Product Description SALSA® MLPA® Probemix P170-C3 APP

To be used with the MLPA General Protocol.

Version C3. As compared to version C2, three reference probes have been replaced and one probe length has been adjusted. For complete product history see page 6.

Catalogue numbers:
- **P170-025R:** SALSA MLPA Probemix P170 APP, 25 reactions.
- **P170-050R:** SALSA MLPA Probemix P170 APP, 50 reactions.
- **P170-100R:** SALSA MLPA Probemix P170 APP, 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 P170 APP is a **research use only (RUO)** assay for the detection of deletions or duplications in the *APP* gene, which is associated with Early-Onset Alzheimer's Disease (EOAD) and Cerebral Amyloid Angiopathy (CAA).

Duplications in the *APP* gene result in accumulation of amyloid-β peptides in the parenchymal and vascular deposits and have been implicated in EOAD and CAA. The size of duplications can vary among patients, ranging from 0.58 to 6.37 Mb.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1161/.

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 *APP* exon numbering used in this P170-C3 APP product description is the exon numbering from the RefSeq transcript NM_000484.4, which is identical to the NG_007376.2 sequence. The exon numbering and NM_ sequence used have been retrieved on 11/2019, we have adjusted the exon numbering of the 218 nt probe from exon 2 to intron 1 as the probe detects a exonic sequence in a different transcript variant (NM_001136016.3). The exon numbering used in previous versions of this product description can be found in between brackets in Table 2. 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 P170-C3 APP contains 38 MLPA probes with amplification products between 128 and 440 nucleotides (nt). This includes 21 probes for the *APP* gene, one probe for each exon of the gene, three probes for exon 1, and one probe for intron 1. Furthermore, five flanking probes upstream of the *APP* gene and three flanking probes downstream of the *APP* gene are included. 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, 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 Alzheimer's 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. 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

Copy number status	Dosage quotient
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 *APP* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P170 APP.
- 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.

***APP* mutation database:** https://databases.lovd.nl/shared/genes/APP and https://www.alzforum.org/mutations. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD) or the Alzforum 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 SNPs and unusual results (e.g., a duplication of *APP* exons 5 and 7 but not exon 6) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P170-C3 APP

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*APP*
64-105	Control fragments – see table in probemix content section for more information		
128	Reference probe 00797-L00093	5q31	
135	**APP probe** 06978-L06578		**Exon 1**
142	Reference probe 16417-L18870	18q21	
148	**APP probe** 18321-L23235		**Exon 8**
154	Reference probe 03857-L03308	17q11	
160	**APP probe** 06197-L05700		**Exon 1**
166 ¬	**NCAM2 probe** 00813-L00636		**Downstream**
170	**APP probe** 18322-L23236		**Exon 10**
177 ¥	**APP probe** 20914-L32071		**Exon 2**
186 *	Reference probe 19885-L29240	11q23	
196	**APP probe** 06203-L05706		**Exon 9**
202	**APP probe** 18323-L23237		**Exon 5**
211	**APP probe** 06199-L05702		**Exon 3**
218	**APP probe** 18324-L23238		**Intron 1**
227 *	Reference probe 15149-L16923	15q24	
238	**APP probe** 06201-L05704		**Exon 6**
247 ¬	**ADAMTS5 probe** 07592-L07277		**Upstream**
255 Ж	**APP probe** 18325-SP0641-L23239		**Exon 11**
265	**APP probe** 06204-L05707		**Exon 12**
274	**APP probe** 18326-L23240		**Exon 1**
282 ¬	**KCNE2 probe** 05073-L04473		**Upstream**
292	**APP probe** 06200-L05703		**Exon 4**
301	**APP probe** 18327-L23241		**Exon 17**
310 ¬	**BACH1 probe** 06983-L06585		**Upstream**
319	Reference probe 03082-L02482	8q23	
328	**APP probe** 20913-L05705		**Exon 7**
335 ¬ «	**RUNX1 probe** 02841-L02272		**Upstream**
346	**APP probe** 00987-L23493		**Exon 16**
355	**APP probe** 18362-L23316		**Exon 13**
362	**APP probe** 18328-L23242		**Exon 14**
373	**APP probe** 06206-L05709		**Exon 18**
382 ¬	**JAM2 probe** 06982-L06584		**Downstream**
391	Reference probe 15395-L17793	3p22	
400 ¬	**CYYR1 probe** 06980-L06582		**Upstream**
409	**APP probe** 18329-L23243		**Exon 15**
421 ¬	**ATP5J probe** 07593-L23492		**Downstream**
427 *	Reference probe 21340-L29746	7q31	
440	Reference probe 17277-L20720	2p13	

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

* New in version C3.
¥ Changed in version C3. 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.

