Product Description SALSA® MLPA® Probemix P223-B3 PHEX

To be used with the MLPA General Protocol.

Version B3. For complete product history see page 7.

Catalogue numbers:
- **P223-025R:** SALSA MLPA Probemix P223 PHEX, 25 reactions.
- **P223-050R:** SALSA MLPA Probemix P223 PHEX, 50 reactions.
- **P223-100R:** SALSA MLPA Probemix P223 PHEX, 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 P223 PHEX is a **research use only (RUO)** assay for the detection of deletions or duplications in the *PHEX* and *FGF23* genes, which are associated with X-linked hypophosphatemia (XLH; HYP).

XLH is a dominant disorder characterised by growth retardation, rachitic and osteomalacic bone disease, hypophosphatemia, renal defects in phosphate reabsorption, and vitamin D metabolism (Sabbagh et al. 2000). XLH is caused by mutations in the phosphate-regulating endopeptidase gene (*PHEX*). A form of autosomal dominant hypophosphatemic rickets (ADHR) is caused by mutation in the *FGF23* gene. The *FGF23* gene encodes a member of the fibroblast growth factor family.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK83985/.

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 *PHEX* and *FGF23* exon numbering used in this P223-B3 PHEX product description is the exon numbering from the RefSeq transcripts NM_000444.6 and NM_020638.3, which are identical to the NG_007563.2 and NG_007087.1 sequences, respectively. The exon numbering and NM_ sequences used have been retrieved on 02/2020. 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 P223-B3 PHEX contains 42 MLPA probes with amplification products between 130 and 463 nucleotides (nt). This includes 28 probes for the *PHEX* gene, one probe for each exon of the gene and two probes for exons 1, 3, 5, 11, 12, and 15, and four probes for the *FGF23 gene,* one probe for each exon and an additional probe for exon 1. 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.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-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 of the same sex 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 X-linked hypophosphatemia. 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. 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 when **reference samples of the same sex** have been used:

Copy number status		Dosage quotient
Autosomal sequences and X chromosome sequences in females	**X chromosome sequences in males**	
Normal	Normal	0.80 < DQ < 1.20
Homozygous deletion	Deletion	DQ = 0
Heterozygous deletion		0.40 < DQ < 0.65
Heterozygous duplication		1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	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 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 most genetic alterations in the *PHEX* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P223 PHEX.
- 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.

PHEX **and** ***FGF23*** **mutation database:** https://databases.lovd.nl/shared/genes/PHEX and https://databases.lovd.nl/shared/genes/FGF23. 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 *PHEX* exons 5 and 7 but not exon 6) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P223-B3 PHEX

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*PHEX*	*FGF23*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 00797-L00463	5q31		
136	**PHEX probe** 07103-L14743		**Exon 12**	
142	**FGF23 probe** 07118-L14932			**Exon 2**
148	**PHEX probe** 07112-L06692		**Exon 19**	
154	**PHEX probe** 07620-L06672		**Exon 3**	
165	**PHEX probe** 07099-L06679		**Exon 9**	
172	**FGF23 probe** 07119-L06699			**Exon 3**
179	**PHEX probe** 07093-L14931		**Exon 4**	
185	**PHEX probe** 07104-L06684		**Exon 12**	
193	Reference probe 03217-L02642	10q25		
199	**PHEX probe** 20664-L29231		**Exon 14**	
204	**PHEX probe** 07100-L29328		**Exon 10**	
211	**PHEX probe** 07114-L06694		**Exon 21**	
219	**PHEX probe** 07089-L06669		**Exon 1**	
227	Reference probe 01732-L01318	14q11		
234	**PHEX probe** 20667-L29233		**Exon 20**	
241	Reference probe 03240-L11136	13q14		
247	**PHEX probe** 07105-L06685		**Exon 13**	
256	**PHEX probe** 07091-L06671		**Exon 3**	
265	**PHEX probe** 20666-L06675		**Exon 5**	
274	Reference probe 13051-L14234	15q14		
283	**PHEX probe** 07107-L06687		**Exon 15**	
292	**PHEX probe** 07101-L06681		**Exon 11**	
301	**PHEX probe** 07096-L08395		**Exon 6**	
310	**PHEX probe** 07110-L06690		**Exon 17**	
319	**PHEX probe** 07622-L06695		**Exon 22**	
328	**PHEX probe** 07097-L06677		**Exon 7**	
343	**FGF23 probe** 07117-L06697			**Exon 1**
351	**PHEX probe** 20669-L08288		**Exon 11**	
358	**PHEX probe** 20670-L29234		**Exon 8**	
364	Reference probe 10802-L11442	2q36		
373	**PHEX probe** 07111-L06691		**Exon 18**	
382	**PHEX probe** 20668-L08287		**Exon 16**	
391	**PQBP1 probe** 03520-L02313		Xp11	
400	**PHEX probe** 07088-L06668		**Exon 1**	
409	**PHEX probe** 07108-L06688		**Exon 15**	
418	Reference probe 15646-L17515	20q13		
427	Reference probe 08370-L08224	17q21		
436	**FGF23 probe** 07116-L06696			**Exon 1**
445	**PHEX probe** 07090-L06670		**Exon 2**	
454	**PHEX probe** 07094-L06674		**Exon 5**	
463	Reference probe 08027-L07808	11q24		

