Product Description
SALSA® MLPA® Probemix P159-A5 GLA

To be used with the MLPA General Protocol.

Version A5
For complete product history see page 8.

Catalogue numbers:
- **P159-025R:** SALSA MLPA Probemix P159 GLA, 25 reactions.
- **P159-050R:** SALSA MLPA Probemix P159 GLA, 50 reactions.
- **P159-100R:** SALSA MLPA Probemix P159 GLA, 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.

Intended purpose
The SALSA MLPA Probemix P159 GLA is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in the *GLA* gene in genomic DNA isolated from human peripheral whole blood specimens. P159 GLA is intended to confirm a potential cause for and clinical diagnosis of Fabry disease and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P159 GLA 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 *GLA* gene are point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with 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 in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).
[2]To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

Clinical background
Fabry disease (OMIM: 301500) (Anderson-Fabry disease, Alpha-galactosidase A deficiency) is an X-linked disorder of glycosphingolipids that is caused by the deficiency of α-galactosidase A caused by defects in the galactosidase alpha (*GLA*) gene and usually has its onset in childhood or adolescence. Fabry disease is associated with the dysfunction of many cell types and includes a systemic vasculopathy (blood vessel disease). As a result, patients have a markedly increased risk of developing small-fiber peripheral neuropathy (disorders of the peripheral nervous system), stroke, myriad cardiac manifestations and chronic renal disease.

Virtually all complications of Fabry disease are non-specific in nature and clinically indistinguishable from similar abnormalities that occur in the context of more common disorders in the general population.

Although this disease was originally thought to be very rare, studies have found a much higher incidence of mutations in the *GLA* gene, suggesting that this disorder is under-diagnosed (Schiffmann 2009).

More information is available at: https://www.ncbi.nlm.nih.gov/books/NBK1292/.

Gene structure
The *GLA* gene (7 exons) spans ~17 kb of genomic DNA and is located on chromosome Xq22.1, about 101 Mb from the p-telomere. The *GLA* LRG_672 is available at www.lrg-sequence.org and is identical to GenBank NG_007119.1.

Transcript variants
For *GLA*, several transcript variants have been described. Transcript variant 1 (NM_000169.3, 1318 nt, coding sequence 23-1312, https://www.ncbi.nlm.nih.gov/gene/2717) is the MANE Select transcript. The ATG translation start site of this transcript is located in exon 1 (23-25) and the stop codon is located in exon 7 (1310-1312).

Exon numbering
The *GLA* exon numbering used in this P159-A5 GLA product description is the exon numbering from the LRG_672 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 P159-A5 GLA contains 25 MLPA probes with amplification products between 146 and 355 nucleotides (nt). This includes 8 probes for the *GLA* gene (one probe for each exon and an additional probe for exon 2), 3 flanking probes for genes located upstream of *GLA* (including *HNRNPH2,* reported to be part of a deletion also comprising *GLA* exons 1 and 2 (Schirinzi et al. 2008)) and 3 flanking probes for downstream genes. In addition, 11 reference probes are included that detect locations on the X-chromosome. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains ten quality control fragments generating amplification products between 64 and 121 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and two chromosome Y-specific fragments (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)
105-121	Y-fragments (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 from peripheral whole blood specimens, 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 Fabry disease. As all probes in P159 target the X chromosome, the gender of the reference samples used in an experiment is not important. Target probes are first normalised to reference probes within a sample. Therefore, a ratio of 1 corresponds to 1 copy in male samples as they have one X chromosome. 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.

Performance characteristics
The majority of Fabry disease patients have pathogenic variants that are easily detected by sequence analysis. It is estimated that approximately ~5% of all Fabry disease patients have deletions or duplications in the *GLA* gene; either involving part of the gene, the whole gene or deletions extending beyond the *GLA* gene. When MLPA is used in addition to sequence analysis of the *GLA* gene, the detection rate generally increases by ~5 percent points. The analytical sensitivity and specificity (based on a 2008-2022 literature review) for the detection of deletions in *GLA* is very high and can be considered >99%.

