Product Description SALSA® MLPA® Probemix P463-A2 MRKH

To be used with the MLPA General Protocol.

Version A2. As compared to version A1, five reference probes have been replaced and one probe length has been adjusted. For complete product history see page 7.

Catalogue numbers:
- **P463-025R:** SALSA MLPA Probemix P463 MRKH, 25 reactions.
- **P463-050R:** SALSA MLPA Probemix P463 MRKH, 50 reactions.
- **P463-100R:** SALSA MLPA Probemix P463 MRKH, 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 P463 MRKH is a **research use only (RUO)** assay for the detection of deletions or duplications in the *TBX6*, *LHX1*, *HNF1B*, and *TBX1* genes, which are associated with Mayer-Rokitansky-Küster-Hauser syndrome (MRKH).

MRKH is characterised by normal physical development of the secondary sexual characteristics and a normal female 46,XX karyotype but with complete aplasia of the uterus, cervix, and superior parts of vagina leading to failure to menstruate and infertility. This syndrome is distinguishable in type I with normally developed fallopian tubes, ovaries, and urinary tract, and type II with fallopian or ovarian abnormalities and additional malformations which involve the urinary tract and spine. MRKH has an incidence of approximately 1 in 5,000 new-born girls. Defects in the *TBX6*, *LHX1*, *HNF1B*, and *TBX1* genes on chromosomes 16, 17, and 22 are some of the causes for the development of MRKH syndrome.

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 *TBX6*, *LHX1*, *HNF1B*, and *TBX1* exon numbering used in this P463-A2 MRKH product description is the exon numbering from the RefSeq transcripts NM_004608.3, NM_005568.5, NM_000458.4, and NM_080647.1, which are identical to the NG_023283.1 (*TBX6*), NG_013019.2 (*HNF1B*), and LRG_226 (*TBX1*) sequences. The exon numbering and NM_ sequences used have been retrieved on 06/2020. 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 P463-A2 MRKH contains 45 MLPA probes with amplification products between 121 and 498 nucleotides (nt). This includes nine probes for the *TBX6* gene, one probe for each exon, five probes for the *LHX1* gene, one probe for each exon, nine probes for the *HNF1B* gene, one probe for each exon, and 12 probes for *TBX1* gene, one probe for each exon of the gene, two probes for

exon 9, and additional two probes downstream of exon 9 targeting exons present in other transcript variants. In addition, ten 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 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 Mayer-Rokitansky-Küster-Hauser syndrome. 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 or in or near the *TBX1* 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 *TBX6*, *LHX1*, *HNF1B*, and *TBX1* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P463 MRKH.
- 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.

LOVD 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 *TBX6* exons 2 and 4 but not exon 3) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix Probemix P463-A2 MRKH