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

Table 2. P223-B3 probes arranged according to chromosomal location

Table 2a. *PHEX*

Length (nt)	SALSA MLPA probe	*PHEX* exon[a]	Ligation site NM_000444.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*682-684 (Exon 1)*		
400	07088-L06668	Exon 1	618-619	ACGCCTCGCTCT-TGAGACCAGCCA	0.1 kb
219	07089-L06669	Exon 1	721-722	AGACTGGAAAGA-AGGCCAACAGAG	5.4 kb
445	07090-L06670	Exon 2	803-804	TTTTTCAGTGAG-TCAAGGTCTCTT	8.7 kb
256	07091-L06671	Exon 3	953-954	CTGGATAAGCAA-TAATCCAATTCC	0.1 kb
154	07620-L06672	Exon 3	9 nt after exon 3	GGGTAAGTTTCT-ACTGGGGTTTGG	29.2 kb
179	07093-L14931	Exon 4	1051-1052	AGAAATCAATCA-GTAGAAGGCGGG	1.1 kb
454	07094-L06674	Exon 5	1167-1168	ATCCTACGGCAT-TCACCTTTCCGC	0.1 kb
265	20666-L06675	Exon 5	1271-1272	GTTTCGTGGTCA-ATACAGCAATTC	12.8 kb
301	07096-L08395	Exon 6	1356-1357	CTGGACCAAGCA-ACACTCTCCCTG	3.6 kb
328	07097-L06677	Exon 7	1460-1461	TGCCGTGCTTTT-AGGAGCTAACAG	3.1 kb
358	20670-L29234	Exon 8	93 nt after exon 8, reverse	AGACTGAGAGCA-GGTTTTACAAGA	1.9 kb
165	07099-L06679	Exon 9	1645-1646	TCAAGAAGGTCA-TTGACACCAGAC	12.5 kb
204	07100-L29328	Exon 10	1801-1802	TGGTTTATTCCA-GAATTCCAAACC	2.9 kb
292	07101-L06681	Exon 11	24 nt before exon 11	AAATGAAGTTTA-ATCTGGATCAAT	0.1 kb
351	20669-L08288	Exon 11	1934-1935	CCCTTATGTTGT-TGGAAAGATGTT	19.0 kb
136	07103-L14743	Exon 12	2020-2021	GCTGGGCCTTTA-TTGACATGCTAG	0.1 kb
185	07104-L06684	Exon 12	84 nt after exon 12	TAGTTGGTTTTT-ATATTGACTGAA	34.6 kb
247	07105-L06685	Exon 13	2132-2131, reverse	CATGAGTATCAT-TCATTATAAACT	10.0 kb
199	20664-L29231	Exon 14	2218-2219	CTCGCAAGTATT-TAGCACAGTCTG	12.1 kb
283	07107-L06687	Exon 15	2273-2274	CTACAGGTGGTT-TACAAATCCGAC	0.1 kb
409	07108-L06688	Exon 15	82 nt after exon 15	AGGGTAAGACAT-TTCTTATTTCCC	22.4 kb
382	20668-L08287	Exon 16	2360-2361	GAAGCCTTTCTT-TTGGGGAACAGA	6.1 kb
310	07110-L06690	Exon 17	2389-2390	ATAGATCTCTGA-GTTATGGTGCTA	2.6 kb
373	07111-L06691	Exon 18	2485-2484, reverse	AGTAGACCACCA-AGGATCCAGGTT	4.9 kb
148	07112-L06692	Exon 19	2642-2643	GCGGGAAGCTTT-TAGGGTATGCGC	1.0 kb
234	20667-L29233	Exon 20	2664-2665	AGGAAATGGATA-AATGACAGAAGG	17.9 kb
211	07114-L06694	Exon 21	2799-2800	GAACAAGTCCAA-ATTGGTGCTCAC	2.5 kb
319	07622-L06695	Exon 22	2888-2889	CTGTCCACCCAA-TTCCACGATGAA	
		stop codon	*2929-2931 (Exon 22)*		

Table 2b. *FGF23*

Length (nt)	SALSA MLPA probe	*FGF23* exon[a]	Ligation site NM_020638.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*131-133 (Exon 1)*		
436	07116-L06696	Exon 1	167-168	GGGTCTGTGCCT-TGTGCAGCGTCT	0.1 kb
343	07117-L06697	Exon 1	302-303	AGATCCACAAGA-ATGGCCATGTGG	6.7 kb
142	07118-L14932	Exon 2	366-367	CAGATCAGAGGA-TGCTGGCTTTGT	2.2 kb
172	07119-L06699	Exon 3	777-776, reverse	CCATCGGGCTGT-TGTCCTCGGCGC	
		stop codon	*884-886 (Exon 3)*		

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.

References

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P223 Product history	
Version	*Modification*
B3	One reference probe has been replaced and several probes have been adjusted in length.
B2	The Y-fragment on 115 nt and one reference probe have been removed. Three reference probes have been replaced. Furthermore, the control fragments have been adjusted (QDX2).
B1	Six reference probes have been replaced. All reference probes are now autosomal. In addition, extra control fragments at 88-96-100-105 nt have been included.
A1	First release.

Implemented changes in the product description

Version B3-01 — 23 March 2020 (02P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the *PHEX* and *FGF23* genes updated according to new versions of the NM_ reference sequences.

Version 09 – 9 May 2016 (55)
- Product description is adapted to a new product version (version number changed, lot number added, tables 1 and 2 adapted and new pictures included).
- Ligation sites in the *PHEX* gene adjusted.
- Various textual changes on page 1 and 2.

Version 08 (52)
- 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).

Version 07 (48)
- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.
- Various minor textual changes

Version 06 (46)
- Remark on transcript variant used added below Table 2.

Version 05 (46) - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. - Partial probe sequences extended to 24 nt. - Data analysis method has been modified. - Various minor textual changes on page 1. - Various minor layout changes.

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