Product Description
SALSA® MLPA® Probemix P207-D1 F9

To be used with the MLPA General Protocol.

Version D1
As compared to version C4, new target probes have been added and several target probes have been replaced. Six reference probes have been replaced and three removed. In addition, multiple lengths have been adjusted. For complete product history see page 8.

Catalogue numbers:
- **P207-025R:** SALSA MLPA Probemix P207 F9, 25 reactions.
- **P207-050R:** SALSA MLPA Probemix P207 F9, 50 reactions.
- **P207-100R:** SALSA MLPA Probemix P207 F9, 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 P207 F9 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *F7*, *F8* and *F9* genes, encoding coagulation factors factor VII, factor VIII and factor IX, respectively.

Coagulation factors play an important role in bleeding control and vessel repair. Deficiency in any of these genes may results in a bleeding disorders with different clinical manifestation: *F7* is involved in factor VII deficiency, *F8* in hemophilia A, and *F9* in hemophilia B. Both hemophilia A and B are inherited in an X-linked manner, while factor VII deficiency is inherited in an autosomal recessive fashion.

More information is available at https://ghr.nlm.nih.gov/gene/F7, https://www.ncbi.nlm.nih.gov/books/NBK1404/ and https://www.ncbi.nlm.nih.gov/books/NBK1495/.

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 *F7* exon numbering used in this P207-D1 F9 product description is the exon numbering from the LRG_554 sequence. The *F8* exon numbering used in this P207-D1 F9 product description is the exon numbering from the LRG_555 sequence. The *F9* exon numbering used in this P207-D1 F9 product description is the exon numbering from the LRG_556 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. 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 P207-D1 F9 contains 38 MLPA probes with amplification products between 130 and 454 nucleotides (nt). This includes 11 probes for the *F7* gene (one for each exon and two for exons 1 and 8), seven probes for the *F8* gene and ten probes for the *F9* gene (one for each exon and two for exons 1 and 8). In addition, 10 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 from the same sex 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 bleeding disorders. It is recommended to use samples of the same sex to facilitate interpretation. 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. Sample ID numbers NA02325, NA03089, NA03330, NA03887 and NA05832 from the Coriell Institute have been tested with this P207-D1 probemix at MRC Holland and can

be used as positive control samples to detect the deletions/duplications shown in the table below. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Sex	Altered target genes in P207-D1	Expected copy number alteration
NA02325	Coriell Institute	Female	*F8*	Heterozygous duplication of *F8* exon 1-22
NA03089	Coriell Institute	Female	*F7*	Heterozygous duplication
NA03330	Coriell Institute	Male	*F7*	Heterozygous duplication
NA03887	Coriell Institute	Female	*F7*	Heterozygous deletion
NA05832	Coriell Institute	Male	*F7*	Heterozygous duplication

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 when **reference samples of the same sex** have been used:

Copy number status		Final ratio (FR)
Autosomal sequences and X chromosome sequences in females	**X chromosome sequences in males**	
Normal	Normal	0.80 < FR < 1.20
Homozygous deletion	Deletion	FR = 0
Heterozygous deletion		0.40 < FR < 0.65
Heterozygous duplication		1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	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 targeted genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P207 F9.
- 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.

EAHAD Coagulation Factor Variant database

https://dbs.eahad.org/. We strongly encourage users to deposit positive results in the EAHAD Coagulation Factor Variant 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 *F7* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P207-D1 F9

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*F7*	*F8*	*F9*
64-105	Control fragments – see table in probemix content section for more information				
130	Reference probe 09978-L10437	19p			
136	**F9** probe 06796-L06388				**Exon 8**
143 ¥	**F7** probe 23138-L32697		**Exon 8**		
148	**F8** probe 05618-L05068			**Exon 7**	
157 ¥	**F9** probe 07280-L32696				**Exon 7**
166 *	Reference probe 20621-L28934	6q			
172	**F9** probe 06797-L06389				**Exon 8**
178 ¥	**F7** probe 23135-L32860		**Exon 2**		
184	**F8** probe 05626-L06057			**Exon 14**	
192	Reference probe 10519-L11073	1p			
198 *	**F7** probe 23151-L32715		**Exon 6**		
208 ¥	**F9** probe 23136-L06383				**Exon 3**
214	**F8** probe 05620-L06927			**Exon 9**	
221 *	Reference probe 19987-L27049	4p			
229 *	**F7** probe 23152-L32862		**Exon 3**		
238 *	**F8** probe 23137-L05080			**Exon 17**	
246 ¥	**F7** probe 23141-L14966		**Exon 9**		
256 *	Reference probe 15329-L17526	8q			
268	**F9** probe 06790-L15258				**Exon 2**
274 *	**F7** probe 23153-L32859		**Exon 1**		
283	**F9** probe 06793-L06385				**Exon 5**
292	Reference probe 03782-L03291	17q			
300 ¥	**F7** probe 23140-L14972		**Exon 8**		
312	**F8** probe 06506-L15887			**Exon 3**	
328 *	**F9** probe 23150-L32714				**Exon 1**
338	Reference probe 12785-L15496	2q			
346	**F7** probe 13501-L14965		**Exon 4**		
355 ¥	**F9** probe 23143-L06384				**Exon 4**
365 ¥	**F7** probe 13503-L32695		**Exon 1**		
377 *	Reference probe 16444-L18897	18q			
384 *	**F9** probe 23149-L32713				**Exon 1**
391	**F7** probe 13505-L14969		**Exon 5**		
410 ¥	**F8** probe 23142-L15888			**Exon 23**	
418 *	**F9** probe 23148-L32712				**Exon 6**
427 *	Reference probe 11659-L16062	5q			
436	**F7** probe 13507-L14971		**Exon 7**		
445 ¥	**F8** probe 23139-L32698			**Exon 1**	
454 *	Reference probe 21342-L29748	7q			

