Product Description SALSA® MLPA® Probemix P177-B3 CASR

To be used with the MLPA General Protocol.

Version B3. As compared to version B2, one reference probe has been replaced. For complete product history see page 6.

Catalogue numbers:
- **P177-025R:** SALSA® MLPA® probemix P177 CASR, 25 reactions.
- **P177-050R:** SALSA® MLPA® probemix P177 CASR, 50 reactions.
- **P177-100R:** SALSA® MLPA® probemix P177 CASR, 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 P177-B3 CASR is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CASR* gene.

Familial Hypocalciuric Hypercalcemia (FHH) is characterized by high levels of calcium in the blood and low levels of calcium in the urine. FHH is primarily caused by inactivating mutations in the *CASR* gene, while activating mutations cause Automosomal Dominant Hypocalcemia that is characterized by low levels of calcium in the blood. The protein encoded by the *CASR* gene is a G protein-coupled receptor expressed in cells of the parathyroid gland and the kidney. This protein senses small changes in circulating calcium concentrations to help maintaining a stable calcium concentration.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1116

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 exon numbering used in this P177-B3 product description is the exon numbering from the RefSeq transcript NM_000388.3. 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 P177-B3 CASR contains 23 MLPA probes with amplification products between 130 and 301 nt. This P177-B3 contains one probe for each exon of the *CASR* gene. Exons 2, 3, 4, 5, 6 and 7 are covered by two probes. Additionally, this probemix includes a probe for intron 1. Finally, nine reference probes are included, that detect several different 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, 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 from peripheral blood, 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 Hypo- or Hypercalcemia. 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 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. 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 *CASR* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P177-B3.
- 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.

***CASR* mutation database:** https://databases.lovd.nl/shared/genes/CASR. 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 *CASR* exons 4 and 6 but not exon 5) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P177-B3 CASR

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*CASR*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 00797-L00463	5q31	
137	**CASR probe** 13526-L14986		**Exon 6**
148	**CASR probe** 05704-L06957		**Exon 7**
154	Reference probe 13377-L14834	6q12	
160	Reference probe 11553-L12300	1q22	
166	**CASR probe** 05705-L05223		**Exon 1**
172	**CASR probe** 05710-L06958		**Exon 7**
178	**CASR probe** 13527-L15343		**Exon 2**
184	Reference probe 02312-L01803	19p13	
194	**CASR probe** 05703-L06959		**Exon 2**
200	**CASR probe** 05706-L06960		**Exon 3**
208	Reference probe 03924-L03379	15q21	
216	**CASR probe** 05707-L06963		**Exon 4**
222	**CASR probe** 05709-L15344		**Exon 6**
231	Reference probe 16398-L18813	17q22	
238	**CASR probe** 05708-L06962		**Exon 5**
247	Reference probe 08828-L08888	2p13	
256 Ø	**CASR probe** 13528-L14988		**Intron 1**
265	**CASR probe** 13529-L15345		**Exon 4**
274	Reference probe 08016-L07797	7q21	
283	**CASR probe** 13530-L14990		**Exon 5**
293	**CASR probe** 13531-L14991		**Exon 3**
301 *	Reference probe 21215-L29590	9p24	

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

* New in version B3 (from lot B3-1119 onwards).

Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of a single intron probe are unlikely to be related to the condition tested.

Table 2. CASR probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*CASR* exon[a]	Ligation site NM_000388.3	Partial sequence [b] (24 nt adjacent to ligation site)	Distance to next probe
		Start Codon	*373-375 (exon 2)*		
166	05705-L05223	Exon 1	109-110	CAGGCAACGCTT-GACCTGAGTCTT	1.1 kb
256 Ø	13528-L14988	Intron 1	1041 nt after exon 1	GTGATATAGTCA-GAAAGGACTCGC	69.1 kb
194	05703-L06959	Exon 2	170-171	GTGGCTTCCAAA-GACTCAAGGACC	0.2 kb
178	13527-L15343	Exon 2	381-382	ACCATGGCATTT-TATAGCTGCTGC	3.0 kb
200	05706-L06960	Exon 3	664-665	GATACAGGATAT-TTGACACTTGCA	0.1 kb
293	13531-L14991	Exon 3	788-789	TCCCTCTACGAT-TGCTGTGGTGGG	4.7 kb
216	05707-L06963	Exon 4	1303-1304	TGCCTCAGTACT-TCCACGTGGTTG	0.2 kb
265	13529-L15345	Exon 4	1529-1530	CAGGTTTAGCAA-CAGCTCGACAGC	13.6 kb
283	13530-L14990	Exon 5	1760-1761	GGTCCTGAAGCA-CCTACGGCATCT	0.1 kb
238	05708-L06962	Exon 5	1886-1887	CTCCATCGTGTT-TAAGGAAGTCGG	6.0 kb
137	13526-L14986	Exon 6	183 nt before exon 6, reverse	TGTGAAAGGGAC-AGATGACTATGG	0.3 kb
222	05709-L15344	Exon 6	2074-2075	AGTGTGTGGAGT-GTCCTGATGGGG	1.5 kb
148	05704-L06957	Exon 7	2137-2138	GCCCAGATGACT-TCTGGTCCAATG	1.4 kb
172	05710-L06958	Exon 7	3536-3537	CCCAGCACTTGT-AGTGTCCAGTTC	
		Stop Codon	*3607-3609 (exon 7)*		

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.

Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of a single intron probe are unlikely to be related to the condition tested.

Related SALSA MLPA probemixes

- P017 MEN1: Contains probes for the *MEN1* gene, involved in multiple endocrine neoplasia.
- P136 Gitelman Syndrome: Contains probes for the *SLC12A3* gene, involved in Gitelman Syndrome.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:57.
- 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.

Selected publications using SALSA MLPA Probemix P177 CASR

- Nissen PH et al. (2010). Multiplex ligation-dependent probe amplification (MLPA) screening for exon copy number variation in the calcium sensing receptor gene: no large rearrangements identified in patients with calcium metabolic disorders. *Clin Endocrinol.* 72:758-762.
- García-Castaño A et al (2018). Identification of a novel large CASR deletion in a patient with familial hypocalciuric hypercalcemia. *Endocrinology, diabetes & metabolism case reports*, 2018.1.

P177 Product history	
Version	*Modification*
B3	One reference probe has been replaced.
B2	Two reference probes has been replaced and the control fragments have been adjusted (QDX2).
B1	The number of CASR probes has been increased to 14. Two probes are now present for each exon. In addition four extra control probes at 88-96-100-105 nt has been included.
A1	First release.

Implemented changes in the product description

Version B3-01 – 16 January 2020 (02P)
- 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).

Version B2-01 – 19 February 2019 (01P)
- Product description restructured and adapted to a new template.
- Product description adapted to a new product lot (version number changed, lot number added, changes in Table 1 and Table 2).

Version 08 – 27 September 2015 (55)
- Various minor textual changes.
- Exon numbering of the *CASR* gene is changed in Table 1 and Table 2.
- Ligation sites of the probes targeting the *CASR* gene updated according to the NM_reference sequence.

Version 07 (53)
- 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).

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