Product Description
SALSA® MLPA® Probemix P176-C3 CAPN3

To be used with the MLPA General Protocol.

Version C3
For complete product history see page 7.

Catalogue numbers:
- **P176-025R:** SALSA MLPA Probemix P176 CAPN3, 25 reactions.
- **P176-050R:** SALSA MLPA Probemix P176 CAPN3, 50 reactions.
- **P176-100R:** SALSA MLPA Probemix P176 CAPN3, 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.

General information
The SALSA MLPA Probemix P176 CAPN3 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CAPN3* gene, which is associated with limb-girdle muscular dystrophy type 2A (LGMD2A).

Limb-girdle muscular dystrophies (LGMD) are a group of phenotypically and genotypically heterogeneous diseases, characterised by progressive weakness and atrophy of the muscles of the pelvic and shoulder girdle. Mutations of the *CAPN3* gene have been associated with limb-girdle muscular dystrophy type 2A (LGMD2A), also called calpainopathy. Patients with LGMD2A have symmetrical and selective involvement of proximal limb-girdle muscles. The disease shows wide intrafamilial and interfamilial clinical variability. The age at onset ranges from 2 to 40 years, but the disease usually first appears in the second or third decade of life, with the development of proximal weakness in the lower limbs. Mutations in *CAPN3* result in a cascade of events leading eventually to muscular dystrophy, but the precise underlying mechanisms have yet to be elucidated. However, a defect in proteolytic activity of calpain 3, the protein encoded by *CAPN3*, is largely recognised as the main pathogenic cause of LGMD2A.

The *CAPN3* gene (24 exons) spans ~53 kb of genomic DNA and is located on chromosome 15q15.1, ~40 Mb from the p-telomere. The gene is predominantly expressed in skeletal muscle where it is present in the cytosol as well as in the nucleus. The protein encoded by this gene (calpain 3) belongs to the superfamily of calcium-activated neutral proteases, which are non-lysosomal intracellular cysteine proteases. Calpains respond to Ca^{2+} signals by cleaving specific proteins, frequently components of signalling cascades, thereby irreversibly modifying their function(s).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1313/.

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 *CAPN3* exon numbering used in this P176-C3 CAPN3 product description is the exon numbering from the NG_008660.1 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 NG 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 P176-C3 CAPN3 contains 36 MLPA probes with amplification products between 130 and 427 nucleotides (nt). This includes 26 probes for the *CAPN3* gene. In addition, 10 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.mrcholland.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.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	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.mrcholland.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 different unrelated individuals who are from families without a history of LGMD2A. 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.

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 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 for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	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 or flanking 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.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *CAPN3* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P176 CAPN3.
- 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.

***CAPN3* mutation database**

We strongly encourage users to deposit positive results in the LOVD Database (https://databases.lovd.nl/shared/genes/CAPN3). 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 *CAPN3* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P176-C3 CAPN3

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*CAPN3*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 00797-L00463	5q	
142	**CAPN3 probe** 05785-L05232		**Exon 1**
148	**CAPN3 probe** 05795-L05242		**Exon 10**
154	Reference probe 03857-L03308	17q	
160	**CAPN3 probe** 05787-L05234		**Exon 2**
166	**CAPN3 probe** 21484-L30121		**Exon 11**
178	**CAPN3 probe** 05788-L05235		**Exon 3**
184	**CAPN3 probe** 05797-L05244		**Exon 13**
190	**CAPN3 probe** 10611-L11162		**Exon 15**
196	Reference probe 03547-L02913	11p	
203	**CAPN3 probe** 05789-L05236		**Exon 4**
211	**CAPN3 probe** 05798-L05245		**Exon 16**
221	Reference probe 01827-L01392	16p	

229	**CAPN3 probe** 05790-L05237		**Exon 5**
238	**CAPN3 probe** 05799-L05246		**Exon 17**
247	Reference probe 02317-L01808	19p	
256	**CAPN3 probe** 05791-L05238		**Exon 6**
265	**CAPN3 probe** 05800-L13102		**Exon 18**
275	Reference probe 12494-L13538	1q	
283	**CAPN3 probe** 05792-L05239		**Exon 7**
292	**CAPN3 probe** 10613-L11164		**Exon 20**
301	**CAPN3 probe** 10615-L11166		**Exon 23**
308	**CAPN3 probe** 17899-L11157		**Exon 24**
315	Reference probe 06741-L24262	8q	
320	**CAPN3 probe** 05802-L20124		**Exon 22**
330	Reference probe 01918-L21732	1q	
337	**CAPN3 probe** 05794-L05241		**Exon 9**
347	**CAPN3 probe** 17898-L11156		**Exon 8**
355	**CAPN3 probe** 10610-L11161		**Exon 14**
364	**CAPN3 probe** 05786-L05233		**Exon 1**
382	**CAPN3 probe** 10614-L11165		**Exon 21**
391 ±	**CAPN3 probe** 10609-L11160		**Exon 12**
400	Reference probe 15766-L24901	14q	
409	**CAPN3 probe** 10606-L11158		**Exon 4**
418	**CAPN3 probe** 10612-L11163		**Exon 19**
427	Reference probe 05561-L04993	7p	

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

± SNP rs28364489 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

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.

