Product Description
SALSA® MLPA® Probemix P028-B2 FHL

To be used with the MLPA General Protocol.

Version B2
As compared to version B1, three reference probes have been replaced and one reference probe has been removed. For complete product history see page 7.

Catalogue numbers:
- **P028-025R:** SALSA MLPA Probemix P028 FHL, 25 reactions.
- **P028-050R:** SALSA MLPA Probemix P028 FHL, 50 reactions.
- **P028-100R:** SALSA MLPA Probemix P028 FHL, 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.mrcholland.com).

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

Precautions and warnings
For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mrcholland.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 P028 FHL is a **research use only (RUO)** assay for the detection of deletions or duplications in the *UNC13D*, *PRF1* and *STX11* genes, which are associated with familial haemophagocytic lymphohistiocytosis (FHL).

FHL is a rare autosomal recessive disorder associated with perforin (*PRF1*) deficiency or a disruption of perforin delivery, which lead to impaired immune activity of cytotoxic T lymphocytes and Natural Killer (NK) cells (Brennan et al. 2010). To compensate for the loss of functional T cells and NK cells, an exaggerated immune response is triggered that is characteristic for FHL. Massive organ infiltration by activated lymphocytes and macrophages can be observed. Other symptoms include persistent fever, hepatosplenomegaly and cytopenia. First episodes occur mostly during infancy, with a rapidly fatal outcome if untreated. Five FHL subtypes (FHL1, FHL2, FHL3, FHL4, FHL5) have been described. Mutations in the *UNC13D* gene are associated with FHL3, defects in the *PRF1* gene cause FHL2, and *STX11* mutations cause FHL4.

The *UNC13D* gene (32 exons) spans ~17 kb of genomic DNA and is located on chromosome 17q25.1, ~71 Mb from the p-telomere. The *PRF1* gene (3 exons) spans ~5.4 kb of genomic DNA and is located on chromosome 10q22.1, 72 Mb from the p-telomere. The *STX11* gene (2 exons) spans ~41 kb of genomic DNA and is located on chromosome 6q24.2, ~145 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1444/.

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 *UNC13D*, *PRF1* and *STX11* exon numbering used in this P028-B2 FHL product description is the exon numbering from the MANE project (release version 1.0) based on MANE Select transcripts NM_199242.3, NM_001083116.3 and NM_003764.4, respectively, as indicated in Table 2. 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 MANE sequence. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content
The SALSA MLPA Probemix P028-B2 FHL contains 44 MLPA probes with amplification products between 130 and 481 nucleotides (nt). This includes 30 probes for the *UNC13D* gene, three probes for the *PRF1* gene and two probes for the *STX11* gene. 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.mrcholland.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.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal 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.mrcholland.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 different unrelated individuals who are from families without a history of FHL. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

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/) have diverse collections of biological resources which may be used as positive

control DNA samples in your MLPA experiments. 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.mrcholland.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 final ratio (FR) 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 FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 2.15
Ambiguous copy number	All other values

Note: The term “dosage quotient”, used in older product description versions, has been replaced by “final ratio” to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

- 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. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also 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.
- 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.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *UNC13D*, *PRF1* and *STX11* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P028 FHL.
- 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. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (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.

***UNC13D*, *PRF1* and *STX11* mutation databases**

https://databases.lovd.nl/shared/genes/UNC13D
https://databases.lovd.nl/shared/genes/PRF1
https://databases.lovd.nl/shared/genes/STX11

We strongly encourage users to deposit positive results in the Leiden Open Variation Database. 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 SNVs and unusual results (e.g., a duplication of *UNC13D* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P028-B2 FHL

