Product Description SALSA® MLPA® Probemix P256-B4 FLCN

To be used with the MLPA General Protocol.

Version B4. As compared to version B3, three reference probes have been replaced. For complete product history see page 6.

Catalogue numbers:
- **P256-025R:** SALSA MLPA Probemix P256 FLCN, 25 reactions.
- **P256-050R:** SALSA MLPA Probemix P256 FLCN, 50 reactions.
- **P256-100R:** SALSA MLPA Probemix P256 FLCN, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, available for various number of reactions. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman 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 P256 FLCN is a **research use only (RUO)** assay for the detection of deletions or duplications in the *FLCN* gene, which is associated with Birt-Hogg-Dube syndrome (BHD). This probemix can also be used to detect the presence of 1285delC and 1285dupC point mutations.

BHD is a rare inherited genodermatosis, characterised by hair follicle hamartomas, kidney tumours, and spontaneous pneumothorax. In a screen of nine BHD families and an additional 53 probands from small BHD families, 27 (44%) were found to have insertion or deletion mutations within a hypermutable C8 tract in exon 11 of the *FLCN* gene, located within the Smith-Magenis syndrome region on chromosome 17 (Nickerson et al. 2002, *Cancer Cell*, 2002, 2:157-64). Eighteen had a 1-bp insertion and nine had a 1-bp deletion in the C8 tract. A slippage-mediated mechanism during DNA replication is thought to be responsible for these frameshift mutations leading to protein truncation.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1522/.

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/

Probemix content: The SALSA MLPA Probemix P256-B4 FLCN contains 26 MLPA probes with amplification products between 127 and 372 nt. This includes one probe for each of the 14 exons of the *FLCN* gene and one probe downstream of the *FLCN* gene. Furthermore, it also contains two probes specific for the 1-bp deletion at 1733C (1285delC, 187 nt probe) and for the 1-bp duplication at 1733C (1285dupC, 194 nt probe) mutations which will only generate a signal when the mutations are present. In addition, nine reference probes are included and detect nine different autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes is 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, 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 or 96 fragment indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

No DNA controls results in only five major peaks shorter than 105 nucleotides: four Q-fragments at 64, 70, 76 and 82 nt, and one 19 nt peak corresponding to the unused portion of the fluorescent PCR primer. Non-specific peaks longer than 105 nt AND with a height >25% of the median of the four Q-fragments should not be observed. Note: peaks below this 25% threshold are not expected to affect MLPA reactions when sufficient amount of sample DNA (50-200 ng) is used.

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

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: 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 Birt-Hogg-Dube syndrome. 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 Biobank (https://catalog.coriell.org) and 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. The quality of cell lines can change, therefore samples should be validated before use.

SALSA Binning DNA SD032: The SD032 Binning DNA provided with this probemix can be used as Binning DNA sample for binning of two mutation-specific probes (187 nt probe 08598-L08600; 1285delC mutation and 194 nt probe 08598-L08601; 1285dupC mutation). SD032 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequences detected by the above mentioned probes. Inclusion of one reaction with 5 µl SD032 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 signals, as for this purpose true mutation positive patient samples or cell lines should be used. It is strongly advised to use DNA sample and reference DNA samples extracted with the same method and derived from the same source of tissue. For further details, please consult the SD032 Binning DNA product description provided. **This product is for research use only (RUO).**

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 all probes in the reference samples should be <0.10 and the dosage quotient (DQ) of the reference probes 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 or pseudo-autosomal chromosomes:

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 (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 are unlikely to have any relation to the condition tested for.

Limitations of the procedure:

- In most populations, the major cause of genetic defects in the *FLCN* gene are small (point) mutations, most of which will not be detected by using SALSA® MLPA® Probemix P256 FLCN.
- 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.

***FLCN* mutation database:** https://databases.lovd.nl/shared/genes/FLCN. 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 *FLCN* exons 6 and 8 but not exon 7) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P256-B4 FLCN

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)	
		Reference	*FLCN*
64-105	Control fragments – see table in probemix content section for more information		
127	Reference probe 15370-L19110	7q11	
136 *	Reference probe 17516-L21415	2q32	
143	**FLCN probe** 08591-L23693		**Exon 5**
153	**FLCN probe** 08595-L08596		**Exon 9**
166 ¬	TNFRSF13B probe 01448-L00932		**Downstream**
172	**FLCN probe** 08599-L08602		**Exon 12**
187 §	**FLCN probe** 08598-L08600		**1285delC (1733delC)** (a small peak is always present)
194 §	**FLCN probe** 08598-L08601		**1285dupC (1733dupC)**
202	**FLCN probe** 08587-L08588		**Exon 1**
214	**FLCN probe** 08594-L08595		**Exon 8**
227	**FLCN probe** 08588-L08589		**Exon 2**
238	**FLCN probe** 08596-L08597		**Exon 10**
250	**FLCN probe** 08590-L08591		**Exon 4**
257	Reference probe 10692-L11274	6p12	
265	**FLCN probe** 08600-L08603		**Exon 13**
283	**FLCN probe** 08593-L08594		**Exon 7**
292	Reference probe 14298-L15968	15q13	
301	**FLCN probe** 08601-L08604		**Exon 14**
310 *	Reference probe 18380-L25673	10q22	
321	**FLCN probe** 08589-L08590		**Exon 3**
328	Reference probe 08741-L08752	9q21	
337	**FLCN probe** 08597-L23694		**Exon 11**
346	Reference probe 05982-L05407	20p12	
355	**FLCN probe** 08592-L08593		**Exon 6**
364 *	Reference probe 06489-L06015	1p13	
372	Reference probe 16852-L19646	18q21	

* New in version B4 (from lot B4-0918 onwards).

