# Product Description
## SALSA® MLPA® Probemix P200-B1 Reference-1

To be used with the MLPA General Protocol.

**Version B1**
For complete product history see page 6.

**Catalogue numbers:**
- **P200-100R:** SALSA MLPA Probemix P200 Reference-1, 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 P200 Reference-1 is a **research use only (RUO)** assay which contains reference probes and control fragments specific for unique human DNA sequences. The P200 probemix offers a collection of thoroughly-tested MLPA reference probes and control fragments to which 'home-made' synthetic (MS-)MLPA probes can be added. Not only do these control fragments and reference probes provide extra assurance about the quality of home-made probes and of the MLPA reaction performed, they also facilitate the data analysis of your synthetic probemix and maximise the number of synthetic probes you can include.

Next to the P200, MRC Holland also offers the P300 Reference-2 probemix. While the P200 leaves the entire range from 82-173 nt open for the inclusion of synthetic probes, the P300 probemix has reference probes distributed over the whole size range of the probemix. This is especially advantageous for correction of the signal sloping of 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 and can disturb the analysis.

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 all targeting the same gene or chromosomal region, and with lengths much smaller than the reference probes. In samples with sloping, it may seem in such cases that all sequences targeted by the synthetic probes are deleted or duplicated. 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 P200-B1 Reference-1 contains 14 MLPA probes with amplification products between 173 and 251 nucleotides (nt). This includes, ten 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, one DNA Denaturation fragment (D-fragment), one digestion control fragment, one chromosome X, and one chromosome Y-specific fragment are included (see Table 1). In addition, it contains four DNA Quantity fragments (Q-fragments) at 64-70-76-82 nt. Interpretation of these control fragments should be done by Coffalyser.Net analysis software. In order to let Coffalyser.Net do this correctly, a few adaptations are necessary. Information on how to make these adaptations can be found here: https://www.mrcholland.com/r/p200/coffalyser-sheet-adjustments. Information about the quality scores in Coffalyser.Net can be found in the Coffalyser.Net Reference Manual (https://www.mrcholland.com/r/ifu/coffalyser-net-reference-manual).

| Length (nt) | Name |
|---|---|
| 64-70-76-82 | Q-fragments (only visible with <100 ng sample DNA) |
| 178 | HhaI-digestion control fragment |
| 208 | X-fragment (X chromosome specific) |
| 239 | Y-fragment (Y chromosome specific) |
| 251 | D-fragment (low signal indicates incomplete denaturation) |

**(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 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 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 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 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 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 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.

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

P200 specific notes:
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. E.g. dissolve 25 nmol oligo in 250 µl (= 100 µM) and then dilute 10 µl of this stock to 1 ml. 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 MLPA reaction, use 1 µl P200 + 0.5 µl synthetic probe mix + 1.5 µl MLPA buffer.
- Always mix thawed oligonucleotide solutions before pipetting!

Methylation-specific MLPA:
- This probemix can also be used in combination with your home-made MS-MLPA probes, as the 178 nt probe contains a HhaI restriction site and can be used as an internal control for complete HhaI digestion. Upon proper HhaI digestion, the 178 nt probe signal will be almost gone. More information on methylation quantification by MS-MLPA can be found on www.mrcholland.com.

Analysis:
- For proper analysis and correct determination of the quality scores the Coffalyser sheet of this product needs to be adjusted. Please see https://www.mrcholland.com/r/p200/coffalyser-sheet-adjustments and contact info@mrcholland.com if additional assistance is required. Please note that we cannot offer additional support on the design of synthetic probes.

**Limitations of the procedure**

- 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.
- 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 P200-B1 Reference-1

| Length (nt) | SALSA MLPA probe | Chromosomal position (hg18) | | |
|---|---|---|---|---|
| | | Other | Reference | hg18 ‡ |
| 64-82 | Q fragments – see table in probemix content section for more information | | | |
| 173 | Reference probe 19185-L27754 | | 3q | 140.44 Mb |
| 178 # | **Digestion control probe** 20190-L27120 | **21q** | | 34.07 Mb |
| 184 | Reference probe 10904-L27810 | | 9q | 134.20 Mb |
| 191 | Reference probe 18767-L28188 | | 10q | 71.87 Mb |
| 196 | Reference probe 11157-L11841 | | 5q | 137.62 Mb |
| 202 | Reference probe 17177-L20402 | | 15q | 46.51 Mb |
| 208 | **Chromosome X** probe 19928-L27808 | **Xq** | | 111.95 Mb |
| 214 | Reference probe 19623-L27807 | | 10p | 34.65 Mb |
| 220 | Reference probe 14967-L27452 | | 6q | 129.68 Mb |
| 226 | Reference probe 20173-L27439 | | 2p | 32.22 Mb |
| 232 | Reference probe 19768-L27755 | | 12q | 41.07 Mb |
| 239 | **Chromosome Y** probe 19927-L27806 | **Yq** | | 13.98 Mb |
| 246 | Reference probe 19985-L27453 | | 4p | 5.67 Mb |
| 251 « | **D-probe** 20039-L18417: Low signal indicates incomplete denaturation | **16q** | | 86.19 Mb |

# When used for methylation quantification (MS-MLPA), this probe can be used as a digestion control probe (warning for insufficient HhaI digestion). Upon digestion of the probe-sample hybrids with HhaI, this probe should not give a signal.
« 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.
‡ 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

P300 Reference-2 Contains control fragments and reference probes distributed over the whole size range of the probemix.