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*UNC13D*	*PRF1*	*STX11*
64-105	Control fragments – see table in probemix content section for more information				
130	Reference probe 00797-L00463	5q			
136	**UNC13D probe** 11678-L12449		**Exon 5**		
142	**UNC13D probe** 11679-L12450		**Exon 23**		
148	**STX11 probe** 11680-L15341				**Exon 2**
154	**UNC13D probe** 11681-L12452		**Exon 8**		
160	**UNC13D probe** 20709-L28599		**Exon 17**		
166 *	Reference probe 05031-L04417	9p			
172	**UNC13D probe** 11683-L12454		**Exon 12**		
177	**UNC13D probe** 11684-L28938		**Exon 28**		
184	**PRF1 probe** 11685-L12456			**Exon 2**	
190	**UNC13D probe** 11686-L12457		**Exon 16**		
196	**UNC13D probe** 11687-L12458		**Exon 30**		
202 *	Reference probe 21832-L31603	2p			
208	**UNC13D probe** 11688-L12459		**Exon 2**		
214	**UNC13D probe** 11689-L15326		**Exon 14**		
220	**UNC13D probe** 11690-L12461		**Exon 6**		
226	**UNC13D probe** 11691-L12462		**Exon 19**		
232	**UNC13D probe** 11692-L15327		**Exon 10**		
238	Reference probe 08109-L07985	11p			
246	**UNC13D probe** 11693-L15328		**Exon 3**		
256	**UNC13D probe** 20710-L28966		**Exon 20**		
264	**UNC13D probe** 11695-L12466		**Exon 32**		
274	**UNC13D probe** 11696-L12467		**Exon 24**		
283	**PRF1 probe** 11697-L12468			**Exon 3**	
292	Reference probe 06443-L05969	3p			
301	**UNC13D probe** 11698-L12469		**Exon 25**		
310	**UNC13D probe** 11699-L12470		**Exon 15**		
319	**UNC13D probe** 11700-L12471		**Exon 1**		
328 Ж	**UNC13D probe** 20711-SP0971-L28560		**Exon 27**		
337	**UNC13D probe** 11702-L12473		**Exon 9**		
346	Reference probe 06015-L07508	19q			
364	**UNC13D probe** 11704-L15329		**Exon 31**		
382	**UNC13D probe** 20714-L28563		**Exon 26**		
391 *	Reference probe 12695-L13773	7q			
400	**UNC13D probe** 11707-L12478		**Exon 4**		
409	**UNC13D probe** 11708-L12479		**Exon 18**		
418 ±	**STX11 probe** 11709-L28448				**Exon 1**
426	**UNC13D probe** 11710-L28937		**Exon 7**		
432	Reference probe 10876-L11546	15q			
445	**UNC13D probe** 11711-L12482		**Exon 13**		
454	**UNC13D probe** 11712-L20235		**Exon 21**		
463	**PRF1 probe** 11713-L12484			**Exon 1**	
472	**UNC13D probe** 20715-L28564		**Exon 29**		
481	Reference probe 13595-L15052	1q			

[a] See section Exon numbering on page 2 for more information.

* New in version B2.

± SNP rs146840517 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. P028-B2 probes arranged according to chromosomal location