[a] See section Exon numbering on page 1 for more information.

* New in version D1.
¥ Changed in version D1. Minor alteration, no change in sequence detected.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 2. P207-D1 probes arranged according to chromosomal location

Table 2a. *F7* gene

Length (nt)	SALSA MLPA probe	*F7* exon[a]	Ligation site NM_000131.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*55-57 (Exon 1)*		
274	23153-L32859	Exon 1	41-42	GGGGCAGCACTG-CAGAGATTTCAT	0.2 kb
365	13503-L32695	Exon 1	140 nt after exon 1	GCTCCTGTTCAA-TTTCTTTCCTTC	0.8 kb
178	23135-L32860	Exon 2	140-141	TAAGGCCTCAGG-AGGAGAAACACG	3.8 kb
229	23152-L32862	Exon 3	187-188	ATTTCCCAGTCT-TCGTAACCCAGG	3.1 kb
346	13501-L14965	Exon 4	363-364	TTCTGGATTTCT-TACAGTGGTGAG	0.2 kb
391	13505-L14969	Exon 5	13 nt after exon 5	TAAGGCCCCACT-TTGGGTCCCATA	1.7 kb
198	23151-L32715	Exon 6	494-495	AGACAAGGATGA-CCAGCTGATCTG	1.2 kb
436	13507-L14971	Exon 7	3 nt after exon 7	CCATGGCAGGTA-AGGCTTCCCCTG	0.6 kb
143	23138-L32697	Exon 8	756-757	TTGGTGAATGGA-GCTCAGTTGTGT	0.1 kb
300	23140-L14972	Exon 8	828-829	TTCGACAAAATC-AAGAACTGGAGG	1.4 kb
246	23141-L14966	Exon 9	1428-1429	CCACGCCCAGGA-GTCCTCCTGCGA	
		stop codon	*1453-1455 (Exon 9)*		

Table 2b. *F8* gene

Length (nt)	SALSA MLPA probe	*F8* exon[a]	Ligation site NM_000132.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*172-174 (Exon 1)*		
445	23139-L32698	Exon 1	24-25	GCACATCCAGTG-GGTAAAGTTCCT	25.7 kb
312	06506-L15887	Exon 3	538-537 reverse	TTTCCAGTAGGA-TACACCAACAGC	27.6 kb
148	05618-L05068	Exon 7	1132-1133	CACTCTTGATGG-ACCTTGGACAGT	3.4 kb
214	05620-L06927	Exon 9	1566-1567	CAGCATGAATCA-GGAATCTTGGGA	35.6 kb
184	05626-L06057	Exon 14	3577-3578	ATGGAAAGAACT-CTCTGAACTCTG	25.9 kb
238	23137-L05080	Exon 17	5818-5819	CTAACACACTGA-ACCCTGCTCATG	41.3 kb
410	23142-L15888	Exon 23	6637-6638	CTGGGATAAAAC-ACAATATTTTTA	
		stop codon	*7225-7227 (Exon 26)*		

