Instructions for Use

SALSA® MLPA® Probemix P256 FLCN

See also the MLPA General Protocol, the product descriptions of the SALSA® MLPA® Reagent Kit, SALSA® Binning DNA SD032, and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P256 FLCN product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P256 FLCN**
Version	C1
Catalogue numbers	P256-025R (25 reactions) P256-050R (50 reactions) P256-100R (100 reactions)
Basic UDI-DI	872021148P25669
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 SD032	SD032

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
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, a second probe for *FLCN* exon 1 has been included, a downstream flanking probe has been removed, and four target probes have been adjusted in length but not in sequence detected. The background signal of the c.1285delC mutation-specific probe has been reduced to 0%. In addition, seven reference probes have been replaced and one additional reference probe has been included.

1. Intended Purpose

The SALSA MLPA Probemix P256 FLCN is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] for the detection of deletions or duplications in the *FLCN* gene, as well as the presence of the two most common point mutations, c.1285delC and c.1285dupC, in genomic DNA isolated from human peripheral whole blood specimens. P256 FLCN is intended to confirm a potential cause for and clinical diagnosis of Birt-Hogg-Dubé syndrome (BHDS) and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P256 FLCN should be confirmed with a different technique. In particular, CNVs detected by only a single probe as well as the two common *FLCN* point mutations always require confirmation by another method. Most defects in the *FLCN* gene are point mutations, which will not be detected by MLPA, with exception of the two aforementioned *FLCN* point mutations. It is therefore recommended to use this assay in combination with sequence analysis.

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, e.g. from DNA extracted from formalin-fixed paraffin embedded (FFPE) or fresh tumour materials.

[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	50-250 ng purified human genomic DNA 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 except for mutation-specific 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 BHDS.	
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 SD032, 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)	• In the validation experiments of this probemix, the peaks of the mutation-specific probes are 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 (*FLCN*)

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.

Possible Results of Mutation-Specific Probes

Signal Strength	Mutation Status
≥20% median peak height reference probes	Mutation c.1285delC or c.1285dupC is detected (expected only in positive samples)
<20% median peak height reference probes	Mutation c.1285delC or c.1285dupC is **not** detected (expected in most samples from healthy individuals)

A background signal of 5-10% can be visible for the *FLCN* 195 nt mutation-specific probe in samples without the c.1285dupC mutation. This background signal indicates the absence of the mutation and might be displayed as an intra ratio percentage instead of a final ratio (see our support portal for more details).

6. Performance Characteristics

<table>
<tr><th>Study</th><th>Description</th></tr>
<tr><td>Expected values for copy numbers 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 can be used.

Cut-off values for copy number determination were verified with SALSA MLPA Probemix P256 FLCN in 30 samples from individuals with normal FLCN copy numbers and nine samples with known CNVs in FLCN. The expected FRs for the corresponding copy number were found in all samples 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 FLCN c.1285delC mutation (188 nt) or FLCN c.1285dupC mutation (195 nt) is present. A clear signal (at least 20% of the median peak height of all reference probes in that sample) indicates that the mutation is present. It is not possible to determine the copy number of mutation-specific probes.

The presence or absence of the FLCN c.1285delC or c.1285dupC mutations were verified with SALSA MLPA Probemix P256 FLCN in 32 mutation-negative samples, 7 mutation-positive samples and SALSA Binning DNA SD032. The expected results for the corresponding mutation were found in all 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 SALSA MLPA Probemix P256 FLCN on one sample with a complete heterozygous FLCN gene deletion, one sample with a complete heterozygous FLCN gene duplication and one wild-type sample. Expected results were obtained 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 (>40 mM), EDTA and hemoglobin) can affect the MLPA reaction.

A study was performed with SALSA MLPA Probemix P256 FLCN to assess the potential for interference of endogenous and exogenous substances on genomic DNA derived from blood on one sample with a complete heterozygous FLCN gene deletion, one sample with a complete heterozygous FLCN gene duplication, one wild-type sample and SALSA Binning DNA SD032. For most copy number probes, expected FRs (FRs within the expected cut-off category) were obtained even in the presence of potential interferents at concentrations shown in the table below. Whereas, in all samples tested, for the mutation-specific probes, the expected signals 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 180/180 measurements
Mutation: Expected signal for 8/8 measurements</td></tr>
<tr><td>NaCl</td><td>Exogenous – DNA extraction</td><td>40 mM</td><td>Copy number: Expected FR for 180/180 measurements
Mutation: Expected signal for 8/8 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 180/180 measurements
Mutation: Expected signal for 8/8 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 180/180 measurements
Mutation: Expected signal for 8/8 measurements</td></tr>
<tr><td>Hemoglobin</td><td>Endogenous – blood sample</td><td>0.02 µg/µl</td><td>Copy number: Expected FR for 174/180 measurements
Mutation: Expected signal for 8/8 measurements</td></tr>
</table>
* Results are summarised for all probes across all four samples tested in triplicate.

