Product Description SALSA® MLPA® Probemix P232-C2 FGD1

To be used with the MLPA General Protocol.

Version C2. As compared to version C1, one probe has been changed in length and the second Y probe has been removed. For complete product history see page 6.

Catalogue numbers:
- **P232-025R:** SALSA MLPA Probemix P232 FGD1, 25 reactions.
- **P232-050R:** SALSA MLPA Probemix P232 FGD1, 50 reactions.
- **P232-100R:** SALSA MLPA Probemix P232 FGD1, 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 P232 FGD1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *FGD1* gene, which is associated with Aarskog-Scott syndrome.

Aarskog-Scott syndrome, also known as faciogenital dysplasia (FGDY), is an X-linked disorder characterised by short stature and facial, skeletal and genital abnormalities. To date, mutations in the *FGD1* gene are the only known genetic cause of Aarskog-Scott syndrome. However, only ~20% of people with this syndrome have identifiable mutations in *FGD1*.

The *FGD1* gene (18 exons), spans ~51 kb of genomic DNA and is located on chromosome Xp11.22, ~55 Mb from the p-telomere.

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 *FGD1* exon numbering used in this P232-C2 FGD1 product description is the exon numbering from the RefSeq transcript NM_004463.3, which is identical to the NG_008054.1 sequence. The exon numbering and NM_ sequence used have been retrieved on 12/2019. 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 P232-C2 FGD1 contains 29 MLPA probes with amplification products between 142 and 463 nucleotides (nt). This includes 19 probes for the *FGD1* gene, one probe for each of the 18 exons and two probes for exon 1, and one flanking probe for the *MAGEH1* gene, which is located upstream of *FGD1*. In addition, nine 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.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 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 unrelated individuals who are from families without a history of Aarskog-Scott syndrome. 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. 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:

Copy Number status: Male samples	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Duplication	1.65 < DQ < 2.25
Ambiguous copy number	All other values

Copy Number status: Female samples	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 *FGD1* 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 *FGD1* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P232 FGD1.
- 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.

***FGD1* mutation database:** https://databases.lovd.nl/shared/genes/FGD1. 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 *FGD1* exons 7 and 9 but not exon 8) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P232-C2 FGD1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*FGD1*
64-105	Control fragments – see table in probemix content section for more information		
142	Reference probe 05610-L05060	Xq28	
153	**FGD1 probe** 07659-L07365		**Exon 15**
158	**FGD1 probe** 07649-L08158		**Exon 5**
165	Reference probe 07099-L06679	Xp22	
178	**FGD1 probe** 07655-L07361		**Exon 11**
190	**FGD1 probe** 07653-L07359		**Exon 9**
202 «	**FGD1 probe** 07645-L07351		**Exon 1**
214	**FGD1 probe** 07648-L07354		**Exon 4**
226	**FGD1 probe** 07654-L07360		**Exon 10**
242	**FGD1 probe** 07660-L23596		**Exon 16**
247 ¥	Reference probe 07665-L32006	Xp22	
257	Reference probe 01958-L01518	Xp21	
269 ¬	MAGEH1 probe 05779-L05217		Upstream
283	**FGD1 probe** 07661-L07367		**Exon 17**
292	Reference probe 10746-L12841	Xq28	
301 «	**FGD1 probe** 07644-L07350		**Exon 1**
319	**FGD1 probe** 07650-L08159		**Exon 6**
337	**FGD1 probe** 07656-L07362		**Exon 12**
346	Reference probe 05125-L04515	Xq26	
355	**FGD1 probe** 07658-L07364		**Exon 14**
373	**FGD1 probe** 07652-L07358		**Exon 8**
382	**FGD1 probe** 07646-L07352		**Exon 2**
391	Reference probe 16698-L19271	Xp11	
399	**FGD1 probe** 07657-L07363		**Exon 13**
418	**FGD1 probe** 07647-L07353		**Exon 3**
427	Reference probe 06188-L02097	Xq13	
436	**FGD1 probe** 07651-L07357		**Exon 7**
454	**FGD1 probe** 07662-L07368		**Exon 18**
463	Reference probe 06475-L06001	Xp22	

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

¥ Changed in version C2. Minor alteration, no change in sequence detected.
« 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.
¬ 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.

