Product Description
SALSA® MLPA® Probemix P112-A4 PROS1

To be used with the MLPA General Protocol.

Version A4
For complete product history see page 7.

Catalogue numbers:
- **P112-025R:** SALSA MLPA Probemix P112 PROS1, 25 reactions.
- **P112-050R:** SALSA MLPA Probemix P112 PROS1, 50 reactions.
- **P112-100R:** SALSA MLPA Probemix P112 PROS1, 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 P112 PROS1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *PROS1* gene, which is associated with hereditary protein S deficiency.

Protein S is a vitamin K-dependent glycoprotein that regulates blood coagulation (Ten Kate et al. 2008). It functions as a non-enzymatic cofactor for activated protein C (APC) in the proteolytic degradation of activated factor V and VIII, resulting in the inhibition of blood coagulation. Protein S circulates in plasma in two forms, a free form and a bound form in complex with C4b-binding protein (Ten Kate et al. 2008). Although both forms can act as cofactors, bound protein S is less effective than free protein S.

Hereditary protein S deficiency is an autosomal dominant disorder associated with an increased risk for venous thrombosis. In fact, while the prevalence of protein S deficiency is 0.2% in the general population, it is about 2% for patients with venous thrombosis (Ten Kate et al. 2008). There are three types of protein S deficiency that can be distinguished based on APC cofactor activity, total protein S and free protein S levels (Ten Kate et al. 2008). Type I is characterized by low levels of both total and free protein S and reduced APC cofactor activity. Type II shows normal levels of free and total protein S, but a reduced APC cofactor activity. Type III deficiency is characterized by normal total protein S levels and reduced free protein S and APC cofactor activity. Large deletions/duplications within the *PROS1* locus have been identified as a relatively common cause of protein S deficiency (Pintao et al. 2009).

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 *PROS1* exon numbering used in this P112-A4 PROS1 product description is the exon numbering from the LRG_572 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 P112-A4 PROS1 contains 26 MLPA probes with amplification products between 136 and 382 nucleotides (nt). This includes 13 probes for the *PROS1* gene and one flanking probe for the *ARL13B* gene located upstream of *PROS1*. Furthermore, this probemix contains one probe for the protein S (beta) pseudogene (*PROSP* or *PROS2* or *PROS2P*), which shows 96.5% homology to exons 3 to 16 of the *PROS1* gene. In addition, 11 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 hereditary protein S deficiency. 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

- 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.

***PROS1* mutation database**

https://databases.lovd.nl/shared/genes/PROS1. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD). Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results (e.g., a duplication of *PROS1* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P112-A4 PROS1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*PROS1*
64-105	Control fragments – see table in probemix content section for more information		
136	Reference probe 00981-L00566	10p	
142	**PROS1 probe** 05294-L04681		**Exon 5**
148	**PROSP probe** 05294-L05462		**Pseudogene**
160	Reference probe 02957-L02389	7q	
166	**PROS1 probe** 05291-L05463		**Exon 1a**
172	**PROS1 probe** 05299-L04687		**Exon 11**
178	Reference probe 02465-L01909	15q	
184	Reference probe 02590-L02590	5q	
190	**PROS1 probe** 05292-L04679		**Exon 1a**
196	**PROS1 probe** 05300-L04688		**Exon 12**
211	Reference probe 02613-L03903	6p	
219	**PROS1 probe** 05293-L04680		**Exon 3**
229	**PROS1 probe** 05301-L04689		**Exon 13**
247	Reference probe 00816-L00334	21q	
256	**PROS1 probe** 05295-L04683		**Exon 6**
265	**PROS1 probe** 05302-L04690		**Exon 14**
283	Reference probe 02414-L01860	16q	
292	**PROS1 probe** 05296-L04684		**Exon 7**
302	Reference probe 05285-L04666	14q	
310	Reference probe 01293-L00838	9p	
328	**PROS1 probe** 05297-L04685		**Exon 8**
337	**PROS1 probe** 06283-L05791		**Exon 16**
355	Reference probe 03895-L01219	18q	
364	**PROS1 probe** 05298-L05464		**Exon 10**
373 ¬	**ARL13B probe** 05305-L04693		**Upstream**
382 *	Reference probe 20537-L28127	1q	

