Instructions for Use
SALSA® MLPA® Probemix P178 F8

See also the MLPA General Protocol, the product description of the SALSA® MLPA® Reagent Kit and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P178 F8 product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P178 F8**
Version	B4
Catalogue numbers	P178-025R (25 reactions) P178-050R (50 reactions) P178-100R (100 reactions)
Basic UDI-DI	872021148P1786E
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

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 B3, one reference probe has been replaced.

1. Intended Purpose

The SALSA MLPA Probemix P178 F8 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 *F8* gene in genomic DNA isolated from human peripheral whole blood specimens. P178 F8 is intended to confirm a potential cause for and clinical diagnosis of hemophilia A, and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P178 F8 should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *F8* gene are intron 22 and intron 1 inversions and point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with targeted intron 22 and intron 1 inversion analysis and 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.

[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. • At least three* independent reference samples required in each experiment for proper data normalisation. Derived from unrelated individuals from families without a history of hemophilia A.	
No-DNA Control (Preferably)	• Provided by user • $TE_{0.1}$ buffer instead of DNA • To check for DNA contamination	
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.

*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	**Female:** 2 **Male**: 1	Normal

Typical Results of X Probes (*F8*)

Final Ratio (FR)	Copy Number Female	Copy Number Male	Description
0	0	0	**Female:** Homozygous deletion **Male**: Deletion
0.40 – 0.65	1	-	**Female:** Heterozygous deletion
0.80 – 1.20	**2**	**1**	**Normal**
1.30 – 1.65	3	-	**Female:** Heterozygous duplication
1.75 – 2.15	4	2	**Female**: Homozygous duplication or Heterozygous triplication **Male:** Duplication
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.

6. Performance Characteristics

Study	Description
Expected values for copy numbers in normal and affected populations	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 in the product description can be used. Cut-off values for copy number determination were verified with SALSA MLPA Probemix P178 F8 in 68 samples from healthy individuals with normal copy number and eight samples with known CNVs. The expected FRs for the corresponding copy number were found in over 99% of measurements across all tested samples. Less than 1% of measurements yielded FRs in the ambiguous range.
Limit of detection	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 recommended 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 P178 F8 on two samples with known CNVs and on one sample without any mutation and expected results were obtained using both the lower and upper input amount of DNA.
Interfering substances	SNVs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl, EDTA and hemoglobin) can affect the MLPA reaction. A study using SALSA MLPA Probemix P178 F8 was performed to assess the potential for interference of endogenous and exogenous substances on genomic DNA on samples with known CNVs. For most 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.

Interferent	Source	Testing Concentration	Results*
EDTA	Exogenous – specimen collection tubes	1.5 mM	Copy number: Expected FR for 290/297 measurements
NaCl	Exogenous – DNA extraction	40 mM	Copy number: Expected FR for 291/297 measurements

	Fe^{3+} ($FeCl_3$)	Exogenous – DNA extraction	1 µM	Copy number: Expected FR for 295/297 measurements
	Heparin	Exogenous – specimen collection tubes	0.02 U/mL	Copy number: Expected FR for 296/297 measurements
	Hemoglobin	Endogenous – blood sample	0.02 µg/µl	Copy number: Expected FR for 277/297 measurements
	* Results are summarised for all probes across all four samples tested. Potential interference with the MLPA reaction was assessed by testing both endogenous (hemoglobin) and exogenous interfering substances (EDTA, heparin, salts (NaCl), and $FeCl_3$) using P178 F8. Hemoglobin had the largest effect on the FRs: an incorrect final ratio was determined and ambiguous results were obtained in all samples. DNA extraction methods from blood remove hemoglobin. Therefore, it is only when hemoglobin is in excess that deviating probe signals can be found. $FeCl_3$, heparin, EDTA and NaCl exhibited minimal interference with P178 F8 assay. In the few instances where interference occurred, the resulting FRs fell within the ambiguous range. Such values are not indicative of false positives and would at most, lead to delayed reporting. 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 to determine whether probes are specific to their target sequence and all probes met the quality criteria for specificity.			
Accuracy	Results of accuracy are derived from trueness and precision studies. For trueness, two previously genotyped samples were tested using SALSA MLPA Probemix P178 F8 and found to have the expected results. Assay precision was tested by repeatedly testing samples with known copy number over multiple days, and by multiple operators. Results showed a correct call in 1448/1485 data points, leading to a precision of >97%.			
Clinical validity*	*F8*: 5-10% of hemophilia A is caused by large deletions and duplications[1,2] (Guo et al. 2018, Lannoy and Hermans 2022, Li et al. 2023, Pezeshkpoor et al. 2015) *Based on a 2008-2024 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.

[1] EAHAD Factor VIII Gene (*F8*) Variant Database
[2] CDC Hemophilia Mutation Projects (CHAMP and CHBMP)

