Product Description
SALSA® MLPA® Probemix P300-B1 Reference-2

To be used with the (MS-)MLPA General Protocol.

Version B1
For complete product history see page 6.

Catalogue numbers:
- **P300-100R:** SALSA MLPA Probemix P300 Reference-2, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, (SALSA HhaI (SMR50)) 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 (MS-)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 P300 Reference-2 is a **research use only (RUO)** assay which is developed to be used in combination with 'home-made' synthetic (MS-)MLPA probes. This probemix contains reference probes and control fragments specific for unique human DNA sequences. The control fragments and reference probes in this P300 probemix facilitate the data analysis of a synthetic probemix with home-made probes.

MRC Holland also offers the SALSA MLPA Probemix P200 Reference-1. While the P200 Reference-1 probemix leaves the entire range from 80-170 nt open for the inclusion of synthetic probes, this P300 Reference-2 probemix has reference probes distributed over the whole size range of the probemix. This is especially advantageous for correction of the signal sloping of (MS-)MLPA amplification products. Signal sloping is the effect that longer probes generate a lower peak height/area on the electropherogram than shorter probes. The exact amount of signal sloping differs between sequencer types and can differ between different samples.

The use of P200 is recommended when a large number of synthetic probes are used, preferably targeting sequences on different chromosomes. Results can be difficult to interpret when P200 is used in combination with a small number of probes that all target the same gene or chromosomal region, or with a small number of probes with lengths much shorter than the reference probes. The use of P300 is recommended when a smaller number of synthetic probes is used. Please note that MRC Holland cannot offer support for custom probe design anymore.

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/

Probemix content
The SALSA MLPA Probemix P300-B1 Reference-2 contains 25 MLPA probes with amplification products between 64 and 274 nt. This includes 14 reference probes 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). In addition, four DNA Quantity Fragments (Q-fragments), two DNA Denaturation Fragments (D-fragments), two HhaI-digestion control fragments, one chromosome X and one chromosome Y-specific fragment are included. The Q-fragments give a warning when insufficient amounts of sample DNA are used. The D-fragments give a warning when sample DNA was not completely denatured. More information on how to interpret observations on these control fragments can be found in the (MS-)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, 252	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
130, 178	HhaI-digestion control fragments
208	X-fragment (X chromosome specific)
239	Y-fragment (Y chromosome specific)

(MS-)MLPA technique
The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mrcholland.com).

It is also possible to use this probemix for the determination of methylation status. The principles of the MS-MLPA technique (Nygren et al. 2005, Schouten et al. 2002) are described in the MS-MLPA General Protocol (www.mrcholland.com). The MS-MLPA technique should always be internally validated before use in your laboratory.

(MS-)MLPA technique validation
Internal validation of the (MS-)MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using (MS-)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 reference probes over the experiment.

Results of MS-MLPA are highly dependent on the HhaI enzyme used. HhaI enzymes that are resistant to heat inactivation are NOT compatible with the MS-MLPA technique and will give aberrant results. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI. We recommend using SALSA HhaI enzyme (SMR50) as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland.

Required specimens
Extracted DNA free from impurities known to affect (MS-)MLPA reactions. For more information please refer to the section on DNA sample treatment found in the (MS-)MLPA General Protocol.

Reference samples
A sufficient number (≥3) of reference samples should be included in each (MS-)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 the disease in question. It is recommended to use samples of the same sex to facilitate interpretation. More information regarding the selection and use of reference samples can be found in the (MS-)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 (MS-)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 (MS-)MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results

The standard deviation of each individual reference 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 (MS-) MLPA results when **reference samples of the same sex** have been used:

Copy number status		**Final ratio (FR)**
Autosomal sequences and X chromosome sequences in females	**X chromosome sequences in males**	
Normal	Normal	0.80 < FR < 1.20
Homozygous deletion	Deletion	FR = 0
Heterozygous deletion		0.40 < FR < 0.65
Heterozygous duplication		1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	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 (MS-)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.
- mRNA levels. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.

- 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.

NOTE: In case digestion control probes are not fully digested (>0.05), please contact info@mrcholland.com for more information.