Exogenous interfering substances (EDTA, heparin, NaCl, and $FeCl_3$) were tested and shown to have no effects on P256 FLCN results. FRs for all interfering substances tested fell within the expected cut-off ranges, except for hemoglobin where FRs in particular for copy number determination were lower than expected in 6/180 probes in total across all samples. At most, hemoglobin can lead to ambiguous ratios</td></tr>
</table>

	and potential delayed results. None of the interferents tested had an effect leading to a potential false result, nor had an effect on the determination of mutation status. Additionally, Coffalyser.Net issues warnings for the samples in which the interferents showed an effect, as well as lowered quality scores. 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.
Cross-reactivity	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 on eight samples with a complete heterozygous *FLCN* gene deletion, one sample with a complete heterozygous *FLCN* gene duplication, seven samples with known *FLCN* c.1285delC or c.1285dupC mutations, SALSA Binning DNA SD032 and 23 normal samples to determine whether probes are specific to their target sequence. As all genotypes were found to match the correct final ratio for their relevant copy number/mutation status indicating that the probes are specific for their target location, SALSA MLPA Probemix P256 FLCN has an analytical specificity of >99%, when free from substances known to affect MLPA reactions.
Accuracy	Results of accuracy are derived from trueness and precision studies. For trueness, nine previously genotyped samples and SALSA Binning DNA SD032 were tested using P256 FLCN and found to have the expected results. Assay precision was tested by repeatedly testing samples with known copy numbers over multiple days, and by multiple operators. Results showed a correct call of 99.6% (897/900 measurements) for copy number and 100% (120/120 measurements) for point mutation status determination. For copy number determination, the overall reproducibility was 99.6% (538/540 measurements) over three operators and three days. For determination of point mutation status, the overall reproducibility over three operators and over three days was 100% (72/72 measurements). Overall, irrespective of replicates, operators, or days, the correct copy number and point mutations status were determined in the tested samples. However, deviations above the upper cut-offs can occur.
Clinical validity*	Deletions or duplications in the *FLCN* gene are the genetic cause of BHDS in <8% of the cases. 20-24% of patients with BHDS have a germline deletion or insertion of a cytosine in exon 11 (c.1285delC or c.1285dupC). *Based on a 2009-2024 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
17p11.2	*FLCN*	Exon 14	1.2 kb		301	08601-L08604	
17p11.2	*FLCN*	Exon 13	0.2 kb		265	08600-L08603	
17p11.2	*FLCN*	Exon 12	1.2 kb		172	08599-L08602	
17p11.2	*FLCN*	Exon 11	0.1 kb	c.1285dupC	195	08598-L31789	§ ±
17p11.2	*FLCN*	Exon 11		c.1285delC	188	08598-L31913	§ ±
17p11.2	*FLCN*	Exon 11	0.6 kb		337	08597-L23694	
17p11.2	*FLCN*	Exon 10	2.1 kb		238	08596-L08597	
17p11.2	*FLCN*	Exon 9	2.4 kb		153	08595-L08596	
17p11.2	*FLCN*	Exon 8	1.0 kb		214	08594-L08595	
17p11.2	*FLCN*	Exon 7	1.5 kb		285	08593-L31925	
17p11.2	*FLCN*	Exon 6	2.2 kb		355	08592-L08593	
17p11.2	*FLCN*	Exon 5	1.7 kb		143	08591-L23693	
17p11.2	*FLCN*	Exon 4	3.9 kb		250	08590-L08591	
17p11.2	*FLCN*	Exon 3	0.9 kb		321	08589-L08590	+
17p11.2	*FLCN*	Exon 2	3.8 kb		227	08588-L08589	
17p11.2	*FLCN*	Intron 1 (Exon 1)	0.5 kb		346	22584-L31783	
17p11.2	*FLCN*	Exon 1			366	22580-L31776	
1p	Reference				275	04489-L03878	
2q	Reference				165	13816-L28133	
4p	Reference				380	16932-L19875	
5p	Reference				129	18709-L26847	
6q	Reference				257	10692-L11274	
10q	Reference				310	18380-L25673	
11q	Reference				220	08940-L31919	
14q	Reference				178	07032-L28099	
15q	Reference				203	17177-L31937	
19q	Reference				328	16275-L22420	

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 *FLCN* 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 one probe with a target at the edge of or slightly outside the coding region, was 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 will only generate a signal when the mutation is present.

± The presence of single nucleotide variant (SNV) rs41459448 could increase the background signal in wildtype DNA for both 188 nt and 195 nt mutation-specific probes. In case of an apparent mutation, it is recommended to sequence the region targeted by these probes.

\+ The ligation site of this probe is >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. 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.
5. The two c.1285delC and c.1285dupC mutation-specific probes are only intended to determine the presence (or absence) of the 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.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Probemix-specific limitations

1. The mutation-specific probes can only detect the presence of the mutation and should not be used to determine zygosity.
2. 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. Only the presence of the two most common point mutations (c.1285dupC and c.1285delC) in the *FLCN* gene can be detected, but other point mutations cannot.
3. The results of mutation-specific probes should always be confirmed visually in the size called peak pattern and/or raw run data. Detection of a background signal in reference or patient samples may lead to false positive results.

Technique-specific limitations
See the MLPA General Protocol.

Implemented changes in the product description

Version C1-03 – 20 June 2025 (03S)

- Product description updated to a new template.
- Intended purpose updated, specifying the assay is manual.
- Reference to SALSA Binning DNA SD032 removed from the intended purpose footnote.
- Binning DNA sample (initial experiment) description rephrased following the removal of SALSA Binning DNA SD032 from the intended purpose.
- Description of probe targets at the edge of or slightly outside the coding region has been adjusted. No change in actual target sites.
- Warning added for probe 08589-L08590 with ligation site >20 nt away from the nearest exon.
- Warning for unknown clinical significance of CNVs for alternative transcript NM_001353229.1 removed for probe 22584-L31783.
- Probemix is now IVDR certified.