## References


- Nygren AO et al. (2005). Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res*. 33:e128.
- 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 P200 Reference-1


- Cheminant M et al. (2022). KIR3DL2 contributes to the typing of acute adult T-cell leukemia and is a potential therapeutic target. Blood, *J Am Soc Hematol*, 140(13), 1522-1532.
- Corrado L et al. (2020). The first case of the TARDBP p. G294V mutation in a homozygous state: is a single pathogenic allele sufficient to cause ALS? *Amyotroph Lateral Scler Frontotemporal Degener*, 21(3-4), 273-279.
- Ebrahimizadeh W et al. (2020). Design and development of a fully synthetic MLPA-based probe mix for detection of copy number alterations in prostate cancer formalin-fixed, paraffin-embedded tissue samples. *J Mol Diagn*.
- García-Fernández J et al. (2021). Detection of Genetic Rearrangements in the Regulators of Complement Activation RCA Cluster by High-Throughput Sequencing and MLPA. In *The Complement System* (pp. 159-178). Humana, New York, NY.
- Gieldon L et al. (2021). Germ cell mosaicism for AUTS2 exon 6 deletion. *Am J Med Genet A*, 185(4), 1261-1265.
- Giorgio E et al. (2019). Design of a multiplex ligation-dependent probe amplification assay for SLC20A2: identification of two novel deletions in primary familial brain calcification. *J Hum Genet*, 1-8.
- Khanam T et al. (2021). Integrative genomic analysis of pediatric T-cell lymphoblastic lymphoma reveals candidates of clinical significance. *Blood*, 137(17), 2347-2359.
- Kondo H et al. (2021). Retinal features of family members with familial exudative vitreoretinopathy caused by mutations in KIF11 gene. *Transl Vis Sci Technol*, 10(7), 18-18.
- Lau NKC, et al. (2021). In-house multiplex ligation-dependent probe amplification assay for citrin deficiency: analytical validation and novel exonic deletions in SLC25A13. *Pathology*, 53(7), 867-874.
- Mellone S et al. (2022). Co-Occurrence of a Pathogenic HSD3B2 Variant and a Duplication on 10q22. 3-q23. 2 Detected in Newborn Twins with Salt-Wasting Congenital Adrenal Hyperplasia. *Genes*, 13(12), 2190.
- Minegishi K et al. (2019). Screening of the copy number increase of AKT in lung carcinoma by custom-designed MLPA. *Int J Clin Exp Pathol*, 12(9), 3344.
- Nasri S et al. (2019). Early Hereditary Diffuse Gastric Cancer (eHDGC) is Characterized by Subtle Genomic Instability and Active DNA Damage Response. *Pathol Oncol Res*, 25(2), 711-721.
- Pingel J et al. (2019). Sequence variants in muscle tissue-related genes may determine the severity of muscle contractures in cerebral palsy. *Am J Med Genet B Neuropsychiatr Genet*, 180(1), 12-24.
- Shah M et al. (2020). Next generation sequencing using phenotype-based panels for genetic testing in inherited retinal diseases. *Ophthalmic Genet*, 41(4), 331-337.
- Shimizu M et al. (2021). Haploinsufficiency of A20 with a novel mutation of deletion of exons 2–3 of TNFAIP3. *Mod Rheumatol*, 31(2), 493-497.
- Soltysova A et al. (2022). Breakpoints characterisation of the genomic deletions identified by MLPA in alkaptonuria patients. *Eur J Hum Genet*, 1-5.
- Touzart A et al. (2020). Low level CpG island promoter methylation predicts a poor outcome in adult T-cell acute lymphoblastic leukemia. *Haematologica*, 105(6), 1575-1581.
- Xie B et al. (2020). Novel compound heterozygous variant of BSCL2 identified by whole exome sequencing and multiplex ligation-dependent probe amplification in an infant with congenital generalized lipodystrophy. *Mol Med Rep*, 21(6), 2296-2302.


| **P200 product history** | |
|---|---|
| *Version* | *Modification* |
| B1 | All reference probes have been replaced. |
| A1 | First release. |

| **Implemented changes in the product description** |
|---|
| *Version B1-03 – 09 March 2023 (04P)*<br>- Product description rewritten and adapted to a new template.<br>- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.<br>- Text that is referring to the Synthetic Probe Design Protocol, which can be used to design synthetic (MS-)MLPA probes, is removed.<br>*Version B1-02 — 25 October 2022 (02P)*<br>- Probemix content paragraph updated and link included to an article in which the P200 Coffalyser sheet adjustments and more information on the analysis are described.<br>- www.mlpa.com was adjusted to www.mrcholland.com and info@mlpa.com to info@mrcholland.com.<br>- Various minor adjustments<br>*Version B1-01 — 28 January 2020 (02P)*<br>- Product description rewritten and adapted to a new template.<br>- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).<br>- SALSA HhaI (SMR50) has been added.<br>- For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36).<br>*Version 17 – 05 April 2019 (55)*<br>- Box added in Data analysis section: For proper analysis and correct determination of the quality scores the Coffalyser sheet of this product needs to be adjusted. Please contact info@mlpa.com for instructions.<br>*Version 16 – 30 October 2018 (55)*<br>- Reference to MLPA General Protocol removed. |

| **More information: www.mrcholland.com; www.mrcholland.eu** | |
|---|---|
| | MRC Holland bv; Willem Schoutenstraat 1<br>1057 DL, Amsterdam, The Netherlands |
| E-mail | info@mrcholland.com (information & technical questions)<br>order@mrcholland.com (orders) |
| Phone | +31 888 657 200 |