P300 specific note:
Method:
- For each synthetic (MS-)MLPA probe oligonucleotide, a 1 µM dilution in TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA) should be made. For example, dissolve 25 nmol oligo in 250 µl (= 100 µM) and then dilute 10 µl of this stock to 1 ml (1 µM). Store the stock and 1 µM dilution at -20 °C.
- Make your synthetic probemix by combining 0.8 µl of each 1 µM oligo solution in a final volume of 200 µl TE. Store at -20 °C.
- For each (MS-)MLPA reaction, use 1 µl P300 + 0.5 µl synthetic probe mix + 1.5 µl MLPA buffer.
- Always mix thawed oligonucleotide solutions thoroughly before pipetting!

- Digestion Control Probes: Upon digestion of the probe-sample hybrids with HhaI, the 130 nt probe should not give a signal, regardless of methylation status of target sequence and the 178 nt probe should not give a signal, because the target sequence is unmethylated in most blood-derived DNA samples. When using the standard MLPA protocol, results of the digestion control probes can be disregarded.

Limitations of the procedure
- (MS-)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 (MS-)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.
- An MS-MLPA probe targets a single specific HhaI site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!
- Rare cases are known in which apparent methylation as detected by an MS-MLPA probe proved to be due to a sequence change in or very near the HhaI site.

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 (MS-)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.

Please report copy number changes detected by the reference probes, false positive results due to SNVs, and unusual results to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P300-B1 Reference-2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)		
		Other	Reference	hg18 ‡
64-82	Q fragments – see table in probemix content section for more information			
88 «	**D-probe 1**: Low signal indicates incomplete denaturation	19p		10.8 Mb
92	**Ligation-dependent control fragment**	2q		113.3 Mb
108	Reference probe S0973-L27812		4p	42.3 Mb
130 #	**Digestion Control probe** S0750-L27811	2q		102.6 Mb
148	Reference probe 17669-L27446		5q	156.6 Mb
173	Reference probe 19185-L27754		3q	140.4 Mb
178 #	**Digestion Control probe** 20190-L27120	21q		34.1 Mb
184	Reference probe 10904-L27810		9q	134.2 Mb
191	Reference probe 18767-L28188		10q	71.9 Mb
196	Reference probe 11157-L11841		5q	137.6 Mb
208	**Chromosome X probe** 19928-L27808	Xq		111.9 Mb
214	Reference probe 19623-L27807		10p	34.6 Mb
220	Reference probe 14967-L27452		6q	129.7 Mb
226	Reference probe 20173-L27439		2p	32.2 Mb
232	Reference probe 19768-L27755		12q	41.1 Mb
239	**Chromosome Y probe** 19927-L27806	Yq		14.0 Mb
246	Reference probe 19985-L27453		4p	5.7 Mb
252 «	**D-probe 2**: 20039-L27756: Low signal indicates incomplete denaturation	16q		86.2 Mb
258	Reference probe 18593-L27454		2q	199.9 Mb
265	Reference probe 13392-L14849		6q	65.4 Mb
274	Reference probe 17450-L21206		16p	9.8 Mb

« 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.
Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal. When using the standard MLPA protocol, results from this probe should be disregarded.
‡ Distance to P-telomere (hg18).

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.

Related SALSA MLPA probemixes

P200 Reference-1 Contains control fragments and reference probes from 173 to 251 nt.

References

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Selected publications using SALSA MLPA Probemix P300 Reference-2

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- Sinha S et al. (2020). Adipose-Derived Mesenchymal Stem Cells do not Affect the Invasion and Migration Potential of Oral Squamous Carcinoma Cells. *Int J Mol Sci*, 21(18), 6455.
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P300 product history	
Version	*Modification*
B1	The probemix has been completely redesigned.
A2	The 88 nt DNA denaturation control fragment is replaced.
A1	First release.

Implemented changes in the product description

Version B1-05 – 09 March 2023 (04P)
- Text that is referring to the Synthetic Probe Design Protocol, which can be used to design synthetic (MS-)MLPA probes, is removed.

Version B1-04 – 17 January 2022 (04P)
- Product description rewritten and adapted to a new template.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

Version B1-03 – 1 October 2020 (01P)
- Control fragment table adjusted; 96 nt corrected to 252 nt.
- Small changes of probe lengths in Table 1 and text in order to better reflect the true lengths of the amplification products.

Version B1-02 – 23 October 2019 (01P)
- Catalogue numbers P300-25R and P300-50R have been removed.
- SALSA HhaI (SMR51) has been added.
- Various textual changes.

Version B1-01 – 31 August 2018 (01P)
- Product description restructured and adapted to a new template.
- Various textual changes.

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