Product Description SALSA® MLPA® Probemix P201-C4 CHARGE

To be used with the MLPA General Protocol.

Version C4. As compared to version C3, two reference probes have been replaced. For complete product history see page 7.

Catalogue numbers:
- **P201-025R:** SALSA MLPA Probemix P201 CHARGE, 25 reactions.
- **P201-050R:** SALSA MLPA Probemix P201 CHARGE, 50 reactions.
- **P201-100R:** SALSA MLPA Probemix P201 CHARGE, 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 P201 CHARGE is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CHD7 gene*, which is associated with CHARGE syndrome.

CHARGE syndrome is an autosomal dominant disorder that affects many areas of the body and has an incidence of approximately 1:10.000. The pattern of malformations varies among individuals with this disorder, and infants often have multiple life-threatening medical conditions. The term **CHARGE** is an acronym for the set of clinical features observed in patients and stands for **C**oloboma, **H**eart disease, **A**tresia choanae, **R**etarded growth and retarded development and/or CNS anomalies, **G**enital hypoplasia, and **E**ar anomalies and/or deafness.

Mutations in the Chromodomain Helicase DNA-Binding Protein 7 *(CHD7)* gene have been found to be responsible for more than half of the CHARGE syndrome cases. *CHD7* is the only gene currently known to be associated with CHARGE syndrome.

The *CHD7* gene has 38 exons, spans ~189 kb of genomic DNA, and is located on chromosome 8q12.2, about 61 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1117/.

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 *CHD7* exon numbering used in this P201-C4 CHARGE product description is the exon numbering from the RefSeq transcript NM_017780.4, which is identical to the LRG_176 sequence. The exon numbering and NM_ sequence used have been retrieved on 07/2019. 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 P201-C4 CHARGE contains 49 MLPA probes with amplification products between 130 and 481 nucleotides (nt). This includes 41 probe(s) for the *CHD7 gene*. In addition, eight 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, 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.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-fragments (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 CHARGE syndrome. 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 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. Sample ID number NA02030 from the Coriell Institute have been tested with this P201-C4 probemix at MRC-Holland and can be used as a positive control sample to detect a duplication of the complete *CHD7* gene. 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
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 in or near the *CHD7* gene. 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 *CHD7* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P201 CHARGE.
- 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.

***CHD7* mutation databases:** https://databases.lovd.nl/shared/genes/CHD7 and https://molgenis51.gcc.rug.nl/menu/main/home. We strongly encourage users to deposit positive results in the LOVD Database and CHD7 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 *CHD7* exons 6 and 8 but not exon 7) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P201-C4 CHARGE

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a] Reference	*CHD7*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 00797-L00463	5q31	
136	**CHD7 probe** 06742-L06346		**Exon 2**
142	**CHD7 probe** 10637-L12898		**Exon 16**
148	**CHD7 probe** 07287-L07511		**Exon 36**
154	**CHD7 probe** 07285-L07512		**Exon 23**
160	**CHD7 probe** 15974-L18507		**Exon 29**
166	**CHD7 probe** 06744-L18508		**Exon 4**
172	Reference probe 09838-L10248	12q24	
178	**CHD7 probe** 06765-L06369		**Exon 38**
184	**CHD7 probe** 06748-L06352		**Exon 9**
190	**CHD7 probe** 06743-L06347		**Exon 3**
196	**CHD7 probe** 06761-L07509		**Exon 32**
202	**CHD7 probe** 10636-L13834		**Exon 12**
208	**CHD7 probe** 10641-L11189		**Exon 25**
214	**CHD7 probe** 06746-L06350		**Exon 7**
220	Reference probe 02452-L01896	15q21	
226 «	**CHD7 probe** 06739-L11139		**Exon 1**
232	**CHD7 probe** 06755-L07510		**Exon 18**
238	Reference probe 02519-L01950	17q11	
244	**CHD7 probe** 15975-L18130		**Exon 27**
250	**CHD7 probe** 06747-L18509		**Exon 8**
256	**CHD7 probe** 06752-L06356		**Exon 15**
263	**CHD7 probe** 15976-L18131		**Exon 20**
269 «	**CHD7 probe** 06740-L18691		**Intron 1**
275	**CHD7 probe** 06759-L19001		**Exon 26**
283	**CHD7 probe** 15977-L19002		**Exon 10**
289	**CHD7 probe** 06754-L29522		**Exon 17**
295	**CHD7 probe** 07286-L19004		**Exon 28**
301	**CHD7 probe** 06749-L19005		**Exon 11**
310	**CHD7 probe** 06741-L06345		**Exon 2**
319 *	Reference probe 06440-L05966	3p12	
326	**CHD7 probe** 06763-L06367		**Exon 35**
337	**CHD7 probe** 10639-L11187		**Exon 22**
346	**CHD7 probe** 06750-L29521		**Exon 13**
355	**CHD7 probe** 06758-L06362		**Exon 24**
363	**CHD7 probe** 06760-L06364		**Exon 30**
373	**CHD7 probe** 06745-L06349		**Exon 5**
383	**CHD7 probe** 21008-L29226		**Exon 14**
391	Reference probe 02846-L02276	18q11	
401	**CHD7 probe** 10638-L12897		**Exon 19**
409	**CHD7 probe** 06756-L18823		**Exon 21**
418	**CHD7 probe** 10643-L11191		**Exon 37**
427	**CHD7 probe** 06762-L13879		**Exon 33**
436	**CHD7 probe** 10634-L11182		**Exon 3**
445 *	Reference probe 16237-L24275	1p32	
454	**CHD7 probe** 10635-L11183		**Exon 6**
463	**CHD7 probe** 10642-L11190		**Exon 34**
472	**CHD7 probe** 15978-L18133		**Exon 31**
481	Reference probe 13835-L15356	5q35	

