Instructions for Use
SALSA® MLPA® Probemix P055 PAH

See also the MLPA General Protocol, the product description of the SALSA® MLPA® Reagent Kit and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P055 PAH product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P055 PAH**
Version	**D1**
Catalogue numbers	**P055-025R (25 reactions)** **P055-050R (50 reactions)** **P055-100R (100 reactions)**
Basic UDI-DI:	**872021148P0555V**
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

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 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 in which the user and/or the patient is located.

Changes in this Product Version

One PAH exon 6 probe added, one PAH exon 12 probe (wild-type sequence at R408W mutation) removed. One reference probe replaced.

1. Intended Purpose

The SALSA MLPA Probemix P055 PAH is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] for the postnatal detection of deletions and duplications in the *PAH* gene in genomic DNA obtained from peripheral whole blood specimens.

P055 PAH is intended to confirm a potential cause for the recessive disorder phenylketonuria (PKU) in patients referred for molecular genetic testing based on clinical presentation. Most defects in the *PAH* gene are point mutations, none of which will be detected by P055 PAH. Therefore it is recommended to use P055 PAH when sequencing analysis did not reveal any defect or when sequencing revealed defect(s) on only one allele of the *PAH* gene. This product can also be used for carrier testing of at-risk family members.

Copy number variations (CNVs) detected with P055 PAH should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. 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, 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, pre-implantation or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1]Please note that this probemix is for IVD use in the countries specified on page 1 of this product description. In all other countries, this is a RUO product.

[2]To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

2. Sample Requirements

Specimen	50-200 ng purified human genomic DNA, free from heparin, dissolved in 5 µl $TE_{0.1}$ buffer, 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. • At least three* independent reference samples required in each experiment for proper data normalisation. Derived from unrelated individuals from families without a history of phenylketonuria.
No-DNA control (preferably)	• Provided by user • $TE_{0.1}$ buffer instead of DNA • To check for DNA contamination
Positive control samples (preferably)	• Provided by user

*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 (*PAH* gene)

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

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.

6. Performance Characteristics

Study	Description
Expected values for copy numbers in normal and affected populations	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 can be used. Cut-off values were verified with SALSA MLPA Probemix P055 PAH. The results of 46 blood derived DNA samples from healthy (mainly) European individuals and three positive blood derived samples, showed that when the correct reference samples are used and the standard deviation of the reference probes is ≤0.10, the proposed cut-off values yielded the expected results and could safely be used to determine the FRs. This was also found using artificial positives samples for all possible target aberrations.
Limit of detection	SALSA MLPA Probemix P055 PAH was tested in house with 50 ng DNA and yielded the expected results. In literature, DNA concentrations of up to 200 ng were used with SALSA MLPA Probemix P055 PAH, and no issues were reported. This supports the use of 50-200 ng of human DNA as the recommend input amount. The use of insufficient or too much sample DNA can affect performance.
Interfering substances	SNVs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl (>40 mM), EDTA and hemoglobin) can affect the MLPA reaction. A study was performed with SALSA MLPA Probemix P055 PAH to assess the potential for interference of endogenous and exogenous substances on genomic DNA derived from blood, shown in the table below. Certain interferents lead to deviating probe results at the concentrations tested.

<table>
<tr><td></td><td>
<table>
<tr><th>Interferent</th><th>Potential source</th><th>Concentration tested</th></tr>
<tr><td>EDTA</td><td>Exogenous – specimen collection tubes</td><td>1.5 mM</td></tr>
<tr><td>NaCl</td><td>Exogenous – DNA extraction</td><td>40 mM</td></tr>
<tr><td>Fe^{3+}</td><td>Exogenous – DNA extraction</td><td>1 µM</td></tr>
<tr><td>Heparin</td><td>Exogenous – specimen collection tubes</td><td>0.02 U/mL</td></tr>
<tr><td>Hemoglobin</td><td>Endogenous – blood sample</td><td>0.02 µg/µL</td></tr>
<tr><td>Bilirubin</td><td>Endogenous – blood sample of newborn</td><td>140 mg/L</td></tr>
<tr><td>Blank (TE)</td><td>n.a.</td><td>10mM Tris, 0.1 mM EDTA</td></tr>
</table>
Interfering substances NaCl and hemoglobin showed more variability in FRs of target and reference probes. The addition of bilirubin, Fe3+ or heparin resulted in several deviating probe results. However none of these interferents had an effect leading to a potential false result.
All samples spiked with EDTA showed major effects on the MLPA results. The quality of these reactions was not found to be sufficient for comparative analysis in Coffalyser.Net. Therefore, these reactions could not be reliably interpreted.
Overall, although several interferents caused more variability in the MLPA results, the concentrations of the interfering substances tested were higher than is expected to occur in MLPA experiments and the results would show multiple warnings, meaning they cannot be interpreted, when the IFU is followed.
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-reactivity</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. All probes in this probemix were found to be specific in hg18/hg19 and the latest hs1 database.

Samples from healthy individuals and three samples from PKU patients with various genotypes were tested with SALSA MLPA Probemix P055 PAH. As all genotypes were found to match the correct final ratio for their relevant copy number status indicating that the probes are specific for their target location, SALSA MLPA Probemix P055 PAH has an analytical specificity of >99%, when free from substances known to affect MLPA reactions.</td></tr>
<tr><td>Accuracy</td><td>Results of accuracy are derived from trueness and precision studies. For trueness, previously genotyped samples were tested and found to have the expected results. For precision studies, results are not affected by operator, probemix lot, day, or laboratory site.

