

Product Description

SALSA® MLPA® Probemix P207-C4 F9

To be used with the MLPA General Protocol.

Version C4

For complete product history see page 7.

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 result in a bleeding disorder 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-C4 F9 product description is the exon numbering from the LRG_554 sequence. The *F8* exon numbering used is the exon numbering from the LRG_555 sequence. The *F9* exon numbering used 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-C4 F9 contains 38 MLPA probes with amplification products between 130 and 454 nucleotides (nt). This includes nine probes for the *F7* gene, seven probes for the *F8* gene and nine

probes for the *F9* gene. In addition, 13 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 of 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 reference and patient 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-C4 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-C4	Expected copy number alteration
NA02325	Coriell Institute	Female	<i>F8</i>	Heterozygous duplication of <i>F8</i> exon 1-22
NA03089	Coriell Institute	Female	<i>F7</i>	Heterozygous duplication
NA03330	Coriell Institute	Male	<i>F7</i>	Heterozygous duplication
NA03887	Coriell Institute	Female	<i>F7</i>	Heterozygous deletion
NA05832	Coriell Institute	Male	<i>F7</i>	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.

P207 specific note:

- When only male samples are to be analysed, turn the slope correction off in Coffalyser.Net analysis, to get the correct copy number interpretation on the target region, because the probes targeting the X-chromosome are located in the first part of the probemix (F8 and F9 genes, see Table 1).

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-C4 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	
142	F8 probe 05610-L05060		Exon 1		
148	F8 probe 05618-L05068		Exon 7		
154	F9 probe 07280-L06904			Exon 7	
160	Reference probe 12741-L13835	21q			
172	F9 probe 06797-L06389			Exon 8	
184	F8 probe 05626-L06057		Exon 14		
192	Reference probe 10519-L11073	1p			
200 +	Reference probe 00976-L07192	11p			
208	F9 probe 06794-L28016			Exon 6	
214	F8 probe 05620-L06927		Exon 9		
220	F9 probe 06791-L06383			Exon 3	
226 #	Reference probe 10267-L10779	6q			
232	F9 probe 06789-L06381			Exon 1	
239	Reference probe 10914-L13898	9q			
247 ±	F8 probe 05617-L05067		Exon 7		
260	Reference probe 13529-L14989	3q			
268	F9 probe 06790-L15258			Exon 2	
274	Reference probe 07233-L06883	1q			
283	F9 probe 06793-L06385			Exon 5	
292	Reference probe 03782-L03291	17q			
301	F9 probe 06792-L06384			Exon 4	
312	F8 probe 06506-L15887		Exon 3		
328	F8 probe 05637-L15888		Exon 23		
338	Reference probe 12785-L15496	2q			
346	F7 probe 13501-L14965		Exon 4		
355	F7 probe 13502-L14966		Exon 9		
364	F7 probe 13503-L14967		Exon 1		
382	F7 probe 13504-L14968		Exon 8		
391	F7 probe 13505-L14969		Exon 5		
402	F7 probe 01753-L01297		Exon 6		
409	Reference probe 10606-L11158	15q			
418	F7 probe 13506-L14970		Exon 2		
427	Reference probe 12576-L13626	20p			
436	F7 probe 13507-L14971		Exon 7		
445	F7 probe 13508-L14972		Exon 8		
454	Reference probe 10090-L10514	8q			

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

± SNP rs34371500 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

+ The region targeted by this probe is reported to be involved in structural variation in healthy individuals. This may lead to a false-positive copy number aberration.

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.

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. P207-C4 probes arranged according to chromosomal locationTable 2a. *F7* gene

Length (nt)	SALSA MLPA probe	<i>F7</i> exon ^a	Ligation site NM_000131.4	Partial sequence ^b (24 nt adjacent to ligation site)	Distance to next probe
		<i>start codon</i>	55-57 (<i>Exon 1</i>)		
364	13503-L14967	Exon 1	140 nt after exon 1	GCTCCTGTTCAA-TTTCTTTCTTTC	0.8 kb
418	13506-L14970	Exon 2	140-141	TAAGGCCTCAGG-AGGAGAAACACG	6.9 kb
	<i>no probe</i>	<i>Exon 3</i>			
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
402	01753-L01297	Exon 6	513-514	ATCTGTGTGAAC-GAGAACGGCGGC	1.2 kb
436	13507-L14971	Exon 7	3 nt after exon 7	CCATGGCAGGTA-AGGCTTCCCCTG	0.6 kb
382	13504-L14968	Exon 8	756-757	TTGGTGAATGGA-GCTCAGTTGTGT	0.1 kb
445	13508-L14972	Exon 8	828-829	TTCGACAAAATC-AAGAACTGGAGG	1.4 kb
355	13502-L14966	Exon 9	1428-1429	CCACGCCCAGGA-GTCCTCTGCGA	
		<i>stop codon</i>	1453-1455 (<i>Exon 9</i>)		

Table 2b. *F8* gene

Length (nt)	SALSA MLPA probe	<i>F8</i> exon ^a	Ligation site NM_000132.4	Partial sequence ^b (24 nt adjacent to ligation site)	Distance to next probe
		<i>start codon</i>	172-174 (<i>Exon 1</i>)		
142	05610-L05060	Exon 1	24-25	GCACATCCAGTG-GGTAAAGTTCTT	25.7 kb
312	06506-L15887	Exon 3	538-537 reverse	TTTCCAGTAGGA-TACACCAACAGC	27.5 kb
247 ±	05617-L05067	Exon 7	984-985	CACAGGAAATCA-GTCTATTGGCAT	0.2 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	67.2 kb
328	05637-L15888	Exon 23	6637-6638	CTGGGATAAAAC-ACAATATTTTTA	
		<i>stop codon</i>	7225-7227 (<i>Exon 26</i>)		

Table 2c. *F9* gene

Length (nt)	SALSA MLPA probe	<i>F9</i> exon ^a	Ligation site NM_000133.4	Partial sequence ^b (24 nt adjacent to ligation site)	Distance to next probe
		<i>start codon</i>	27-29 (<i>Exon 1</i>)		
232	06789-L06381	Exon 1	144 nt after exon 1	AGGTTGGTAAGT-ACTGGTTCTTTG	6.1 kb
268	06790-L15258	Exon 2	187-188	TAAATTGGAAGA-GTTTGTTC AAGG	0.3 kb
220	06791-L06383	Exon 3	4 nt after exon 3	GTATGTTGGTAA-GCAATTCATTTT	3.7 kb
301	06792-L06384	Exon 4	321-322	ATCAGTGTGAGT-CCAATCCATGTT	7.3 kb
283	06793-L06385	Exon 5	465-466	AGTTTTGTAAAA-ATAGTGCTGATA	2.8 kb
208	06794-L28016	Exon 6	722-723	GGTGGAGAAGAT-GCCAAACCAGGT	9.5 kb
154	07280-L06904	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	
		<i>stop codon</i>	1410-1412 (<i>Exon 8</i>)		

^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.

± SNP rs34371500 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

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.

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.
- Payne AB et al. (2012). Utility of multiplex ligation-dependent probe amplification (MLPA) for hemophilia mutation screening. *J Thromb Haemost.* 10:1951.
- Pavlova A et al. (2015). Congenital combined deficiency of coagulation factors VII and X—different genetic mechanisms. *Haemophilia.* 21:386-391.
- 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
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 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 Table 1 for specificity of a reference probe relying on a single nucleotide difference between its targeted gene and related gene or pseudogene.
- Warning added to Table 1 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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