Table 2. CAPN3 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_000070.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*306-308 (Exon 1)*		
142	05785-L05232	Exon 1	454-455	CATCAGCCGCAA-TTTTCCTATTAT	0.1 kb
364	05786-L05233	Exon 1	572-573	TCTCTCTTTTAT-AGCCAGAAGTTC	24.5 kb
160	05787-L05234	Exon 2	1 nt after exon 2	GGAGAGCTAGGT-AGGAAAGTGCCT	1.7 kb
178	05788-L05235	Exon 3	758-759	GTCATACCCCAT-GATCAAAGTTTC	1.4 kb
409	10606-L11158	Exon 4	102 nt before exon 4	TCCAGGAAATGA-TGCTGCTTTGGG	0.1 kb
203	05789-L05236	Exon 4	815-816	TTCTGGCGCTAT-GGAGAGTGGGTG	1.3 kb
229	05790-L05237	Exon 5	1034-1035	GAGATCAGGGAT-GCTCCTAGTGAC	1.0 kb
256	05791-L05238	Exon 6	1147-1148	TCCTTCTGGTCT-GAACATGGGGGA	2.7 kb
283	05792-L05239	Exon 7	1271-1272	CCGGTTCAGTAT-GAGACAAGAATG	1.6 kb
347	17898-L11156	Exon 8	25 nt before exon 8	GGCTGCAGAGCA-TGAGAGCTCTTT	2.6 kb
337	05794-L05241	Exon 9	1441-1442	CTGGAGCTTTGT-GGACAAAGATGA	2.7 kb
148	05795-L05242	Exon 10	1548-1549	TGGAGATCTGCA-ACCTCACGGCCG	2.1 kb
166	21484-L30121	Exon 11	1699-1700	TCTGAAGCTCCT-GGAGGAGGACGA	0.4 kb
391 ±	10609-L11160	Exon 12	21 nt before exon 12	TCTGAAGCATCT-TCCTTTCTGTTT	0.8 kb
184	05797-L05244	Exon 13	1916-1917	AGCAAAACCTAC-ATCAACATGCGG	0.9 kb
355	10610-L11161	Exon 14	2057-2058	CACAGGGAAGTT-GAAAATACCATC	2.3 kb
190	10611-L11162	Exon 15	84 nt after exon 15	GTGTGAGCTCAT-ATGCATCCATGC	2.2 kb
211	05798-L05245	Exon 16	2136-2137	ACAGAGCAAACA-GCAACAAGGAGC	1.1 kb
238	05799-L05246	Exon 17	2261-2262	GAGGAACAGCAA-CAATTCCGGAAC	0.5 kb

265	05800-L13102	Exon 18	2318-2319	ATCTGTGCAGAT-GAGCTCAAGAAG	0.2 kb
418	10612-L11163	Exon 19	2386-2387	CGGGTTCACACT-GGAGTCCTGCCG	0.5 kb
292	10613-L11164	Exon 20	6 nt after exon 20	GGCAGGTGGGAA-GAGAAAATGAAG	0.1 kb
382	10614-L11165	Exon 21	2550-2551	ACGAGATGCGAA-ATGCAGTCAACG	0.3 kb
320	05802-L20124	Exon 22	2594-2595	AACCAGCTCTAT-GACATCATTACC	0.4 kb
301	10615-L11166	Exon 23	2706-2707	ATGCATTTGACA-AGGATGGAGATG	0.5 kb
308	17899-L11157	Exon 24	2761-2762	GCAGCTCACCAT-GTATGCCTGAAC	
		stop codon	*2769-2771 (Exon 24)*		

[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.

± SNP rs28364489 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

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.

Complete probe sequences are available at www.mrcholland.com.

Related SALSA MLPA probemixes

- P268 DYSF: contains probes for the *DYSF* gene involved in LGMD2B.
- P116 SGC: contains probes for the *SGCA*, *SGCB*, *SGCD*, *SGCG* and *FKRP* genes involved in LGMD2D, 2E, 2F, 2C, and 2I.
- P061 Lissencephaly: contains probes for the *POMT1* gene involved in LGMD2K.
- P048 LMNA/MYOT/ZMPSTE24: contains probes for the *LMNA* and *CAV3* genes involved in LGMD1B and 1C.
- P436 ANO5: contains probes for the *ANO5* gene, involved in LGMD2L.
- P034/P035 DMD: Duchenne muscular dystrophy and Becker muscular dystrophy. Contains probes for all *DMD* exons.
- P326 LARGE1: contains probes for the *FKTN* and *POMT2* genes, involved in LGMD2M and 2N.

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.

Selected publications using SALSA MLPA Probemix P176 CAPN3

- Stehlíková, K et al. (2014). Autosomal recessive limb-girdle muscular dystrophies in the Czech Republic. *BMC Neurol* 14.1:154.
- Ten Dam L et al. (2019). Autosomal recessive limb-girdle and Miyoshi muscular dystrophies in the Netherlands: The clinical and molecular spectrum of 244 patients. *Clinical Genetics* 96:126–133.

P176 product history	
Version	*Modification*
C3	Three reference probes have been replaced and three probe lengths have been adjusted.
C2	One reference probe has been removed.
C1	One reference probe has been removed.
B2	QDX2 control fragments have been added.
B1	Several CAPN3 probes have been added. All exons are now covered.
A1	First release.

Implemented changes in the product description
Version C3-01 – 11 December 2020 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *CAPN3* 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). - New reference added to Selected Publications. Version 11 – 27 June 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). - Various minor textual changes on pages 1 and 2. - New reference added on page 2. - Exon numbering of *CAPN3* has changed

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