To demonstrate precision, a study was performed including experiments by an external lab and in-house experiments. Irrespective of probemix lot, day, or laboratory site, the correct genotypes for all samples were determined, when using SALSA MLPA Probemix P055-D1 PAH, in combination with the SALSA MLPA Reagent Kit.

Overall the expected FRs of PAH probes were found in 1155/1190 total probe results, resulting in a correct call of >97%. Therefore, MLPA, as a semi-quantitative/categorical multiplex assay, is able to repeatably and reproducibly provide the correct result.</td></tr>
<tr><td>Clinical validity*</td><td>Deletions or duplications in the PAH gene are the genetic cause of PKU in 1-5% of the cases. However, this percentage varies strongly between populations.

*Based on a 2006-2023 literature review</td></tr>
</table>

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	Length (nt)	Probe number	Warnings
12q23.2	*IGF1*		363.4 kb	310	02340-L01834	¬
12q23.2	*PAH*	Exon 13	1.3 kb	292	02339-L01829	
12q23.2	*PAH*	Exon 12	3.3 kb	265	02337-L02469	
12q23.2	*PAH*	Exon 11	0.6 kb	242	02335-L14055	
12q23.2	*PAH*	Exon 10	2.5 kb	211	02333-L01826	
12q23.2	*PAH*	Exon 9	4.8 kb	187	02331-L23231	
12q23.2	*PAH*	Exon 8	1.1 kb	161	02328-L11413	
12q23.2	*PAH*	Exon 7	0.1 kb	352	12256-L14058	+
12q23.2	*PAH*	Exon 7	2.4 kb	142	02326-L01823	
12q23.2	*PAH*	Exon 6	0.1 kb	227	17737-L21083	
12q23.2	*PAH*	Intron 5 (Exon 6)	11.2 kb	283	16491-L18947	Ø
12q23.2	*PAH*	Exon 5	0.2 kb	256	02336-L01821	
12q23.2	*PAH*	Intron 4 (Exon 5)	10.7 kb	346	16492-L18948	Ø
12q23.2	*PAH*	Exon 4	0.1 kb	400	12260-L14061	
12q23.2	*PAH*	Exon 4	17.2 kb	235	02334-L23232	
12q23.2	*PAH*	Exon 3	0.4 kb	201	16489-L18945	+
12q23.2	*PAH*	Intron 2 (Exon 3)	17.6 kb	365	16493-SP0373-L18949	Ж Ø
12q23.2	*PAH*	Exon 2	0.4 kb	180	16488-L23230	+
12q23.2	*PAH*	Intron 1 (Exon 2)	4.1 kb	373	16494-L18950	Ø
12q23.2	*PAH*	Exon 1	0.4 kb	168	16487-L23233	
12q23.2	*PAH*	Upstream (Exon 1)	1.0 kb	337	12254-L14056	Ø
12q23.2	*PAH*	Upstream	2.0 kb	154	12251-L14053	Ø
12q23.2	*PAH*	Upstream	38.9 kb	418	12261-L13203	Ø
12q23.2	*ASCL1*			149	02327-L01835	¬
1p	Reference			274	15473-L17313	
3p	Reference			319	06440-L05966	
3q	Reference			194	05703-L06959	
5q	Reference			128	00797-L00093	
6q	Reference			136	07292-L06929	
7q	Reference			382	13974-L15543	
8q	Reference			409	10063-L10487	
13q	Reference			220	01782-L01346	
14q	Reference			427	05915-L17921	
15q	Reference			359	13731-SP0136-L15212	Ж
16p	Reference			174	01823-L23229	
18q	Reference			391	12522-L13572	
21q	Reference			247	07695-L07419	
22q	Reference			300	01575-L01147	

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 *PAH* exon numbers 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. Annotations of several probes with targets at the edge of or slightly outside the coding region, were altered. The exon numbering from the previous version of this product description is disclosed between brackets.
Chromosomal bands are based on: hg18

7. Precautions and Warnings

Probe warnings

¬	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.
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 a sequence outside of the known coding region. Copy number alterations of only these probes are of unknown clinical significance.
+	The ligation sites of these probes are >20 nt away from the nearest exon. For more information, download the probe sequences document available on the product page at www.mrcholland.com.

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.
4. Copy number changes of reference probes are unlikely to be related to the condition tested.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Probemix-specific limitations
1. The significance of deletions or duplications in the *PAH* upstream region is not clear. DNaseI hypersensitive sites regulatory elements were previously described [1].

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. Bristeau A et al. (2001). Conserved as well as divergent regulatory elements account for expression of the human and rodent phenylalanine hydroxylase genes. Gene. 274:283-291.

Implemented changes in the product description

Version D1-07 – 7 January 2025 (03S)
- Updated to new template.
- Intended purpose updated, specifying assay is manual and the function of the device is clarified. Device no longer intended to be used on buccal swab or saliva samples.
- Description of probe targets at the edge of or slightly outside the coding region has been adjusted. No change in actual target sites.
- Warning has been removed for incomplete denaturation of the 149 nt probe.
- SNVs rs192439649 and rs542737289 can affect the probe signal. However, the warnings for these SNVs present in previous product description versions have been replaced by a general warning for small sequence changes, included in section Precautions and Warnings.
- This probemix is now IVDR certified.