Product Description SALSA® MLPA® Probemix P453-A2 GAA

To be used with the MLPA General Protocol.

Version A2. For complete product history see page 5.

Catalogue numbers:
- **P453-025R:** SALSA MLPA Probemix P453 GAA, 25 reactions.
- **P453-050R:** SALSA MLPA Probemix P453 GAA, 50 reactions.
- **P453-100R:** SALSA MLPA Probemix P453 GAA, 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.mlpa.com).

Certificate of Analysis: Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mlpa.com.

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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 P453 GAA is a **research use only (RUO)** assay for the detection of deletions or duplications in the lysosomal acid alpha-glycosidase (*GAA*) gene, which is associated with Pompe disease.

Acid alpha-glycosidase is active in lysosomes where it breaks down glycogen. Mutations in the *GAA* gene cause Pompe disease, a condition where the activity of acid alpha-glycosidase is significantly reduced. As a results, glycogen builds up to toxic levels in the lysosome. This accumulation damages multiple organs and tissues, particularly muscles, leading to progressive muscle weakness, heart problems, and other features.
Pompe disease can be classified by age of onset and severity. Infantile-onset Pompe disease (IOPD) begins before the age of 12 months, usually within a few months after birth, and is characterized by cardiomyopathy. Late-onset Pompe disease (LOPD) includes individuals with onset before 12 months of age without cardiomyopathy and individuals with onset after 12 months.
Pompe disease is inherited in an autosomal recessive manner. Approximately 5-13% of pathogenic *GAA* variants are intragenic deletions or duplications.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1261/

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 *GAA* exon numbering used in this P453-A2 GAA product description is the exon numbering from the RefSeq transcript NM_000152.5, which is identical to the LRG_673 sequence. The exon numbering and NM_ sequence used have been retrieved on 03/2020. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P453-A2 GAA contains 27 MLPA probes with amplification products between 130 and 400 nucleotides (nt). This includes 18 probes for the *GAA* gene, one probe for each exon, with the exception of exons 2 and 11. In addition, nine 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.mlpa.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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment 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.mlpa.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 unrelated individuals who are from families without a history of Pompe disease. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample in your MLPA experiments. Sample ID number NA11661 and NA16445 from the Coriell Institute have been tested with this P453-A2 probemix at MRC-Holland and can be used as a positive control samples to detect a heterozygous deletion of *GAA* exon 18 or heterozygous duplication of the complete *GAA* gene, respectively. 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.mlpa.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 dosage quotient (DQ) 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 DQ of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

- 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. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the *GAA* gene. 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.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. 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: lower injection voltage / injection time settings, 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 *GAA* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P453 GAA.
- 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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.

***GAA* mutation database:** https://databases.lovd.nl/shared/genes/GAA. 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 SNPs and unusual results (e.g., a duplication of *GAA* exons 3 and 5 but not exon 4) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P453-A2 GAA

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	GAA
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 18835-L24359	3p14	
136	Reference probe 17174-L20399	15q21	
148 «	**GAA probe** 19863-L26719		**Exon 6**
157 «	**GAA probe** 19864-L26720		**Exon 1**
166	**GAA probe** 19865-L26721		**Exon 16**
184	**GAA probe** 19866-L27067		**Exon 19**
193 «	**GAA probe** 20050-L27374		**Exon 4**
214	Reference probe 07733-L07423	20q13	
221 «	**GAA probe** 19869-L27066		**Exon 5**
229	**GAA probe** 19870-L26726		**Exon 18**
239 «	**GAA probe** 19871-L26727		**Exon 8**
247	Reference probe 17856-L26079	19q13	
254	**GAA probe** 19872-L26728		**Exon 15**
265 «	**GAA probe** 19873-L26729		**Exon 13**
274 «	**GAA probe** 19874-L26730		**Exon 7**
283	Reference probe 19759-L26542	9q34	
292 «	**GAA probe** 19875-L26731		**Exon 3**
301 «	**GAA probe** 19876-L26732		**Exon 14**
310 «	**GAA probe** 19877-L26733		**Exon 10**
319	Reference probe 17521-L21420	2q32	
328 «	**GAA probe** 19878-L26734		**Exon 12**
349 «	**GAA probe** 19880-L26736		**Exon 20**
361	Reference probe 07034-L22804	14q11	
371 Ж «	**GAA probe** 19881-SP0879-L27065		**Exon 17**
380 «	**GAA probe** 19882-L26738		**Exon 9**
391	Reference probe 14984-L16720	6q22	
400	Reference probe 16282-L18574	11q13	

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

a) See above section on exon numbering for more information.

