUC7L*		33.5 kb		277	15859-L21960	¬
16p13.3	*ITFG3*		31.9 kb		445	17227-L20554	¬
16p13.3	*RGS11*		16.3 kb		472	18102-L20488	¬ «
16p13.3	*AXIN1*		119.4 kb		418	17212-L13393	¬ «
16p13.3	*DECR2*				262	17613-L23601	¬
1q	Reference				238	11435-L12163	
2q	Reference				481	15318-L17117	
3q	Reference				409	03272-L02709	
5p	Reference				269	03075-L19996	
5q	Reference				130	00797-L13645	
8p	Reference				142	07641-L07326	
10q	Reference				196	05846-L11214	
11q	Reference				355	00547-L00116	
12p	Reference				208	11331-L12056	
13q	Reference				300	03250-L02687	
15q	Reference				454	07607-L07292	

Probe lengths may vary slightly depending on capillary electrophoresis instrument settings. Please see the most up to date Coffalyser sheet for exact probe lengths obtained at MRC Holland.

The exon numbers of the alpha-globin (*HBA*) gene cluster are derived from MANE project and are based on MANE Select transcript. For more information, see the probe sequences document available on the product page at www.mrcholland.com.
Chromosomal bands are based on: hg18.

7. Precautions and Warnings

Probe warnings

§	This probe will only generate a signal when the Hb Constant Spring mutation is present (*HBA2*:c.427T>C, p.*143Glnext*31).
¬	These probes are flanking probes, included to help determine the extent of a deletion/duplication. Copy number alterations of flanking probes are unlikely to be related to the condition tested.
±	In addition to the single nucleotide variants (SNVs) present in Table 1 of the interpretation-aid document, available on the Product Page, the presence of SNV rs370305736 and rs555255920 (both frequently found in African populations) can affect the signal of the 220 nt and 256 nt probes respectively.
Δ	This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
«	These probes are located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation.
Ж	These probes consist of three parts and have two ligation sites. A low signal of these probes can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.
Ø	These probes target sequences outside of the known coding region. Copy number alterations of only one of these probes are of unknown clinical significance.
»	These probes detect sequences that are present in both *HBA1* and *HBA2*. Deletion of a single target site results in a 20-25% decrease in the signal intensity of this probe.
~	This probe has been reported to be deleted/duplicated in several samples from Southeast Asia. This is probably a benign polymorphism referred to as the 'Asian polymorphism' (see Table 1 in the interpretation-aid document available on the Product Page, with final ratios for a selected subset of frequent polymorphisms). The exact deletion boundaries are currently unknown, but seem to cover at least position 20361-23609 of the Genbank NG_000006.1 reference sequence (www.ncbi.nlm.nih.gov/nuccore/NG_000006.1).
£	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.
#	In the sequence detected by these probes, 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.

Probemix-specific precautions

1. This product is not known to contain any harmful agents. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. **A Safety Data Sheet (SDS) is not required for this product**: none of the ingredients contain dangerous substances at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments).
2. Sample or technical artefacts may appear as a (mosaic) copy number change of the whole/partial gene. Whole/partial gene deletions or duplications should therefore be confirmed by analysis of an independent DNA sample, to exclude false positive results.
3. Small changes (e.g. SNVs, small indels) in the sequence targeted by a probe can cause false positive results, even when >20 nt from the probe ligation site. Sequence changes 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. Deviations detected by this product should be confirmed, and single-probe deviations always require confirmation. Sequencing of the target region is recommended. Please contact MRC Holland for more information: info@mrcholland.com. Please note that any probe can be affected by known or novel SNVs. See Table 1 in the interpretation-aid document available on the Product Page (www.mrcholland.com), for final ratios for a selected subset of frequent polymorphisms. Furthermore, SNVs rs370305736 and rs555255920 mentioned in this document require additional caution.
4. Copy number alterations of reference probes are unlikely to be related to the condition tested.
5. Large deletions are often detected with this probemix. The slope correction algorithm in Coffalyser.Net may confuse a large deletion for sloping, leading to an incorrectly applied slope correction and a false warning or ambiguous results for multiple probes. Contact MRC Holland if you suspect that this happened.
6. 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 leads to an α^{0}-thalassaemia phenotype, even though the *HBA* genes are present and intact. Several studies indicate that HS40 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 HS40 does not lead to the lethal Hb Bart's hydrops fetalis syndrome (Sollaino et al. 2010).
7. 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 regions.
8. Not all deletions in the alpha-globin gene cluster can be discriminated from each other using this probemix:
   - 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 can be a combination of two different $-\alpha^{3.7}$ deletions. To the best of our knowledge, distinguishing the various $-\alpha^{3.7}$ or the various $-\alpha^{4.2}$ deletions has no clinical significance. The final ratios for different deletions and duplications can be found in Table 2 in the interpretation-aid document available on the Product Page (www.mrcholland.com).
   - The $--^{FIL}$ and $--^{THAI}$ deletions will display identical final ratios. The same accounts for the $--^{MED2}$ and $--^{Dutch1}$ deletions (final ratios for different deletions and duplications can be found in Table 3 in the interpretation-aid document available on the Product Page (www.mrcholland.com.)
9. Presence of three *HBA* genes on one allele (α-triplication) is relatively common. The α-triplication can be detected by P140 HBA, but should be considered as a polymorphism as it is not associated with an alpha-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).