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

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 expected results for the GLA probes in female DNA samples are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication) and occasionally 4 (heterozygous triplication). For male DNA samples, copy numbers of 1 (normal), 0 (deletion), or 2 (duplication) can be expected.

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:

Copy Number status: Male samples	**Final ratio (FR)**
Normal	0.80 < FR < 1.20
Deletion	FR = 0
Duplication	1.65 < FR < 2.25
Ambiguous copy number	All other values

Copy Number status: Female samples	**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 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.

Notes regarding interpretation of results for the SALSA MLPA probemix P159 GLA

- Deletion or duplication of only the flanking probes are not expected to be the cause of Fabry disease. These probes have only been included to delineate the extent of large deletions and duplications.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *GLA* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P159 GLA.
- 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.

***GLA* mutation database**

https://databases.lovd.nl/shared/genes/GLA. We strongly encourage users to deposit positive results in the *GLA* gene 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 *GLA* exons 2 and 4 but not exon 3) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P159-A5 GLA

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	Flanking	*GLA*
64-121	Control fragments – see table in probemix content section for more information			
146	Reference probe 01394-L01042	Xp21		
154 ¬	Flanking probe 2 01274-L01640		Xq22.2; 2369 kb upstream GLA	
161	Reference probe 16675-L20191	Xp11		
166	Reference probe 02927-L03721	Xq27		
178	**GLA probe** 05153-L04557			**Exon 1**
185 ±	**GLA probe** 05158-L08283			**Exon 5**
190	Reference probe 07653-L14466	Xp11		
196 ¬	Flanking probe 6 06926-L06506		Xq22.1; 48 kb downstream GLA	
206	**GLA probe** 05154-L28096			**Exon 2**
217	**GLA probe** 05159-L28097			**Exon 6**
222	**GLA probe** 05178-L28098			**Exon 2**
229	Reference probe 02922-L04201	Xp21		
238	**GLA probe** 05160-L04564			**Exon 7**
247	Reference probe 03650-L03063	Xp22		
256	**GLA probe** 05156-L04560			**Exon 3**
265 ¬ ≈	Flanking probe 3 (HNRNPH2) 05181-L04553		Xq22.1; 0.3 kb upstream GLA	
275	Reference probe 04124-L03481	Xq22		
283 ¬	Flanking probe 4 05183-L04555		Xq22.1; 2 kb downstream GLA	
291 ¬	Flanking probe 1 05856-L05256		Xq22.3; 7144 kb upstream GLA	
298	Reference probe 06476-L26217	Xp22		
310	Reference probe 03493-L02870	Xq28		
319	**GLA probe** 06500-L04561			**Exon 4**
328	Reference probe 12605-L13689	Xq12		
337 ¬	Flanking probe 5 06911-L06491		Xq22.1; 26 kb downstream GLA	
355	Reference probe 13524-L14330	Xq21		

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

¬ 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.

± SNP rs104894841 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

≈ This flanking probe has been reported to be part of a deletion also comprising *GLA* exons 1 and 2 (Schirinzi et al. 2008).

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 table above. Single probe aberration(s) must be confirmed by another method.