Table 2a. *UNC13D*

Length (nt)	SALSA MLPA probe	*UNC13D* exon[a]	Ligation site NM_199242.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*68-70 (Exon 1)*		
319	11700-L12471	Exon 1	25-26	GCTTCGCTGTCT-TCACCCAGCACC	0.9 kb
208	11688-L12459	Exon 2	4 nt before exon 2	CCACCTTGCTCT-GCAGATCCAGCC	0.3 kb
246	11693-L15328	Exon 3	288-287 *reverse*	CCTCCGTCACAT-GGTTGGGCTCAG	0.2 kb
400	11707-L12478	Exon 4	385-386	GTCAGGGAGCTT-GAGGTAACCTGG	0.1 kb
136	11678-L12449	Exon 5	435-436	GGCCAAGGGCAT-TCTGGGCAAAGA	0.4 kb
220	11690-L12461	Exon 6	557-556 *reverse*	CTCGGGGATGGT-GTGCCTCACCAC	1.5 kb
426	11710-L28937	Exon 7	651-650 *reverse*	TCGCATTGGTGA-TGTCCTCAAACT	0.2 kb
154	11681-L12452	Exon 8	712-713	TCTGTCCGACAG-AAGCTTGGGGAG	0.3 kb
337	11702-L12473	Exon 9	808-807 *reverse*	TGCAGCCTCAGA-ACCACGTTCCCC	0.3 kb
232	11692-L15327	Exon 10	914-915	AGTTCCAACTCA-TCCATAAGCGGG	0.4 kb
	No probe	Exon 11			
172	11683-L12454	Exon 12	1091-1092	ACGCCACACAGA-AGGACCTATCCG	3.0 kb
445	11711-L12482	Exon 13	1199-1200	CCATCACCAGCA-TCGAGTACCAGT	0.3 kb
214	11689-L15326	Exon 14	14 nt after exon 14	CAGTGGCTGCCT-TCCTCTTCCTCG	0.2 kb
310	11699-L12470	Exon 15	1404-1405	GGCCTTTGGAGA-ACTGTGCCCCAA	0.2 kb
190	11686-L12457	Exon 16	1497-1498	GCAGCAGCACCA-TCAACCCATGGT	0.2 kb
160	20709-L28599	Exon 17	1599-1600	CACATGGGACAA-GATCTTCCACAA	0.1 kb
409	11708-L12479	Exon 18	1619-1920	CCAGTACCCTCA-AGATCCACCTCT	0.2 kb
226	11691-L12462	Exon 19	1740-1741	GAGTCTGTTCCA-GCTCTACATCAG	0.2 kb
256	20710-L28966	Exon 20	1859-1860	CCTCCTGGCTGC-AGAAGACGTACA	0.4 kb
454	11712-L20235	Exon 21	1946-1947	TGACCAAGCACA-GCACATCAGCGG	0.5 kb
	No probe	Exon 22			
142	11679-L12450	Exon 23	2187-2188	TGACATGGAGCA-GCTGCGGCTGGT	0.4 kb
274	11696-L12467	Exon 24	2397-2398	GCACATCCAGAA-ACTGGTGGGCGT	1.1 kb
301	11698-L12469	Exon 25	2473-2474	GAGGTGGAGCTT-TGCTACATGAAC	1.8 kb
382	20714-L28563	Exon 26	17 nt after exon 26	AGAACCGGTCAC-AGCAATGATAAC	0.2 kb
328 Ж	20711-SP0971-L28560	Exon 27	14 nt after exon 27; 44 nt after exon 27	AGGGGCTTTGGG-30 nt spanning oligo-TGCCTTTTGGCA	0.4 kb
177	11684-L28938	Exon 28	2748-2747 *reverse*	TGCAGAAGTACT-TCCGGATGAGTT	0.2 kb
472	20715-L28564	Exon 29	2817-2818	TGTGACAGTCAA-GGCCTCCTACCG	0.3 kb
196	11687-L12458	Exon 30	2913-2914	CAGCGACCCCTT-TGTCCAGCTGAC	1.1 kb
364	11704-L15329	Exon 31	3027-3026 *reverse*	GCTCAGCAGGCA-CCAGGCTGCGGG	1.7 kb
264	11695-L12466	Exon 32	3972-3973	CAGAAAGGACAG-TTTGGCTGCTGT	
		stop codon	*3338-3340 (Exon 32)*		

Table 2b. *PRF1*

Length (nt)	SALSA MLPA probe	*PRF1* exon[a]	Ligation site NM_001083116.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*128-130 (Exon 2)*		
463	11713-L12484	Exon 1	60-61	GAAGAGAGGATA-TCCATCTGTGTA	2.2 kb
184	11685-L12456	Exon 2	597-598	GGCAGCCAACTT-TGCAGCCCAGAA	2.2 kb
283	11697-L12468	Exon 3	1645-1646	TCTGGTTCCCAT-GAGGTGAGATGC	
		stop codon	*1793-1795 (Exon 3)*		

Table 2c. *STX11*

Length (nt)	SALSA MLPA probe	*STX11* exon[a]	Ligation site NM_003764.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*193-195 (Exon 2)*		
418 ±	11709-L28448	Exon 1	107-108	AACAGGTTTCCT-TCTCCATCGCTG	36.8 kb
148	11680-L15341	Exon 2	942-943	CTCAACGTACAA-AAGACGGTCGAC	
		stop codon	*1054-1056 (Exon 2)*		

[a] See section Exon numbering on page 2 for more information.

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

± SNP rs146840517 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

References

- Brennan AJ et al. (2010). Perforin deficiency and susceptibility to cancer. *Cell Death Differ.* 17:607-615.
- 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 P028 FHL

- Ježová M and Gaillyová R (2017). Familial hemophagocytic lymphohistiocytosis: from autopsy to prenatal diagnosis. Report of a case. *Cesk Patol.* 53:29-34.

P028 product history	
Version	*Modification*
B2	Three reference probes have been replaced and one reference probe has been removed.
B1	Probes for *UNC13D* exons 17, 20 and 26 have been included and the probe for exon 22 has been removed. Probes for *UNC13D* exons 27 and 29 have been redesigned. In addition, one reference probe has been replaced.
A1	First release.

Implemented changes in the product description
Version B2-01 – 08 November 2023 (04P) - 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). - Changed the Exon numbering section to include the MANE project instead of LRG sequences. - Ligation sites of the probes targeting the *UNC13D* gene updated according to new version of the NM_ reference sequence. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. Version B1-01 — 03 March 2020 (02P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *PRF1* gene updated according to new version of the NM_ reference sequence. - The NM_ reference sequence for the *STX11* gene was updated.

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