Content – Probe Details Sorted by Chromosomal Position

Chr. position	Target	Exon	Distance to next probe	Length (nt)	Probe number
Xq28	*F8*	Exon 26	1.5 kb	229	05641-L05091
Xq28	*F8*	Exon 26	22.8 kb	468	13708-L15182
Xq28	*F8*	Exon 25	1.3 kb	418	13707-L15181
Xq28	*F8*	Exon 24	1.4 kb	372	14164-L15766
Xq28	*F8*	Exon 23	32.9 kb	328	05637-L05087
Xq28	*F8*	Exon 22	3.8 kb	391	07045-L05898
Xq28	*F8*	Exon 21	1.5 kb	197	05634-L17547
Xq28	*F8*	Exon 20	0.7 kb	160	05633-L05083
Xq28	*F8*	Exon 19	1.9 kb	459	05632-L14950
Xq28	*F8*	Exon 18	0.5 kb	409	05631-L05081
Xq28	*F8*	Exon 17	0.5 kb	364	05630-L05080
Xq28	*F8*	Exon 16	1.6 kb	319	05629-L05079
Xq28	*F8*	Exon 15	22.3 kb	265	05628-L05078
Xq28	*F8*	Exon 14	1.5 kb	154	05627-L06055
Xq28	*F8*	Exon 14	0.9 kb	191	05626-L17546
Xq28	*F8*	Exon 14	16.5 kb	452	05625-L14949
Xq28	*F8*	Exon 13	6.1 kb	402	05624-L05074
Xq28	*F8*	Exon 12	0.1 kb	274	07044-L05899
Xq28	*F8*	Exon 12	3.1 kb	355	05623-L05893
Xq28	*F8*	Exon 11	4.1 kb	305	06287-L06058
Xq28	*F8*	Exon 10	4.9 kb	256	05621-L05071
Xq28	*F8*	Exon 9	0.6 kb	215	05620-L17549
Xq28	*F8*	Exon 8	2.7 kb	179	05619-L14947
Xq28	*F8*	Exon 7	0.2 kb	148	05618-L05068
Xq28	*F8*	Exon 7	15.2 kb	247	05617-L05067
Xq28	*F8*	Exon 6	2.5 kb	186	06290-L17545
Xq28	*F8*	Exon 5	5.7 kb	221	06288-L05892
Xq28	*F8*	Exon 4	4.0 kb	298	05614-L05891
Xq28	*F8*	Exon 3	0.1 kb	313	06506-L06340
Xq28	*F8*	Exon 3	2.5 kb	444	05613-L14948
Xq28	*F8*	Exon 2	22.9 kb	172	05612-L05062
Xq28	*F8*	Exon 1	0.3 kb	209	05611-L17548
Xq28	*F8*	Exon 1		142	05610-L05060
Xp	Reference			166	04423-L05579
Xp	Reference			136	07103-L14743
Xp	Reference			292	02920-L02314
Xp	Reference			477	01391-L01039
Xp	Reference			426	13207-L14528
Xq	Reference			202	13203-L14524
Xq	Reference			434	13118-L15558
Xq	Reference			238	06910-L06490
Xq	Reference			382	02908-L02302
Xq	Reference			346	05125-L04515

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 *F8* 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.
Chromosomal bands are based on: hg18.

7. Precautions and Warnings

Probe warnings
There are no probe warnings.

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. Copy number alterations of reference probes are unlikely to be related to the condition tested.

5. Before testing patient samples, testing of samples from healthy individuals is required to identify suitable reference samples for proper data analysis.
6. Skewed inactivation of the X-chromosome may significantly affect factor VIII expression (Garagiola et al. 2021, Shen et al. 2022). Additional testing is recommended to determine the clinical significance of heterozygous findings in symptomatic females.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Probemix-specific limitations
1. Duplication of *F8* exon 1-22 is unlikely to result in a risk of hemophilia A (Lannoy et al. 2013, Lannoy and Hermans 2018). Nevertheless, duplications in the *F8* gene warrant careful consideration in genetic counseling, while efforts to identify the underlying mutation responsible for hemophilia continue.
2. In most populations, the major cause of genetic defects in the *F8* gene are intron 22 inversions, intron 1 inversions and small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P178 F8.
3. DNA samples with gross deletions or duplications of the X-chromosome, including the sequences detected by the reference probes, are not suitable for analysis with SALSA MLPA Probemix P178 F8.

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. Guo Z et al. (2018). Spectrum of Molecular Defects in 216 Chinese Families With Hemophilia A: Identification of Noninversion Mutation Hot Spots and 42 Novel Mutations. *Clin Appl Thromb Hemost.* 24:70-78
2. Lannoy N et al. (2022). Accessibility and visibility of genetic testing for haemophilia across Europe: Where do we stand? *Haemophilia.*
3. Li F et al. (2023). Variant spectrum of F8 and F9 in hemophilia patients from southern China and 26 novel variants. *Front Genet.* 14:1254265
4. Pezeshkpoor B et al. (2015). Novel characterization of a breakpoint in F8: an individualized approach to gene analysis when PCR and MLPA results contradict. *Haemophilia.* 21:392-397.
5. Garagiola I et al. (2021). X Chromosome inactivation: a modifier of factor VIII and IX plasma levels and bleeding phenotype in Haemophilia carriers. *Eur J Hum Genet.* 29:241-249.
6. Shen MC et al. (2022). Skewed X-Chromosome Inactivation and Parental Gonadal Mosaicism Are Implicated in X-Linked Recessive Female Hemophilia Patients. *Diagnostics (Basel).* 12.
7. Lannoy N et al. (2013). Intron 22 homologous regions are implicated in exons 1-22 duplications of the F8 gene. *Eur J Hum Genet.* 21:970-976.
8. Lannoy N et al. (2018). Review of molecular mechanisms at distal Xq28 leading to balanced or unbalanced genomic rearrangements and their phenotypic impacts on hemophilia. *Haemophilia.* 24:711-719.

Implemented changes in the product description
Version B4-03 – 22 January 2026 (03S) - Product Description adapted to a new template. - Intended Purpose updated to clarify that the MLPA assay is manual. - Salt warning removed for F8 probes 07045-L05898, 05634-L17547, 05633-L05083, and 05632-L14950. - New probe notification removed for Reference probe 04423-L05579. - Precaution about skewed inactivation of the X-chromosome added to section 7. Precautions and Warnings. - Specific Note regarding *F8* exon 1-22 duplication from the previous Product Description version was modified and added to section 8. Limitations. - Probemix is now IVDR certified.