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

* New in version C4.

« Probe located in or near a GC-rich region. A low signal can be caused in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Table 2. CHD7 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	CHD7 exon[a]	Ligation site NM_017780.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		Start codon	*518-520 (exon 2)*		
226 «	06739-L11139	Exon 1	133-134	CGGATTAAACTT-GAATCGAGTGAA	1.8 kb
269 «	06740-L18691	Intron 1	1567 nt after exon 1	GGGTCGGACTCT-TTTCCTGGAGGT	60.7 kb
310	06741-L06345	Exon 2	460-461	CACCTCAGTGAA-GTGAAGCACAGG	1.5 kb
136	06742-L06346	Exon 2	1995-1996	AGCAATCCAGGA-ACGACTGATACC	38.1 kb
190	06743-L06347	Exon 3	2225-2224 reverse	AACCTGAGTCAT-ATCCGGCACTGG	0.1 kb
436	10634-L11182	Exon 3	2355-2356	GGATCCCAGTAA-AGGTTTTGGTAA	13.9 kb
166	06744-L18508	Exon 4	2731-2730 reverse	TCCTCATCTTCT-TCAGGAGGAGGA	5.4 kb
373	06745-L06349	Exon 5	2824-2825	AAGATTTCTGAT-GAGGAGGCAGAT	1.1 kb
454	10635-L11183	Exon 6	2944-2945	ATGAGCAGTCGT-TCAGTAAAAAAG	6.7 kb
214	06746-L06350	Exon 7	2976-2977	ATCTGGAGAGGA-GGTAGAAATTGA	8.3 kb
250	06747-L18509	Exon 8	3127-3126 reverse	TGTCGTACCTCT-GAAAGGAACTTG	3.6 kb
184	06748-L06352	Exon 9	3186-3187	GATAATGGACTT-TGCACGTAGCAC	1.8 kb
283	15977-L19002	Exon 10	3309-3310	AAAGATCGAGGA-GTTTGAGAAACT	0.2 kb
301	06749-L19005	Exon 11	3377-3378	CTGATGATTGGA-AGAAATCGGAGA	0.6 kb
202	10636-L13834	Exon 12	3666-3667	TCATGGGAGTCA-AGCTAGTCGTCG	1.3 kb
346	06750-L29521	Exon 13	12 nt after exon 13	GTCAGTGACCAT-ATTGGTGATTGC	4.7 kb
383	21008-L29226	Exon 14	3950-3951	TGGAAGAACTCT-TCAGCTTGCTTC	1.8 kb
256	06752-L06356	Exon 15	4242-4243	ACCTAACCTATT-AAACACTATGAT	5.7 kb
142	10637-L12898	Exon 16	4420-4421	CTGCTGCCAAAA-CTGAAGGCTGGT	0.8 kb
289	06754-L29522	Exon 17	4653-4654	CACTGCTGCTGA-TACCTGCATCAT	0.8 kb
232	06755-L07510	Exon 18	4761-4762	CTACAGGCTGAT-TACAAGAAATTC	0.5 kb
401	10638-L12897	Exon 19	5011-5010 reverse	GACTCAATGGTA-ATGGTGTGGGTT	3.5 kb
263	15976-L18131	Exon 20	5150-5151	ATATTGATGCCT-TAAATGGGAGGG	0.2 kb
409	06756-L18823	Exon 21	5230-5231	GTGAAGGAAGAT-GAGCTGATGGAG	3.0 kb
337	10639-L11187	Exon 22	5408-5409	ACGGACGCTATA-AACGCCAACTCA	0.4 kb
154	07285-L07512	Exon 23	5682-5681 reverse	TGTCCTCCTGGA-ACAGGGCATCTG	3.2 kb
355	06758-L06362	Exon 24	5761-5762	CTGTACTACCTA-AGACAAGAAGTG	0.5 kb
208	10641-L11189	Exon 25	5854-5855	CCTTTCCATGCT-GAAGTTCCTGCA	1.5 kb
275	06759-L19001	Exon 26	6030-6031	AGGAACAGACAT-GCTAGCAGATGG	0.5 kb
244	15975-L18130	Exon 27	6088-6089	GACCCAGAATAT-AAACCAACCAGA	0.2 kb
295	07286-L19004	Exon 28	6159-6160	GGATAAGGAAGA-ATCCATGGAAAT	0.9 kb
160	15974-L18507	Exon 29	6378-6379	TCTGGAAGCGGA-AAGGGAAGCTAT	0.3 kb
363	06760-L06364	Exon 30	6445-6446	GATTTTTACCGT-GTGGTATCCACC	0.7 kb
472	15978-L18133	Exon 31	7026-7027	GGTACTGGAACA-AGCCGAAGGCAA	1.2 kb
196	06761-L07509	Exon 32	7394-7395	TGGAGGACGGAG-ATCCTTCAGTAG	1.7 kb
427	06762-L13879	Exon 33	7610-7611	GGAGCTTTGCTG-AGCTCTCCATGG	0.6 kb
463	10642-L11190	Exon 34	7960-7961	TTTAAGACTCAA-ATGGAACTGCTC	4.2 kb
326	06763-L06367	Exon 35	8159-8160	CTTTTGAAGAAG-ATATAGAGACCC	1.5 kb
148	07287-L07511	Exon 36	53 nt after exon 36	AGCCCAGAAGGA-AGTGTTTTATCC	0.2 kb
418	10643-L11191	Exon 37	8554-8553 reverse	GGAGCAACTGCA-AATTCAGGATTT	2.8 kb
178	06765-L06369	Exon 38	9004-9005	AACACCACTACT-GCTTCTAGTCAA	
		Stop codon	*9509-9511 (exon 38)*		