Table 2. GAA probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	GAA exon[a]	Ligation site NM_000152.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*343-368 (exon 2)*		
157 «	19864-L26720	Exon 1	200-201	GACAGTGACCTC-GGTGACGCGAAG	4.0 kb
	No probe	Exon 2			
292 «	19875-L26731	Exon 3	969-970	TCCCCACTCTAC-AGCGTGGAGTTC	1.8 kb
193 «	20050-L27374	Exon 4	1140-1139 reverse	CTGAGCATCAGG-GGACTGAGGTGC	0.2 kb
221 «	19869-L27066	Exon 5	1288-1289	TGCTAAACAGCA-ATGCCATGGGTA	0.4 kb
148 «	19863-L26719	Exon 6	1328-1329	CCCTGCCCTTAG-CTGGAGGTCGAC	0.2 kb
274 «	19874-L26730	Exon 7	1423-1424	CTGCAGGATACC-CGTTCATGCCGC	0.3 kb
239 «	19871-L26727	Exon 8	1595-1596	CACGTTCAACAA-GGATGGCTTCCG	1.3 kb
380 «	19882-L26738	Exon 9	1776-1777	CCGCTGATTGGG-AAGGTAGGGCGA	0.8 kb
310 «	19877-L26733	Exon 10	1856-1857	CATGGTGGCTGA-GTTCCATGACCA	1.2 kb
	No probe	Exon 11			
328 «	19878-L26734	Exon 12	2054-2053 reverse	AGAGGTTGTGCA-GGTTGTAGTGTG	0.7 kb
265 «	19873-L26729	Exon 13	2224-2225	TCGCCTCCTCCG-TGCCAGGTGAGC	0.3 kb
301 «	19876-L26732	Exon 14	2353-2352 reverse	GTGGTTCCGCAT-GAAGGGGTAGAA	0.4 kb
254	19872-L26728	Exon 15	2528-2529	GCCCCTCTTCCT-GGAGTGAGTGAC	3.7 kb
166	19865-L26721	Exon 16	2628-2629	GGGAAGGCCGAA-GTGACTGGCTAC	0.6 kb
371 Ж «	19881-SP0879-L27065	Exon 17	2688-2689 and 2714-2715	CCAGTAGAGGCC-26 nt spanning oligo-AGCTCCCCGTGA	0.7 kb
229	19870-L26726	Exon 18	2960-2961	GCGAGGGGCCTA-CACACAGGTCAT	0.4 kb
184	19866-L27067	Exon 19	3112-3113	GTGTCCCTGTCT-CCAACTTCACCT	0.8 kb
349 «	19880-L26736	Exon 20	3462-3463	AATAAGATTGTA-AGGTTTGCCCTC	
		stop codon	*3199-3201 (exon 20)*		

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

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.

P453 Product history	
Version	*Modification*
A2	One reference probe has been removed and one has been replaced.
A1	First release.

Implemented changes in the product description
Version A2-01 — 08 May 2020 (02P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *GAA* gene updated according to new version of the NM_ reference sequence. *Version 05 – 16 March 2018 (55)*

- Information added on positive control DNA samples on page 2.
- Various minor textual changes.

Version 04 – 18 July 2017 (55)

- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).
- New reference added on page 1.
- Various minor textual changes.

Version 03 – 03 March 2017 (55)

- Minor change: the LPO of the *GAA* exon 4 probe was erroneously named L26735. This has been updated to L27374. No difference in sequence detected between these two LPOs.

Version 02 – 07 January 2016 (55)

- Various minor textual changes on page 1.

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