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.
¬ 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. *APP* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*APP* exon[a]	Ligation site NM_000484.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
335 ¬ «	02841-L02272	*RUNX1*		ATTTCACCTGGA-CGTGCCAGCGGC	428.8 kb
282 ¬	05073-L04473	*KCNE2*		GCCAGAACACAA-CAGCTGAGCAAG	5049.2 kb
310 ¬	06983-L06585	*BACH1*		TAGCACCAATGT-TTTACTCAGCCT	2386.6 kb
247 ¬	07592-L07277	*ADAMTS5*		GACCTACCACGA-AAGCAGATCCTG	454.4 kb
400 ¬	06980-L06582	*CYYR1*		TTGCCATATGCA-TCTGCATGTGCA	309.3 kb
		start codon	*151-153 (Exon 1)*		
274	18326-L23240	Exon 1	NM_001136131.2; 44-45	GAGCGCTCTCGA-CTTTTCTAGAGC	0.3 kb
160	06197-L05700	Exon 1	32 nt before exon 1	GCTGACTCGCCT-GGCTCTGAGCCC	0.2 kb
135	06978-L06578	Exon 1	163-164	TGCTGCCCGGTT-TGGCACTGCTCC	30.3 kb
218	18324-L23238	Intron 1 (Exon 2)	NM_001136016.3; 40-41	GAAGTTGGGTTA-GTGTTGACATGC	28.3 kb
177	20914-L32071	Exon 2	262-263	TTGCCATGTTCT-GTGGCAGACTGA	22.1 kb
211	06199-L05702	Exon 3	411-412	AATGTGGTAGAA-GCCAACCAACCA	36.7 kb
292	06200-L05703	Exon 4	552-553	GACAAGTGCAAA-TTCTTACACCAG	2.2 kb
202	18323-L23237	Exon 5	689-690	TGACAAGTTCCG-AGGGGTAGAGTT	29.1 kb
238	06201-L05704	Exon 6	845-846	AGCAGAGGAGGA-AGAAGTGGCTGA	21.9 kb
328	20913-L05705	Exon 7	1076-1077	CCGCTGGTACTT-TGATGTGACTGA	2.7 kb
148	18321-L23235	Exon 8	1217-1218	TACCCAGGAACC-TCTTGCCCGAGA	15.0 kb
196	06203-L05706	Exon 9	1283-1284	CGTTGACAAGTA-TCTCGAGACACC	6.5 kb
170	18322-L23236	Exon 10	1410-1411	GAACGTCAAGCA-AAGAACTTGCCT	0.8 kb
255 Ж	18325-SP0641-L23239	Exon 11	1493-1494 and 1520-1521	GGAAGCAGCCAA-27nt spanning oligo-CATGGCCAGAGT	19.4 kb
265	06204-L05707	Exon 12	1637-1638	TATGCTAAAGAA-GTATGTCCGCGC	1.1 kb
355	18362-L23316	Exon 13	1800-1801	CTGCTCTACAAC-GTGCCTGCAGTG	42.8 kb
362	18328-L23242	Exon 14	2005-2004 reverse	ATGCCACGGCTG-GAGATCGTCCAG	6.8 kb
409	18329-L23243	Exon 15	2112-2113	ACCACTCGACCA-GGTATCAGAACC	7.4 kb
346	00987-L23493	Exon 16	2170-2171	AGATGGATGCAG-AATTCCGACATG	5.9 kb
301	18327-L23241	Exon 17	2342-2343	GTACACATCCAT-TCATCATGGTGT	10.1 kb
373	06206-L05709	Exon 18	2505-2504 reverse	GTAGTGAAGCAA-TGGTTTTGCTGT	146.4 kb
		stop codon	*2461-2463 (Exon 18)*		
421 ¬	07593-L23492	*ATP5J*		CCTTTATACACT-TCGATAACCCTA	41.4 kb
382 ¬	06982-L06584	*JAM2*		TCGTTGTGAAGT-TAGTGCCCCATC	4261.7 kb
166 ¬	00813-L00636	*NCAM2*		AGAGCTGTCGCA-GACCACGGCCAA	

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.
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.
¬ 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.

Related SALSA MLPA probemixes

P254 PSEN1	Contains probes for the *PSEN1* gene, implicated in Alzheimer's disease.
P275 MAPT-GRN	Contains probes for the *MAPT* and *GRN* genes, implicated in Alzheimer's disease/dementia.
P471 EOFAD	Contains probes for the *PSEN1*, *PSEN2*, and *APP* genes, implicated in early-onset familial Alzheimer's 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.
- 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 P170 APP

- Llado A et al. (2014). Large APP locus duplication in a sporadic case of cerebral haemorrhage. *Neurogenetics* 15(2):145-9.
- Luedecke D et al. (2014). A novel presenilin 1 mutation (Ala275Val) as cause of early-onset familial Alzheimer disease. *Neurosci lett* 566:115-9.

P170 Product history

Version	*Modification*
C3	Three reference probes have been replaced and one probe length has been adjusted.
C2	Two probes have a small change in length.
C1	Compared to previous version, the mutation specific probes have been removed. Probes for exon 1, 3, 7, 10, 12, 13, 15, 17 and 19 of the *APP* gene have been added, and the probe for exon 16 has been replaced. Six reference probes have been removed.
B2	One reference probe (427 nt) has been replaced. In addition, four extra control fragments at 88-96-100 and 105 nt have been added.
B1	Two *APP* probes specific for certain promoter mutations/polymorphisms have been added. In addition, several probes in genes close to *APP* have been included.
A1	First release.

Implemented changes in the product description

Version C3-01 — 29 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 and Table 2).
- APP exon numbering for the 218 nt probe has been adjusted from exon 2 to intron 1.
- Ligation sites of the probes targeting the *APP* gene updated according to new version of the NM_ reference sequence.

Version 13 (55) – 05 February 2016
- Product description adapted to a new product version (version number changed, lot number added, new pictures included).
- APP exon numbering has been adjusted.
- Manufacturer's address adjusted.

Version 12 (54) – 22 July 2015
- Figure based on the use of old MLPA buffer (replaced in December 2012) removed.

Version 11 (49)
- Product description adapted to a new product version (version number changed, lot number added, new pictures included).
- Various textual changes on page 1 and 2.

- Various layout changes.
- Changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

Version 10 (48)

- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

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