Product Description SALSA® MLPA® Probemix P198-A4 FH

To be used with the MLPA General Protocol.

Version A4. As compared to version A3, two reference probes have been replaced. For complete product history see page 5.

Catalogue numbers:
- **P198-025R:** SALSA MLPA Probemix P198 FH, 25 reactions.
- **P198-050R:** SALSA MLPA Probemix P198 FH, 50 reactions.
- **P198-100R:** SALSA MLPA Probemix P198 FH, 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.mlpa.com).

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

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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.

General information: The SALSA MLPA Probemix P198 FH is a **research use only (RUO)** assay for the detection of deletions or duplications in the fumarate hydratase (*FH*) gene, which is associated with *FH* tumour predisposition syndrome and FH deficiency.

FH tumour predisposition syndrome (also known as hereditary leiomyomatosis and renal cell cancer, HLRCC) is an autosomal dominant tumour syndrome characterized by leiomyomas in the skin and uterus. A subset of patients develops renal cell cancer (Smit et al. 2011). In the exceptional case of autosomal recessive inheritance, homozygous *FH* mutations result in FH deficiency (also known as fumarase deficiency or fumaric aciduria), which causes neonatal and early infantile encephalopathy. FH deficiency is characterized by poor feeding, failure to thrive, hypotonia, lethargy and seizures. FH deficiency is often fatal in early childhood.

The *FH* gene (10 exons) spans ~22 kb of genomic DNA and is located on chromosome 1q43, about 240 Mb from the p-telomere. The protein encoded by *FH* is an enzymatic component of the Krebs cycle. It catalyzes the conversion of fumarate to malate.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1252/ (*FH* tumour predisposition syndrome) and https://www.ncbi.nlm.nih.gov/books/NBK1506/ (FH deficiency).

This SALSA MLPA Probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Gene structure and transcript variants:
Entrez Gene shows transcript variants of each gene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering: The *FH* exon numbering used in this P198-A4 FH product description is the exon numbering from the LRG_504 sequence. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. The exon numbering and NM_ sequence used have been retrieved on 10/2020. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P198-A4 FH contains 19 MLPA probes with amplification products between 142 and 300 nucleotides (nt). This includes 10 probes for the *FH* gene, one probe for each exon. In addition, nine 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.mlpa.com).

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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment 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.mlpa.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, free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

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 unrelated individuals who are from families without a history of *FH* tumour predisposition syndrome or FH deficiency. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample in your MLPA experiments. Sample ID numbers NA03648 (heterozygous duplication), NA05347 (heterozygous duplication), NA06473 (heterozygous deletion) and NA10020 (heterozygous deletion) from the Coriell Institute have been tested with this P198-A4 probemix at MRC-Holland and can be used as positive control samples to detect whole gene deletions and duplications of the *FH* gene. The quality of cell lines can change; therefore samples should be validated before use.

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.mlpa.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.

Interpretation of results: The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the dosage quotient (DQ) 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 DQ of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

- 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. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. 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.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. 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: lower injection voltage / injection time settings, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure:

- In most populations, the major cause of genetic defects in the *FH* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P198 FH.
- 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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.

***FH* mutation database:** https://databases.lovd.nl/shared/genes/FH. We strongly encourage users to deposit positive results in 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 SNPs and unusual results (e.g., a duplication of *FH* exons 4 and 6 but not exon 5) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P198-A4 FH

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	FH
64-105	Control fragments – see table in probemix content section for more information		
142	Reference probe 10699-L11281	6p12	
152	**FH probe** 06640-L06208		**Exon 1**
160 *	Reference probe 22518-L31981	14q32	
166	**FH probe** 06646-L06214		**Exon 7**
172	Reference probe 05037-L04423	9p13	
178	**FH probe** 06642-L06210		**Exon 3**
184	**FH probe** 06649-L07122		**Exon 10**
191	Reference probe 06057-L06042	4p16	
202	**FH probe** 19700-L26453		**Exon 9**
214	Reference probe 03259-L07116	3q29	
220	**FH probe** 06645-L06213		**Exon 6**
229	Reference probe 01828-L01393	16p13	
238 #	**FH probe** 06647-L06215		**Exon 8**
247	**FH probe** 06641-L06209		**Exon 2**
256	**FH probe** 06643-L06211		**Exon 4**
265 *	Reference probe 19015-L25096	21q21	
283	Reference probe 08214-L08147	2q14	
291	**FH probe** 06644-L06212		**Exon 5**
300	Reference probe 03250-L02687	13q14	

* New in version A4.
# This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

a) See above section on exon numbering for more information.

Table 2. *FH* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	FH exon[a]	Ligation site NM_000143.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*34-36 (Exon 1)*		
152	06640-L06208	Exon 1	34-35	CCCTCAGCACCA-TGTACCGAGCAC	2.5 kb
247	06641-L06209	Exon 2	270-271	ACGATGAACTTT-AAGATTGGAGGT	3.6 kb
178	06642-L06210	Exon 3	29 nt after exon 3 *reverse*	GGGTCTGAGGTT-ATTAAGCAAACA	1.6 kb
256	06643-L06211	Exon 4	555-556	AGCAAGATACCT-GTGCATCCCAAC	3.3 kb
291	06644-L06212	Exon 5	687-688	CATGATGCTCTT-GATGCAAAATCC	2.6 kb
220	06645-L06213	Exon 6	889-890	GTTTAAATACTA-GAATTGGCTTTG	2.0 kb
166	06646-L06214	Exon 7	41 nt after exon 7 *reverse*	TGCCTAGGACCT-AGTCAAGTTTTA	1.5 kb
238 #	06647-L06215	Exon 8	1215-1214 *reverse*	CCTCCGACAGTG-ACAGCAACATGG	2.4 kb
202	19700-L26453	Exon 9	293 nt after exon 9	TATTCTATGCAG-ACCATTGGGGAA	2.4 kb
184	06649-L07122	Exon 10	1671-1670 *reverse*	GCTAGAGATGCT-TAAGTTCAATAG	
		stop codon	*1564-1566 (Exon 10)*		

This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Smit DL et al. (2011). Hereditary leiomyomatosis and renal cell cancer in families referred for fumarate hydratase germline mutation analysis. *Clin Genet*. 79:49-59.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

Selected publications using SALSA MLPA Probemix P198 FH

- Ahvenainen T et al. (2008). Mutation screening of fumarate hydratase by multiplex ligation-dependent probe amplification: detection of exonic deletion in a patient with leiomyomatosis and renal cell cancer. *Cancer Genet Cytogenet*. 183:83-88.
- Cardot Bauters C et al. (2020). Genetic predisposition to neural crest-derived tumors: revisiting the role of KIF1B. *Endocr Connect*. 9:1042-1050.
- Castro-Vega LJ et al. (2014). Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas. *Hum Mol Genet*. 23:2440-2446.
- Smit DL et al. (2011). Hereditary leiomyomatosis and renal cell cancer in families referred for fumarate hydratase germline mutation analysis. *Clin Genet*. 79:49-59.

P198 Product history	
Version	*Modification*
A4	Two reference probes have been replaced.
A3	Two reference probes have been replaced and the control fragments have been adjusted.
A2	Three reference probes have been replaced and four new control fragments have been included.
A1	First release.

Implemented changes in the product description
Version A4-01 — 11 November 2020 (02P) - Product description rewritten and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Ligation sites of the probes targeting the *FH* gene updated according to new version of the NM_ reference sequence. - Warning added to Table 1 and Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. *Version 11 – 21 November 2017 (55)* - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).

More information: www.mlpa.com; www.mlpa.eu	
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