Table 2. *GLA* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_000169.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
291 ¬	05856-L05256	Flanking probe 1		TGATGGAATTCC-AGGGCCACCAGG	4775.3 kb
154 ¬	01274-L01640	Flanking probe 2		AGCCACAAAGCA-GACTAGCCAGCC	2368.6 kb
265 ¬ ≈	05181-L04553	Flanking probe 3 (*HNRNPH2* gene)		TTAGAGGTTGGT-GTGTGGGTGGGA	0.5 kb
GLA probes					
		start codon	*23-25 (Exon 1)*		
178	05153-L04557	Exon 1	133-134	AATGGATTGGCA-AGGACGCCTACC	3.8 kb
206	05154-L28096	Exon 2	241-242	ATGGAGATGGCA-GAGCTCATGGTC	0.1 kb
222	05178-L28098	Exon 2	339-340	AGAAGGCAGACT-TCAGGCAGACCC	2.1 kb
256	05156-L04560	Exon 3	476-477	GTTTTGGATACT-ACGACATTGATG	1.0 kb
319	06500-L04561	Exon 4	609-610	TAGGACTGGCAG-AAGCATTGTGTA	1.8 kb
185 ±	05158-L08283	Exon 5	701-702	GCAATCACTGGC-GAAATTTTGCTG	0.5 kb
217	05159-L28097	Exon 6	969-970	GGATAAGGACGT-AATTGCCATCAA	0.5 kb
238	05160-L04564	Exon 7	1149-1150	GGGTAAAGGAGT-GGCCTGTAATCC	2.1 kb
		stop codon	*1310-1312 (Exon 7)*		
283 ¬	05183-L04555	Flanking probe 4		AGAGCCATCAAT-ACAATTCCGCTT	24.2 kb
337 ¬	06911-L06491	Flanking probe 5		AAATTTCAATCA-TTGAAAGGTTCC	21.7 kb
196 ¬	06926-L06506	Flanking probe 6		TCCCACTTTCAA-AATTCTTCTGAG	

[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.

± SNP rs104894841 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

≈ This flanking probe has been reported to be part of a deletion also comprising *GLA* exons 1 and 2 (Schirinzi et al. 2008).

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

P164 IDS Contains probes for the *IDS* gene, involved in another X-linked lysosomal storage disease.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schiffmann R (2009). Fabry disease. *Pharmacol Ther.* 122:65-77.
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- 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.

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- De Schoenmakere G et al. (2008). Two-tier approach for the detection of alpha-galactosidase A deficiency in kidney transplant recipients. *Nephrol Dial Transplant*. 23:4044-4048.
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- Torra R & Ortíz A (2012). Fabry disease: the many faces of a single disorder. *Clin Kidney J*. 5:379-382.
- Yoshimitsu M et al. (2011). Identification of novel mutations in the alpha-galactosidase A gene in patients with Fabry disease: pitfalls of mutation analyses in patients with low alpha-galactosidase A activity. *J Cardiol*. 57:345-353.

P159 product history	
Version	*Modification*
A5	The length of the Y control fragment has been elongated from 118 nt to 121 nt.
A4	Four reference probes have been replaced.
A3	The 88, 96 and 118 nt control fragments have been replaced.
A2	Two reference probes have been replaced. Three extra reference probes, probes for genes nearby *GLA* and extra control fragments at 88, 96, 100 and 105 nt have been added.
(A)	First release.

Implemented changes in the product description

Version A5-04 – 24 November 2022 (04P)
- Adjusted probe length (161 nt) in Table 1 in order to better reflect the true length of the amplification product.
- Reference probe location adjusted in Table 1.
- Adjusted flanking probe numbering in Table 1.
- Various minor textual and layout changes.
- Adjusted section 'Transcript variants'.

Version A5-04 – 12 October 2021 (04P)
- Product description adapted to a new template.
- Warning for SNP rs104894841 added to Table 1 and 2.
- Chapter 'Reference samples' updated (using samples from different genders is possible).
- Small textual changes.
- Additional information added on the HNRNPH2 flanking probe, including relevant literature reference.
- UK added to the list of European countries that accept the CE-mark.

Version A5-03 – 22 September 2020 (02P)
- Updated NM_ sequence from NM_000169.2 to NM_000169.3
- Product description adapted to a new template.

Version A5-02 – 10 April 2020 (04) - Israel was added as a country where P159 GLA is registered as an IVD.

More information: www.mrcholland.com; www.mrcholland.eu	
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	EUROPE*  ISRAEL
	ALL OTHER COUNTRIES

*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.