Product Description SALSA® MLPA® Probemix P215-B4 EXT

To be used with the MLPA General Protocol.

Version B4. As compared to version B3, two reference probes have been removed. For complete product history see page 6.

Catalogue numbers:
- **P215-025R:** SALSA MLPA Probemix P215 EXT, 25 reactions.
- **P215-050R:** SALSA MLPA Probemix P215 EXT, 50 reactions.
- **P215-100R:** SALSA MLPA Probemix P215 EXT, 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 P215 EXT is a **research use only (RUO)** assay for the detection of deletions or duplications in the *EXT1* and *EXT2* genes, which are associated with Multiple Osteochondromas (MO). MO, also known as hereditary multiple exostoses, is inherited in an autosomal dominant manner. It is characterised by the development of multiple cartilage capped bony outgrowths, (mostly) on the metaphyses of the long bones, predominantly around the knee. Flat bones may also be affected, whereas facial bones usually remain unaffected. Osteochondromas are benign lesions with a risk of malignant transformation. Defects in the *EXT1* and *EXT2* genes on chromosomes 8 and 11, respectively, are the main cause of MO. These genes appear to function as tumour suppressor genes. The proteins encoded by the *EXT1* and *EXT2* genes are exostosin glycosyltransferases, involved in the chain elongation step of heparan sulfate biosynthesis in the Golgi apparatus. *EXT1* and *EXT2* have also been associated with other diseases. Langer-Giedion syndrome is caused by the deletion or mutation of at least two genes: *EXT1* and *TRPS1*. The loss of *EXT1* causes the multiple exostoses seen in people with Langer-Giedion syndrome. Potocki-Shaffer syndrome is caused by a deletion at the short arm of chromosome 11 resulting in, amongst others, the deletion of the *EXT2* gene. The loss of *EXT2* is responsible for multiple exostoses in these patients.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1235/.

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 *EXT1* and *EXT2* exon numberings used in this P215-B4 EXT product description are the exon numberings from the RefSeq transcripts NM_000127.2 and NM_000401.3, which are identical to the LRG_493 and LRG_494 sequences respectively. Please note that the *EXT2* exon numbering has changed. From description version B4-01 onwards, we have adopted the NCBI exon numbering that is present in the LRG_494 sequence for *EXT2* which combines both NM_000401.3 (transcript variant 1) and NM_207122.1 (transcript variant 2) and states that the *EXT2* gene consists of 15 exons. 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 P215-B4 EXT contains 37 MLPA probes with amplification products between 130 and 441 nucleotides (nt). This includes 13 probes for the *EXT1* gene, one probe for each exon of the gene and two probes for exon 1 and exon 11, and 16 probes for the *EXT2* gene, one probe for each exon of the gene and one probe in intron 2. 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, 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 Multiple Osteochondromas. 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
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 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 *EXT1* and *EXT2* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P215 EXT.
- 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.

***EXT1* and *EXT2* 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 SNPs and unusual results (e.g., a duplication of *EXT1* exons 5 and 7 but not exon 6) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P215-B4 EXT

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*EXT1*	*EXT2*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 00797-L00463	5q31		
137	**EXT2 probe** 06846-L06440			**Exon 12**
142	**EXT2 probe** 06847-L06441			**Exon 13**
148	**EXT1 probe** 06828-L07403		**Exon 6**	
154	**EXT2 probe** 06834-L06428			**Exon 1**
160	**EXT1 probe** 06829-L06423		**Exon 7**	
166	**EXT2 probe** 06843-L06437			**Exon 9**
172	Reference probe 07339-L05904	16q23		
178	**EXT1 probe** 06830-L06424		**Exon 8**	
184	**EXT2 probe** 06838-L06432			**Exon 4**
190	**EXT2 probe** 07791-L23351			**Exon 8**
196	Reference probe 10723-L11305	6p12		
202	**EXT2 probe** 12566-L13616			**Exon 7**
209	**EXT1 probe** 07794-L07530		**Exon 5**	
217	**EXT1 probe** 06831-L07404		**Exon 9**	
223	**EXT2 probe** 06835-L08769			**Exon 2**
229	**EXT2 probe** 06848-L06442			**Exon 14**
240	Reference probe 06211-L05178	19q12		
247	**EXT2 probe** 07466-L06431			**Exon 3**
256	Reference probe 00518-L00098	2q13		
265	**EXT1 probe** 06833-L08485		**Exon 11**	
274	**EXT1 probe** 12567-L13617		**Exon 2**	
283	**EXT2 probe** 12568-L13992			**Intron 2**
292 «	**EXT1 probe** 06822-L06416		**Exon 1**	
310	**EXT1 probe** 07793-L07529		**Exon 3**	
319	Reference probe 17454-L21210	12p13		
328	**EXT2 probe** 20888-L06443			**Exon 15**
336 «	**EXT1 probe** 07792-L22369		**Exon 1**	
346	**EXT2 probe** 06839-L22370			**Exon 5**
355	**EXT1 probe** 03080-L22371		**Exon 11**	
364	**EXT1 probe** 06832-L22372		**Exon 10**	
372	**EXT2 probe** 06845-L22373			**Exon 11**
382	**EXT1 probe** 06826-L06420		**Exon 4**	
391	Reference probe 04837-L04221	5p13		
409	**EXT2 probe** 06840-L06434			**Exon 6**
418	**EXT2 probe** 06844-L06438			**Exon 10**
441	Reference probe 05916-L05361	21q11		