Table 2. *FGD1* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*FGD1* exon[a]	Ligation site NM_004463.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
269 ¬	05779-L05217	*MAGEH1* gene		CAAACTGAAAGT-CATGCATTTTGT	957.0 kb
		start codon	*803-805 (Exon 1)*		
301 «	07644-L07350	Exon 1	349-350	GCCCAGATCTAT-TCCCATTCGGGG	0.7 kb
202 «	07645-L07351	Exon 1	1079-1080	TGCGCTTCTCTT-ACCACCTGGAGG	23.9 kb
382	07646-L07352	Exon 2	17 nt after exon 2	TGGTCCTGGAAA-AGAGCAGAGAAG	0.7 kb
418	07647-L07353	Exon 3	1447-1448	TCCTCACTGATT-GAGAAGTTTGAA	0.5 kb
214	07648-L07354	Exon 4	1864-1865	AAGGACAGAGAA-ATCCCAGTGCCC	1.2 kb
158	07649-L08158	Exon 5	1932-1933	GGTGTTTCACAT-TGCCAATGAGCT	1.0 kb
319	07650-L08159	Exon 6	2079-2080	CTCTAACATCTG-CTCCATCTATTG	2.1 kb
436	07651-L07357	Exon 7	2215-2216	GGTGAGTATGTG-AAGAACTTTGAC	0.3 kb
373	07652-L07358	Exon 8	2395-2396	AAGGACTATCTG-TTAAAGCTGCCC	8.9 kb
190	07653-L07359	Exon 9	2452-2453	TCTCTGGAGCTG-ATCGCCACAGCA	0.2 kb
226	07654-L07360	Exon 10	2527-2528	CTGAAGGTATAT-GAGCTGTTAGGG	0.6 kb
178	07655-L07361	Exon 11	2662-2663	GACCGCCTCCTT-TACTGCGTGCCC	0.3 kb
337	07656-L07362	Exon 12	2775-2776	GCCTCGAACCTT-CCTGGTGTCAGG	5.2 kb
399	07657-L07363	Exon 13	2837-2838	AGGAGAAGAAAG-ACTGGGTCCAGG	0.5 kb
355	07658-L07364	Exon 14	2855-2856	CCCAGGCCATCA-ACTCCACCCTCC	0.6 kb
153	07659-L07365	Exon 15	3035-3036	AGCCCTTCAATT-CTATCACCAAAC	0.3 kb
242	07660-L23596	Exon 16	3126-3127	CGTCTATGACAA-CAACCGCTCCAA	1.6 kb
283	07661-L07367	Exon 17	3346-3347	GTCCCTGAAAAT-GAACCCTTGGTG	1.0 kb
454	07662-L07368	Exon 18	3477-3478	TGTCTTCAAGAT-CACCCAGAGCCA	
		stop codon	*3686-3688 (Exon 18)*		

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.

« 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.
¬ 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.

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.

P232 Product history	
Version	*Modification*
C2	One probe has been changed in length and the second Y probe has been removed.
C1	All reference probes and the Y-chromosome specific probe have been replaced. QDX2 fragments have been added.
B1	Extra reference probes and extra control fragments have been added. All reference probes now target autosomal chromosomes.
A1	First release.

Implemented changes in the product description
Version C2-02 – 19 February 2020 (02P) - Probe remark about influence of SNP rs142114385 on the FGD1 exon 9 probe (190 nt; 07653-L07359) removed. Testing of a positive sample showed no influence of SNP rs142114385 on the probe signal. - Various minor textual or layout changes. *Version C2-01 – 9 January 2020 (02P)* - Product description rewritten and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1). - Ligation sites of the probes targeting the *FGD1* gene updated according to new version of the NM_ reference sequence. - For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36). *Version 09 – 25 May 2016 (55)* - Product description adapted to a new lot (lot number added). - Various textual changes on page 1 and 2, and minor textual and layout changes throughout the document. - Ligation sites and exon numbers of the probes targeting the *FGD1* gene are checked according to NM_004463.2. *Version 08 (48)* - Electropherogram pictures of the old buffer (introduced Dec. 2012) removed.

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