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

* New in version A4.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

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. *PROS1* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*PROS1* exon[a]	Ligation site NM_000313.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
373 ¬	05305-L04693	*ARL13B*		ATCTGCGTCTTT-GGTCAGGTTGTT	6.5 kb
		start codon	*148-150 (Exon 1a)*		
166	05291-L05463	Exon 1a	223-224	CAGAGGCAAACT-GTGAGTAATCAA	0.1 kb
190	05292-L04679	Exon 1a	66 nt after exon 1a	GTGCCCTGGTTG-GTAGGATTTTCT	46.3 kb
	No probe	Exon 2			
219 #	05293-L04680	Exon 3	347-348	CAATAAAGAAGA-AGCCAGGGAGGT	16.6 kb
	No probe	Exon 4			
142 #	05294-L04681	Exon 5	451-452	CTGCTGCACGTC-AGTCAACTAATG	4.5 kb
256 #	05295-L04683	Exon 6	546-545, reverse	CCATCTTTGCAG-CTCATATATCCA	0.3 kb
292 #	05296-L04684	Exon 7	710-711	TTCCTGTAAAAA-TGGTTTTGTTAT	5.0 kb
328 #	05297-L04685	Exon 8	834-835	TGTGAATGCCCC-GAAGGCTACAGA	4.2 kb
	No probe	Exon 9			
364	05298-L05464	Exon 10	1066-1067	CGGAGCAGTTTG-CAGGGGTTGTTT	3.5 kb
172 #	05299-L04687	Exon 11	1167-1166, reverse	ATAGATTCTGCG-TACAGTATCACG	7.0 kb
196 #	05300-L04688	Exon 12	217 nt after exon 12	CATTTAAATCCC-CAGCATAAATCA	1.2 kb
229 #	05301-L04689	Exon 13	1498-1497, reverse	ATTCCAGCTTCG-TATACATCCATC	5.6 kb
265 #	05302-L04690	Exon 14	1700-1701	TCGTCCATCCAC-GGGCACTGGTGT	5.7 kb
	No probe	Exon 15			
337 #	06283-L05791	Exon 16	2831-2830, reverse	GATATCTCATCC-TGACAGACTGCA	4.7 **M**b
		stop codon	*2176-2178 (Exon 16)*		
148 + #	05294-L05462	*PROSP*		CTGCTGCACATT-AGTCAACTAATG	

[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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.
+ The 148 nt PROSP probe is expected to give also a small (20-25%) signal on the *PROS1* exon 5 sequence. The *PROSP* gene is located on chromosome 3p, close to the centromere. The *PROS1* gene is also located close to the centromere but on the 3q arm.
# 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. Single probe aberration(s) must be confirmed by another method.

Related SALSA MLPA probemixes

P265 PROC	Protein C deficiency. Contains probes targeting the *PROC* gene.
P227 SERPINC1	Antithrombin deficiency. Contains probes targeting the *SERPINC1* gene.

References

- Pintao MC et al. (2009). Gross deletions/duplications in *PROS1* are relatively common in point mutation-negative hereditary protein S deficiency. *Hum Genet.* 126:449-456.
- 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.
- Ten Kate MK et al. (2008). Protein S deficiency: a clinical perspective. *Haemophilia*. 14:1222-1228.
- 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 P112 PROS1

- Castoldi E et al. (2010). Similar hypercoagulable state and thrombosis risk in type I and type III protein S-deficient individuals from families with mixed type I/III protein S deficiency. *Haematologica*. 95:1563-1571.
- Duebgen S et al. (2012). Genotype and laboratory and clinical phenotypes of protein S deficiency. *Am J Clin Pathol.* 137:178-184.
- Kim HJ et al. (2014). Distinct frequencies and mutation spectrums of genetic thrombophilia in Korea in comparison with other Asian countries both in patients with thromboembolism and in the general population. *Haematologica*. 99:561-569.
- Pintao MC et al. (2009). Gross deletions/duplications in *PROS1* are relatively common in point mutation-negative hereditary protein S deficiency. *Hum Genet*. 126:449-456.
- Seo JY et al. (2014). The genomic architecture of the *PROS1* gene underlying large tandem duplication mutation that causes thrombophilia from hereditary protein S deficiency. *Gene*. 547:295-299.
- Tang L et al. (2013). Molecular basis of protein S deficiency in China. *Am J Hematol*. 88:899-905.
- Wypasek E et al. (2017). Genetic characterization of antithrombin, protein C, and protein S deficiencies in Polish patients. *Pol Arch Intern Med.* 127:512-523.

P112 product history	
Version	*Modification*
A4	One reference probe has been replaced and three reference probes have been removed.
A3	Two reference probes have been removed.
A2	The 88, 96, 100 and 105 nt control fragments (QDX2) have been included.
A1	First release.

Implemented changes in the product description
Version A4-02 – 01 December 2021 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *PROS1* gene updated according to new version of the NM_ reference sequence. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. Version A4-01 – 07 May 2018 (01P) - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

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