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

« 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.

Table 2. P215-B4 probes arranged according to chromosomal location

Table 2a. *EXT1*

Length (nt)	SALSA MLPA probe	*EXT1* exon[a]	Ligation site NM_000127.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*774-776 (Exon 1)*		
292 «	06822-L06416	Exon 1	793-794	CAAAAAACGCTA-TTTCATCCTGCT	0.7 kb
336 «	07792-L22369	Exon 1	1449-1450	GTACTGAAAACT-TCCGACCCAACT	273.3 kb
274	12567-L13617	Exon 2	62 nt after exon 2, reverse	GGTGATAATGTT-AAACCCACTTAA	1.6 kb
310	07793-L07529	Exon 3	1889-1888, reverse	TGGTTCCAATTA-ATCACTTCAGAG	5.2 kb
382	06826-L06420	Exon 4	2004-2005	AGACACAATTCT-TGTGGGAGGCTT	7.8 kb
209	07794-L07530	Exon 5	2147-2148	CTACCACAGTAT-TCATCTTATCTG	2.8 kb
148	06828-L07403	Exon 6	2265-2266	CCCAGCCAGTGT-TGAAGCTTCTCG	1.2 kb
160	06829-L06423	Exon 7	2341-2342	TGACAAGCCCCT-ACCAGCCAAACA	5.6 kb
178	06830-L06424	Exon 8	2436-2437	TGCCCTACGACA-ACATCATCACAG	5.7 kb
217	06831-L07404	Exon 9	2604-2605	GATACACATCAA-AGTGGACGAACG	2.4 kb
364	06832-L22372	Exon 10	2718-2719	TGGTGGACCAAT-TGGCCAATTGTG	5.0 kb
265	06833-L08485	Exon 11	2896-2897	CATGAATACGTT-TGCCAGCTGGTT	0.2 kb
355	03080-L22371	Exon 11	3086-3087	TCCCAGTGCAGA-TCCACTCATCAG	
		stop codon	*3012-3014 (Exon 11)*		

Table 2b. *EXT2*

Length (nt)	SALSA MLPA probe	*EXT2* exon[a]	Ligation site NM_000401.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*57-59 (Exon 2)*		
154 $	06834-L06428	Exon 1	601 nt before exon 2; 47-48 in NM_207122.1	CTGCCTGGGAAA-ACACTGCAGCGG	0.7 kb
223	06835-L08769	Exon 2 (1)	72-73	CCTGCGCCTCAG-GGTCCGGTGGTG	7.2 kb
283 Ø	12568-L13992	Intron 2 (1)	4.2 kb before exon 2	CAAGAACCAGTG-TTCTAAGATTTT	4.4 kb
247	07466-L06431	Exon 3 (2)	314-315	ATCGAGTCCTCA-AATGACTGGAAT	1.3 kb
184	06838-L06432	Exon 4 (3)	721-722	TCACCTGTTGTT-CAACATGTTGCC	5.0 kb
346	06839-L22370	Exon 5 (4)	809-810	GGCGGCTTTTCT-ACGTGGACTTAC	10.7 kb
409	06840-L06434	Exon 6 (5)	1037-1038	GAGGGTGTCCTT-TCTGTCCGTAAG	2.0 kb
202	12566-L13616	Exon 7 (6)	1233-1234	TTGACTGGAAGA-GGTGGGTAGTAC	3.1 kb
190	07791-L23351	Exon 8 (7)	1247-1248	GCATCTGTGGTT-GTACCAGAAGAA	41.6 kb
166	06843-L06437	Exon 9 (8)	1416-1417	GGATCTATCCAT-ATGCTGCCATCT	26.2 kb
418	06844-L06438	Exon 10 (9)	1517-1518	CCACAGTCTCAA-GGGTTCACCGCC	8.9 kb
372	06845-L22373	Exon 11 (10)	1683-1684	GGGTTCCATTAA-AAGTTGTGAGGA	25.6 kb
137	06846-L06440	Exon 12 (11)	1843-1844	TCCTGACCGGTT-GGTGGGTTACCC	1.8 kb
142	06847-L06441	Exon 13 (12)	2038-2039	GAACTGTGAAGA-TATTGCCATGAA	2.1 kb
229	06848-L06442	Exon 14 (13)	2134-2135	GTGCACAGCCAT-AGATGGGCTTTC	7.8 kb
328	20888-L06443	Exon 15 (14)	2205-2206	TTGCTTCAGTCT-TCGGGACCATGC	
		stop codon	*2310-2312 (Exon 15)*		