§ Mutation-specific probes. The probes at 187 nt and 194 nt will only generate a signal when the 1285delC or 1285dupC mutations are present, respectively. These probes have been tested on artificial DNA **but not on positive human samples!** Please note that a small peak is always present at the length of the 1285delC mutation specific probe, and that the 1285dupC mutation specific probe may give a very small background signal. The heights of these peaks are clearly distinguishable from the peak heights in a positive sample.

¬ 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.

Note: The exon numbering used in this P256-B4 FLCN product description is the exon numbering from the RefSeq transcript NM_144997.5, which is identical to the LRG_325 sequence. The exon numbering and NM sequence used is from 10/2018, but can be changed (e.g. by NCBI) after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2. FLCN probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*FLCN* exon	Ligation site NM_144997.5	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*505-507 (Exon 4)*		
202	08587-L08588	Exon 1	62-63	GTCGCTCCTGGT-TCTGCCAGCTCC	4.3 kb
227	08588-L08589	Exon 2	379-380	AAGCGTGATTCT-GCTGAGTGTCAG	0.9 kb
321	08589-L08590	Exon 3	32 nt before exon 3	TTCTCTTCAAAT-GTCTGTTTTCTG	3.9 kb
250	08590-L08591	Exon 4	665-666	TGGCATTCAGAT-GAACAGTCGGAT	1.7 kb
143	08591-L23693	Exon 5	790-791	ACCCGGGATATA-TCAGCCATGATA	2.2 kb
355	08592-L08593	Exon 6	977-978	CACCTTCTTCAT-CAAGGACAGCCT	1.5 kb
283	08593-L08594	Exon 7	1171-1172	CTCAGAGGATGA-ACACAGCCTTCA	1.0 kb
214	08594-L08595	Exon 8	1329-1330	AAGCTCCTGGAA-GGTGCTCCGACC	2.4 kb
153	08595-L08596	Exon 9	Intron 8-1376	TCTGTCTTGCAG-ATTTAGAAGAGG	2.1 kb
238	08596-L08597	Exon 10	1676-1677	TTTTGAAGTACT-TCGGGTGAGAAC	0.6 kb
337	08597-L23694	Exon 11	11 nt before exon 11	CTGAGTCCTGCT-GTCCTCCTCAGA	0.1 kb
194 §	08598-L08601	Exon 11	1789-1788 reverse	GAGAGCACGTGG-GGGGGGGATCTG	-
187 §	08598-L08600	Exon 11	1789-1788 reverse	AGGAGAGCACGT-GGGGGGGATCTG	1.2 kb
172	08599-L08602	Exon 12	1898-1899	CAAGTACGAGTT-TGTGGTGACCAG	0.2 kb
265	08600-L08603	Exon 13	2027-2028	CGTCTGCCTCAA-GGAGGAGTGGAT	1.2 kb
301	08601-L08604	Exon 14	2105-2106	GGACACACAGAA-GCTGCTGAGCAT	264.9 kb
		stop codon	*2242-2244 (Exon 14)*		
166 ¬	01448-L00932	*TNFRSF13B*		GAGCAAGGCAAG-TTCTATGACCAT	

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References

- Nickerson ML et al. (2002). Mutations in a novel gene lead to kidney tumors, lung wall defects, and benign tumors of the hair follicle in patients with the Birt-Hogg-Dube syndrome. *Cancer cell*, *2*(2), 157-164.
- 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.
- 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 P256 FLCN

- Benhammou JN et al. (2011). Identification of Intragenic Deletions and Duplication in the FLCN Gene in Birt-Hogg-Dube Syndrome. *Genes Chromosomes Cancer*. 50:466-77.
- Ding Y et al. (2015). FLCN intragenic deletions in Chinese familial primary spontaneous pneumothorax. *Am J Med Genet A, 167*(5), 1125-33.
- Houweling AC et al. (2011). Renal cancer and pneumothorax risk in Birt–Hogg–Dubé syndrome; an analysis of 115 FLCN mutation carriers from 35 BHD families. *Br J Cancer*. 105:1912-19.
- Menko FH et al. (2013). A de novo FLCN mutation in a patient with spontaneous pneumothorax and renal cancer; a clinical and molecular evaluation. *Familial Cancer*. 12:373-9.
- Rossing M et al. (2017). Genetic screening of the FLCN gene identify six novel variants and a Danish founder mutation. *J Hum Genet*, *62*(2), 151.

P256 Product history	
Version	*Modification*
B4	Three reference probes have been replaced.
B3	One reference probe has been replaced and one has been added.
B2	Three reference probes have been replaced and the control fragments have been adjusted (QDX2).
B1	Three new reference probes have been added and one flanking probe has been removed.
A1	First release.

Implemented changes in the product description

Version B4-01 – 23 October 2018 (01P)
- Product description restructured and adapted to a new template.
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).

Version 11 – 12 December 2016 (55)
- Description of background signal for the mutation-specific probes clarified.

Version 10 – 18 February 2016 (55)
- Product description adapted to a new version (lot number added, small changes in Table 1, new pictures included).
- Manufacturer's address adjusted.

Version 09 (51)
- Product description adapted to a new version (lot number added, small changes in Table 1 and 2, new picture included).

Version 08 (48)
- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

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