10. 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 ~0.50), while the other has a ~50% increased probe signal (ratio ~1.50), 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 (see Table 1 in the interpretation-aid document available on the Product Page, with final ratios for a selected subset of frequent polymorphisms).
11. 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), (see Table 1 in the interpretation-aid document available on the HBA Product Page, with final ratios for a selected subset of frequent polymorphisms).
12. A combination of polymorphism 3B, polymorphism 4B (see Table 1 in the interpretation-aid document available on the Product Page, with final ratios for a selected subset of frequent polymorphisms) and SNV rs555255920 affecting the 256 nt probe has frequently been reported by users of P140 HBA.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Probemix-specific limitations

1. The mutation-specific probe can only detect the presence of the mutation and should not be used to determine zygosity.
2. 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.
3. Very rarely, two different types of deletions are detected by P140 HBA 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.

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. Camaschella C et al. (1997). Different haematological phenotypes caused by the interaction of triplicated alpha-globin genes and heterozygous beta-thalassemia. Am J Hematol. 55(2):83-8.
2. Goossens M et al. (1980). Triplicated alpha-globin loci in humans. Proc Natl Acad Sci USA. 77:518-21.
3. Harteveld CL et al. (2008). Segmental duplications involving the alpha-globin gene cluster are causing betathalassemia intermedia phenotypes in beta-thalassemia heterozygous patients. Blood Cells Mol Dis. 40(3):312-6.
4. Sollaino MC et al. (2010). Homozygous deletion of the major alpha-globin regulatory element (MCS-R2) responsible for a severe case of haemoglobin H disease. Blood. 116(12):2193-4.
5. Viprakasit V et al. (2006). A novel deletion causing alpha thalassemia clarifies the importance of the major human alpha globin regulatory element. Blood. 107(9):3811-2.
6. Zhang HB et al. (2002). The control of expression of the alpha-globin gene cluster. Int J Hematol. 76(5):420-6.

Implemented changes in the product description

Version C1-09 – 15 July 2026 (03S)

- Intended purpose was updated specifying the assay is manual. Duplications of *HBA* region are only relevant for disease severity assessment when co-inherited with other haemoglobinopathies.
- TE concentration to dissolve RNase added to Sample Requirements.
- In the table 'Typical Results of Probes Targeting Four Copies', the descriptions of FR ~ 0.75 and FR ~ 1.25 were extended.
- Performance characteristics table added.
- Targets renamed for probes 04624-L04004, 04637-L04018, 18096-L22520 and 18880-L24428 to align with Coffalyser.Net.
- Exon renamed for probes 04624-L04004 and 04637-L04018.
- Probe warnings for RNase sensitivity removed for probes 18099-L22524, 18881-L06288, 15857-L21812 and S0585-SP0043-L09493.
- Corrected 'RNAse' with 'RNase' throughout the document.
- Appendix: specified RNase recommendations based on internal analytical performance testing.
- Appendix: added a sentence specifying SD031 does not require RNase treatment.
- Minor textual changes.
- Probemix is now IVDR certified.

10. Appendix

RNase sample treatment (recommended for P140 HBA and essential for P102 HBB probemixes)

Since *HBA* is heavily expressed in red blood cells, an RNase treatment of samples is recommended 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, probes may bind to HBA mRNA, thereby reducing the effective concentration of probes. Please note that some automatic DNA purification methods (e.g. Roche MagNA Pure) do not include an RNase treatment.

Although analytical performance testing for P140 HBA showed no effect of RNA (0.8 ng/µl) on probe ratios or mutation signal in the samples tested, these results were obtained using a limited number of samples and a single RNA concentration and therefore cannot be generalised. As RNA content may vary substantially between blood-derived samples, RNase treatment remains recommended to ensure reliable assay performance. This recommendation is supported by observations with the SALSA MLPA Probemix P102 HBB, for which RNA interference can affect probe signals when samples are not treated with RNase.

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.

When using between 50-250 ng of DNA, the DNA concentration after the addition of RNase A should be 10-50 ng/µl.

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.

**Please note that RNase treatment is not needed for SALSA Binning DNA SD031.*