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]				
		Reference	*TBX6*	*LHX1*	*HNF1B*	*TBX1*
64-105	Control fragments – see table in probemix content section for more information					
121	Reference probe 19616-L27455	4p13				
130 *	Reference probe 14118-L15719	7p22				
142	**TBX6 probe** 20417-L28418		**Exon 8**			
147	**HNF1B probe** 09858-L27515				**Exon 7**	
154	**TBX6 probe** 20413-L27855		**Exon 2**			
160	**HNF1B probe** 07699-L12885				**Exon 2**	
166 « Ж	**TBX1 probe** 09404-SP0975-L29167					**Exon 1**
178	**HNF1B probe** 20396-L27838				**Exon 6**	
184 «	**TBX1 probe** 05408-L29326					**Exon 2**
190	Reference probe 16365-L18758	12q13				
196 «	**TBX1 probe** 09411-L10879					**Exon 8**
202	**LHX1 probe** 20400-L27842			**Exon 1**		
211	**TBX6 probe** 20415-L27857		**Exon 7**			
219	**LHX1 probe** 20399-L28951			**Exon 3**		
225 Ж	**TBX6 probe** 20412-SP0972-L28952		**Exon 6**			
231	**HNF1B probe** 20395-L28900				**Exon 8**	
237	Reference probe 14756-L16453	11q23				
247 «	**TBX1 probe** 10810-L24823					**Exon 7**
256	**TBX6 probe** 20409-L27851		**Exon 5**			
265	**LHX1 probe** 20402-L27844			**Exon 4**		
274	**TBX1 probe** 20407-L27849					**Downstream**
283 *	Reference probe 18166-L22686	3p21				
289	**TBX6 probe** 20410-L27852		**Exon 4**			
301	**HNF1B probe** 08299-L09335				**Exon 9**	
310	**LHX1 probe** 20401-L27843			**Exon 5**		
318 «	**TBX1 probe** 20406-L27848					**Exon 6**
328 *	Reference probe 14301-L15971	15q13				
335	**HNF1B probe** 07698-L18619				**Exon 1**	
346	**LHX1 probe** 20403-L27845			**Exon 2**		
355 «	**TBX1 probe** 09407-L27518					**Exon 4**
364	**HNF1B probe** 07701-L07460				**Exon 4**	
373 «	**TBX1 probe** 20408-L27850					**Exon 9**
382 *	Reference probe 20768-L28670	1q24				
391	**TBX6 probe** 20414-L27856		**Exon 9**			
399	**TBX1 probe** 09413-L09699					**Downstream**
409 «	**TBX1 probe** 09408-L09694					**Exon 5**
418	**HNF1B probe** 20397-L27839				**Exon 5**	
425	Reference probe 12789-L13924	2q13				
445	**TBX6 probe** 20416-L27858		**Exon 1**			
454	**HNF1B probe** 20398-L27840				**Exon 3**	
463 *	Reference probe 20114-L27638	8q24				
472 «	**TBX1 probe** 20404-L27846					**Exon 3**
481	**TBX6 probe** 20411-L28610		**Exon 3**			
490 «	**TBX1 probe** 20405-L27847					**Exon 9**
498 ¥	Reference probe 19022-L24835	21q22				

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

* New in version A2.
¥ Changed in version A2. Minor alteration, no change in sequence detected.
« 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.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

Table 2. P463-A2 probes arranged according to chromosomal location

Table 2a. *TBX6* gene

Length (nt)	SALSA MLPA probe	***TBX6*** **exon[a]**	Ligation site NM_004608.3	**Partial sequence[b] (24 nt adjacent to ligation site)**	Distance to next probe
		start codon	*61-63 (Exon 2)*		
445	20416-L27858	Exon 1	18 nt after exon 1	GCCTTCGAACTT-TGGAGGCTTGGC	0.7 kb
154	20413-L27855	Exon 2	56-57	CAGACGGAACTA-CAACATGTACCA	0.4 kb
481	20411-L28610	Exon 3	387-388	TCTGTGGGAACA-GAAATGATCATC	1.8 kb
289	20410-L27852	Exon 4	661-662	TCACCAACAGCA-CGCTGGACCCCC	0.2 kb
256	20409-L27851	Exon 5	711-712	CACAAGTACCAA-CCCCGCATACAC	0.3 kb
225 Ж	20412-SP0972-L28952	Exon 6	851-852 & 886-887	GAAGATTGCAGC-35 nt spanning oligo-GAAACTGTAAGA	1.8 kb
211	20415-L27857	Exon 7	956-957	AGCAGCCACAGA-GGCCTATGGGAG	0.2 kb
142	20417-L28418	Exon 8	1030-1031	GTGAATCAGATC-CAGAACAGGCCC	0.4 kb
391	20414-L27856	Exon 9	1315-1314, reverse	GGTGTATGGTAG-AGGGAAGGGGCC	
		stop codon	*1369-1371 (Exon 9)*		

Table 2b. *LHX1* gene

Length (nt)	SALSA MLPA probe	***LHX1*** **exon[a]**	Ligation site NM_005568.5	**Partial sequence[b] (24 nt adjacent to ligation site)**	Distance to next probe
		start codon	*724-726 (Exon 1)*		
202	20400-L27842	Exon 1	829-830	GTGAATGTAAAT-GCAACCTGACCG	2.1 kb
346	20403-L27845	Exon 2	1039-1040	AGGAACTCTACA-TCATCGACGAGA	0.3 kb
219	20399-L28951	Exon 3	1211-1212	CAACGTGTCGGA-CAAGGAAGCGGG	1.5 kb
265	20402-L27844	Exon 4	5 nt before exon 4	GCCGCCATGTGC-TGCAGGTCTGGT	1.1 kb
310	20401-L27843	Exon 5	2063-2064	CAGCCTCGAGAA-CCATTCTCCTTC	
		stop codon	*1942-1944 (Exon 5)*		