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.
$ The sequence detected by this probe is present in exon 1 of NM_207122.1, which represents transcript variant 2 of *EXT2*. The significance of exon 1 deletions in this transcript variant is not clear as this exon is non-coding.
Ø Intron probe. Only included to facilitate the determination of the extent of a deletion/duplication. Copy number alterations of a single intron probe are unlikely to be related to the condition tested.

Related SALSA MLPA probemixes

P228 TRPS1-EXT1 Contains probes for the *TRPS1* and *EXT1* genes, involved in Langer-Giedion syndrome.

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 P215 EXT

- Akbaroghli S et al. (2017). Identification of a new mutation in an Iranian family with hereditary multiple osteochondromas. *Ther Clin Risk Manag*, *13*, 15.
- Delgado MA et al. (2014). A broad spectrum of genomic changes in latinamerican patients with EXT1/EXT2-CDG. *Sci rep*, *4*, 6407.
- Jamsheer A et al. (2014). Mutational screening of EXT1 and EXT2 genes in Polish patients with hereditary multiple exostoses. *J Appl Genet*, *55*(2), 183-188.
- Li Y et al. (2017). A genotype-phenotype study of hereditary multiple exostoses in forty-six Chinese patients. *BMC med genet*, *18*(1), 126.
- Li Y et al. (2018). Heterogeneous spectrum of EXT gene mutations in Chinese patients with hereditary multiple osteochondromas. *Medicine*, *97*(42).
- Long X et al. (2019). Identification of pathogenic mutations in 6 Chinese families with multiple exostoses by whole-exome sequencing and multiplex ligation-dependent probe amplification: Case series. *Medicine*, *98*(20).
- Medek K et al. (2017). Hereditary multiple exostoses: clinical, molecular and radiologic survey in 9 families. *Prague Med Rep*, *118*(2-3), 87-94.
- Oliver GR et al. (2019). RNA-Seq detects a SAMD12-EXT1 fusion transcript and leads to the discovery of an EXT1 deletion in a child with multiple osteochondromas. *Mol genet genom med*, *7*(3), e00560.
- Wang Y et al. (2015). Characterization of 26 deletion CNVs reveals the frequent occurrence of micro-mutations within the breakpoint-flanking regions and frequent repair of double-strand breaks by templated insertions derived from remote genomic regions. *Hum genet*, *134*(6), 589-603.

P215 Product history	
Version	*Modification*
B4	Two reference probes have been removed.
B3	Two reference probes have been removed.
B2	Four reference probes have been replaced and the control fragments have been adjusted (QDX2).
B1	*EXT1* exon 2, *EXT2* exons 3 and 8, and several reference probes have been replaced. In addition, extra control fragments at 88-96-100-105 nt have been included.
A1	First release.

Implemented changes in the product description
Version B4-01 — 07 November 2019 (02P) - Product description rewritten and adapted to a new template. - Exon numbering of *EXT2* has been adjusted. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). *Version 13 – 03 March 2017 (55)* - Warning added in Table 1 and Table 2, 292 nt probe 06822-L06416. - Minor textual changes. *Version 12 – 18 May 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).
- Exon numbering of the *EXT2* gene has been changed on page 4 and 5.
- Various minor textual changes and layout changes.
- Warning added below Table 2 for the 154 nt probe 06834-L06428 and the 283 nt probe 12568-L13992.
- Manufacturer's address adjusted.
- New reference added on page 1.
- Related product P096 added.

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