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 in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

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 P201 CHARGE

- Busa T et al. (2016). Prenatal findings in children with early postnatal diagnosis of CHARGE syndrome. *Prenat Diagn.* 36:561-7.
- Lee SJ et al. (2015). Non-homologous end joining repair mechanism-mediated deletion of CHD7 gene in a patient with typical CHARGE syndrome. *Ann Lab Med.* 35:141-5.
- Magini P et al. (2019). Challenges in the clinical interpretation of small de novo copy number variants in neurodevelopmental disorders. *Gene.* 706:162-71.
- Schulz Y et al. (2014). CHARGE and Kabuki syndromes: a phenotypic and molecular link. *Hum Mol Genet.* 23:4396-4405.
- Sohn YB et al. (2016). Cerebellar vermis hypoplasia in CHARGE syndrome: clinical and molecular characterization of 18 unrelated Korean patients. *J Hum Genet.* 61:235-9.

P201 Product history	
Version	*Modification*
C4	Two reference probes have been replaced.
C3	One reference probe has been replaced and the length of several probes has been adjusted.
C2	QDX2 fragments have been added.
C1	5 new CHD7 probes and 5 reference probes have been added.
B1	CHD7 exon 16 probe has been replaced and 7 new CHD7 probes have been added. In addition, two extra control fragments at 100 and 105 nt have been included.
A1	First release.

Implemented changes in the product description

Version C4-01 — 03 December 2019 (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).
- Two references added to the section selected publications.
- Start and stop codon, as well as ligation sites of the probes targeting the *CHD7* gene updated according to new version of the NM_ reference sequence.
- Warning added to Table 1 for new probe in version C4, the warning for new probe in version C3 was removed from Table 1.
- Symbol for warning of probe located in or near a GC-rich region changed from ± to «.

Version 12 – 31 August 2016 (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).
- New references added on page 1.

Version 11 – 15 July 2015 (54)
- Figure(s) based on the use of old MLPA buffer (replaced in December 2012) removed.

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