Product Description
SALSA® MLPA® Probemix P196-B2 TNNT2-BAG3

To be used with the MLPA General Protocol.

Version B2
As compared to version B1, one reference probe has been replaced. For complete product history see page 7.

Catalogue numbers:
- **P196-025R:** SALSA MLPA Probemix P196 TNNT2-BAG3, 25 reactions.
- **P196-050R:** SALSA MLPA Probemix P196 TNNT2-BAG3, 50 reactions.
- **P196-100R:** SALSA MLPA Probemix P196 TNNT2-BAG3, 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 P196 TNNT2-BAG3 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *TNNT2* and *BAG3* genes, which are associated with dilated cardiomyopathy (DCM).

Defects in the *TNNT2* gene are associated with familial hypertrophic cardiomyopathy as well as DCM. The protein encoded by *TNNT2* gene is the tropomyosin-binding subunit of the troponin complex, which regulates muscle contraction in response to alterations in intracellular calcium ion concentration. Copy number changes of the *BAG3* gene have also been found to be a cause for some cases of DCM (Norton et al. 2011). The *BAG3* gene is a member of the Bcl2-associated athanogene (BAG) family proteins, which causes cardiomyopathy and myofibrillar myopathy and is characterized by myofibril and Z-disc disruption.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1768/ and https://www.ncbi.nlm.nih.gov/books/NBK1309/.

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 *TNNT2* and *BAG3* exon numbering used in this P196-B2 TNNT2-BAG3 product description is the exon numbering from the LRG_431 and LRG_742 sequences, respectively. 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 LRG 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 P196-B2 TNNT2-BAG3 contains 30 MLPA probes with amplification products between 130 and 373 nucleotides (nt). This includes 17 probes for the *TNNT2* gene, one probe for each exon with the exception of exon 13 and two probes for exon 17, and four probes for the *BAG3* gene, one probe for each exon. 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.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 DCM. 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 *TNNT2* and *BAG3* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P196 TNNT2-BAG3.

- 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.

LOVD mutation database

https://databases.lovd.nl/shared/genes/. 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 SNVs and unusual results (e.g., a duplication of *TNNT2* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P196-B2 TNNT2-BAG3

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*TNNT2*	*BAG3*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 00797-L19287	5q		
137	**TNNT2 probe** 16804-L19450		**Exon 2**	
143	**TNNT2 probe** 16805-L19451		**Exon 5**	
148	**TNNT2 probe** 16806-L19452		**Exon 16**	
154 «	**BAG3 probe** 16753-L19395			**Exon 1**
160	**BAG3 probe** 16754-L19396			**Exon 2**
166 *	Reference probe 12741-L21552	21q		
172	**TNNT2 probe** 06556-L19388		**Exon 6**	
178	**TNNT2 probe** 06561-L19389		**Exon 11**	
184	**BAG3 probe** 16755-L19397			**Exon 4**
190	Reference probe 11686-L16733	17q		
200	**TNNT2 probe** 06557-L19390		**Exon 7**	
214	**TNNT2 probe** 06558-L19813		**Exon 8**	
220 Ж	**TNNT2 probe** 16807-SP0399-L19895		**Exon 4**	
229	Reference probe 16553-L19044	11q		
240	**TNNT2 probe** 17009-L20056		**Exon 12**	
246	**TNNT2 probe** 17011-L20058		**Exon 15**	
256	Reference probe 02469-L01913	15q		
265	**TNNT2 probe** 07776-L07532		**Exon 17**	
274	**TNNT2 probe** 06555-L06113		**Exon 3**	
283	**TNNT2 probe** 07775-L07531		**Exon 14**	
294	Reference probe 15474-L19814	1p		
301	**BAG3 probe** 16757-L19815			**Exon 3**
315	**TNNT2 probe** 06553-L19391		**Exon 1**	
324	**TNNT2 probe** 06559-L19392		**Exon 9**	
337	Reference probe 07237-L06887	3p		
346	**TNNT2 probe** 06560-L06118		**Exon 10**	
355	**TNNT2 probe** 07777-L07533		**Exon 17**	
364	Reference probe 12449-L13450	14q		
373	Reference probe 06745-L06349	8q		

[a] See section Exon numbering on page 1 for more information.