Table 2c. *F9* gene

Length (nt)	SALSA MLPA probe	*F9* exon[a]	Ligation site NM_000133.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*27-29 (Exon 1)*		
328	23150-L32714	Exon 1	18-19	ACAATCTGCTAG-CAAAGGTTATGC	0.1 kb
384	23149-L32713	Exon 1	95-94 reverse	TCAGCACTGAGT-AGATATCCTAAA	6.3 kb
268	06790-L15258	Exon 2	187-188	TAAATTGGAAGA-GTTTGTTCAAGG	0.3 kb
208	23136-L06383	Exon 3	4 nt after exon 3	GTATGTTGGTAA-GCAATTCATTTT	3.7 kb
355	23143-L06384	Exon 4	321-322	ATCAGTGTGAGT-CCAATCCATGTT	7.3 kb
283	06793-L06385	Exon 5	465-466	AGTTTTGTAAAA-ATAGTGCTGATA	2.7 kb
418	23148-L32712	Exon 6	573-574	GAAGAGTTTCTG-TTTCACAAACTT	9.7 kb
157	07280-L32696	Exon 7	778-779	AGTTGATGCATT-CTGTGGAGGCTC	0.8 kb
136	06796-L06388	Exon 8	955-956	TGCAGCTATTAA-TAAGTACAACCA	0.3 kb
172	06797-L06389	Exon 8	1241-1242	GGAGGTAGAGAT-TCATGTCAAGGA	
		stop codon	*1410-1412 (Exon 8)*		

[a] See section Exon numbering on page 1 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Related SALSA MLPA probemixes

- P011 VWF mix 1: Contains probes for the *vWF* gene, involved in von Willebrand factor deficiency.
- P012 VWF mix 2: Contains probes for the *vWF* gene, involved in von Willebrand factor deficiency.
- P178 F8: Contains probes for the *F8* gene, involved in Hemophilia A.
- P440 F10 + F11: Contains probes for the *F10* and the *F11* gene, involved in bleeding disorders.
- P469 F5: Contains probes for the *F5* gene.

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.
- 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 P207 F9

- Belvini D et al. (2017). Multiplex ligation-dependent probe amplification as first mutation screening for large deletions and duplications in haemophilia. *Haemophilia*. 23:e124-e132.
- Kihlberg K et al. (2017). Discrepancies between the one-stage clotting assay and the chromogenic assay in haemophilia B. *Haemophilia*. 23:620-627.
- Kwon M-J et al. (2008). Identification of mutations in the F9 gene including exon deletion by multiplex ligation-dependent probe amplification in 33 unrelated Korean patients with haemophilia B. *Haemophilia*. 14:1069-1075.
- Li T et al. (2014). Mutation analysis of a cohort of US patients with hemophilia B. *Am J Hematol*. 89:375-379.
- Mårtensson A et al. (2016). Mutation analysis of Swedish haemophilia B families–high frequency of unique mutations. *Haemophilia*. 22:440-445.
- Nakamura Y et al. (2016). Distinct X chromosomal rearrangements in four haemophilia B patients with entire F9 deletion. *Haemophilia*. 22:433-439.
- Pavlova A et al. (2015). Congenital combined deficiency of coagulation factors VII and X–different genetic mechanisms. *Haemophilia*. 21:386-391.
- Payne AB et al. (2012). Utility of multiplex ligation-dependent probe amplification (MLPA) for hemophilia mutation screening. *J Thromb Haemost*. 10:1951.
- Rath M et al. (2015). Large deletions play a minor but essential role in congenital coagulation factor VII and X deficiencies. *Hamostaseologie*. 35 Suppl 1:S36-42.
- Wheeler RB et al. (2015). The first report of a multi-exon duplication in the F9 gene causative of severe haemophilia B. *Haemophilia*. 21:e433-5.
- Zahari M et al. (2018). Mutational Profiles of F8 and F9 in a Cohort of Haemophilia A and Haemophilia B Patients in the Multi-ethnic Malaysian Population. *Mediterr J Hematol Infect Dis*. 10.

P207 product history	
Version	*Modification*
D1	New target probes have been added and several target probes have been replaced. Six reference probes have been replaced and three removed. In addition, multiple lengths have been adjusted.
C4	The 118 nt Y-fragment has been removed.
C3	Three reference probes have been removed.
C2	QDX2 fragments have been added.
C1	F7 gene has been included and reference probes have been replaced.
B1	Two reference probes have been replaced, three extra reference probes have been added and four extra control fragments at 88-96-100-105 nt have been included.
A1	First release.

Implemented changes in the product description

Version D1-01 – 10 March 2022 (04P)
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- Product-specific warning about sloping correction removed.

Version C4-03 – 05 May 2021 (04P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the F8 and F9 genes updated according to new versions of the NM_ reference sequence.
- Positive samples added that have been tested with the probemix at MRC Holland.
- Warning added to **Error! Reference source not found.** for specificity of a reference probe relying on a single nucleotide difference between its targeted gene and related gene or pseudogene.
- Warning added to **Error! Reference source not found.** on reported structural variation in healthy individuals in the region that is targeted by a reference probe.

Version C4-02 – 05 August 2019 (01P)
- Adjusted a sentence in 'Reference sample'-section in order to clarify that the use of reference and target samples facilitates data interpretation.

Version C4-01 – 01 February 2019 (01P)
- Product description restructured and adapted to a new template.
- Product description adapted to a new product version.

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