* New in version B2.
« 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 2. P196-B2 probes arranged according to chromosomal location

Table 2a. *TNNT2*

Length (nt)	SALSA MLPA probe	*TNNT2* exon[a]	Ligation site NM_000364.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*73-75 (Exon 2)*		
315	06553-L19391	Exon 1	35-36	CGCCTCCAGGAT-CTGTCGGCAGCT	4.4 kb
137	16804-L19450	Exon 2	7 nt after exon 2	GAGGAGTGAGTA-TCTGGAGCATCT	1.1 kb
274	06555-L06113	Exon 3	18 nt after exon 3	GTAAACGTGTGT-ACTCATTTGGAT	0.1 kb
220 Ж	16807-SP0399-L19895	Exon 4	8 nt and 33 nt after exon 4	GAAGGTATGTGC-25 nt spanning oligo-GGGCAGGCTCAG	2.2 kb
143	16805-L19451	Exon 5	5 nt after exon 5	GAAGACGGTAGT-ACAGCCTTTCCT	1.6 kb
172	06556-L19388	Exon 6	194-195	AGCGGAAGAGGA-TGCTGAAGCAGA	0.4 kb
200	06557-L19390	Exon 7	249-248, reverse	TTTGCTTCCTCT-TCTTCTTCATCT	0.9 kb
214	06558-L19813	Exon 8	280-281	CAGATGGCCCAA-TGGAGGAGTCCA	1.2 kb
324	06559-L19392	Exon 9	335-336	GGTGCCTCCCAA-GATCCCCGATGG	0.4 kb
346	06560-L06118	Exon 10	462-461, reverse	TTGAGAGAAACG-AGCTCCTCCTCC	0.9 kb
178	06561-L19389	Exon 11	538-539	ATGAGCGGGAGA-AGGAGCGGCAGA	1.1 kb
240	17009-L20056	Exon 12	52 nt after exon 12	TAGCCCTGAGGA-ATGAGGTGTCCA	1.2 kb
	No probe	*Exon 13*			
283	07775-L07531	Exon 14	692-693	GAAAAGTGGGAA-GAGGCAGACTGA	1.2 kb
246	17011-L20058	Exon 15	442 nt after exon 15	TTTCGGAGTCCT-ATGTGCACTAAT	1.2 kb
148	16806-L19452	Exon 16	5 nt before exon16	TCCCACTTTTCT-TGCAGATCAATG	0.5 kb
265	07776-L07532	Exon 17	973-974	GCCTGGCCTCCT-TCACCAAAGATC	0.2 kb
355	07777-L07533	Exon 17	1136-1137	CACACCAGTAAT-AAAAAGCCACCA	
		stop codon	*958-960 (Exon 16)*		

Table 2b. *BAG3*

Length (nt)	SALSA MLPA probe	*BAG3* exon[a]	Ligation site NM_004281.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*297-299 (Exon 1)*		
154 «	16753-L19395	Exon 1	427-428	GGACCACAACAG-CCGCACCACTAC	18.2 kb
160	16754-L19396	Exon 2	618-619	AGAACCGGCAGG-TGCACCCTTTCC	2.7 kb
301	16757-L19815	Exon 3	16 nt after exon 3	GGGAAGTTAGTC-GTCAGCAGACTG	4.3 kb
184	16755-L19397	Exon 4	1679-1680	TTGACCAAAGAG-CTGCTGGCCCTG	
		stop codon	*2022-2024 (Exon 4)*		

[a] See section Exon numbering on page 1 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.

« 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

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

Related SALSA MLPA probemixes

P100 MYBCP3	Contains probes for the *MYBCP3* gene.
P418 MYH7	Contains probes for the *MYH7* gene.

References

- Norton N et al. (2011). Genome-wide studies of copy number variation and exome sequencing identify rare variants in BAG3 as a cause of dilated cardiomyopathy. *Am J Hum Genet*, 88(3), 273-282.
- 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 P196 TNNT2-BAG3

- Bagnall RD et al. (2010). The role of large gene deletions and duplications in MYBPC3 and TNNT2 in patients with hypertrophic cardiomyopathy. *Int J Cardiol*, 145(1), 150-153.
- Pezzoli L et al. (2012). A new mutational mechanism for hypertrophic cardiomyopathy. *Gene*, 507(2), 165-169.

P196 product history	
Version	*Modification*
B2	One reference probe has been replaced.
B1	Three probes for TNNT2 have been removed and six new TNNT2 probes, four probes for the *BAG3* gene and four control fragments have been included.
A1	First release.

Implemented changes in the product description

Version B2-01 – 16 April 2021 (04P)
- 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).
- Ligation sites of the probes targeting the *TNNT2* and *BAG3* genes updated according to new version of the NM_ reference sequences.

Version 12 – 23 September 2017 (55)
- Product description adapted to a new lot number (lot number added, minor changes in Table 1 and 2, new picture included).
- Small textual and layout changes throughout the document.

Version 11 – 15 September 2017 (55)
- Warning added in Table 1, 154 nt probe 16753-L19395.

Version 10 – 14 January 2016 (55)
- Product description adapted to a new lot (lot number added, new picture is included).

Version 09 – 15 July 2015 (54)
- Figure based on the use of old MLPA buffer (replaced in December 2012) removed.

Version 08 (48)
- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

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