Table 2c. *HNF1B* gene

Length (nt)	SALSA MLPA probe	***HNF1B*** **exon[a]**	Ligation site NM_000458.4	**Partial sequence[b] (24 nt adjacent to ligation site)**	Distance to next probe
		start codon	*176-178 (Exon 1)*		
335	07698-L18619	Exon 1	180-181	TTGGAAAATGGT-GTCCAAGCTCAC	5.3 kb
160	07699-L12885	Exon 2	572-573	TGCAGCAACACA-ACATCCCCCAGA	5.8 kb
454	20398-L27840	Exon 3	732-733	ATTCAACCAGAC-AGTCCAGAGTTC	2.0 kb
364	07701-L07460	Exon 4	1051-1050, reverse	ACACGGACCTCA-GTGACCAAGTTG	21.2 kb
418	20397-L27839	Exon 5	1316-1317	GCCAGTCGGTTT-TACAGCAAGTCT	5.6 kb
178	20396-L27838	Exon 6	1459-1460	CATAATCCCCAG-CAATCTCAAAAC	3.9 kb
147	09858-L27515	Exon 7	1634-1633, reverse	GCTCTGCTGCAT-GAGGGGCTGCTG	2.0 kb
231	20395-L28900	Exon 8	1798-1799	AGCAGCATCAGT-ACACTCACCAAC	12.0 kb
301	08299-L09335	Exon 9	2079-2080	CTCTCCCACGAT-GTCAAGGACTCC	
		stop codon	*1847-1849 (Exon 9)*		

Table 2d. *TBX1* gene

Length (nt)	SALSA MLPA probe	*TBX1* exon[a]	Ligation site NM_080647.1	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*130-132 (Exon 2)*		
166 Ж «	09404-SP0975-L29167	Exon 1	27-28 & 15 nt after exon 1	AGGAGGAAGGGA-31 nt spanning oligo-ACACTTGCCGCG	2.8 kb
184 «	05408-L29326	Exon 2	101-102	CCGGGTGAAGCT-TCGCTGGCTGCC	1.6 kb
472 «	20404-L27846	Exon 3	462-463	GCCGGTGTGAGC-GTGCAGCTAGAG	2.1 kb
355 «	09407-L27518	Exon 4	560-561	TCCCACCTTCCA-AGTGAAGCTCTT	1.0 kb
409 «	09408-L09694	Exon 5	752-753	GCAGTGGATGAA-GCAAATCGTGTC	0.7 kb
318 «	20406-L27848	Exon 6	863-864	CCACGTGGTCTA-TGTGGACCCACG	0.8 kb
247 «	10810-L24823	Exon 7	1007-1008	TCCCTTCGCGAA-AGGCTTCCGGGA	0.2 kb
196 «	09411-L10879	Exon 8	1136-1137	CGGCACGGAGAA-AGGTAGGGCCGG	0.6 kb
373 «	20408-L27850	Exon 9	1397-1398	GGCCGCCGCCTA-CGACCACTATCT	0.7 kb
490 «	20405-L27847	Exon 9	12 nt after exon 9	AAGCCGCCTGCG-TGTCCATTTATT	12.0 kb
274 +	20407-L27849	Downstream	NM_080646.1; 1250-1249, reverse	CTGCGGGGCAGG-GGAGCCCCAGGT	4.0 kb
399 +	09413-L09699	Downstream	NM_005992.1; 1282-1283	AAGTCAGGAGGT-CAAGTGTGCATG	
		stop codon	*1615-1617 (Exon 9)*		

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.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.
+ The 274 nt probe detects exon 9 in transcript variant A (NM_080646.1); the 399 nt probe detects exon 10 in transcript variant B (NM_005992.1).

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 P463 MRKH

- Morisada N et al. (2019). Clinical characteristics of HNF1B-related disorders in a Japanese population. *Clin Exp Nephrol*, 23(9), 1119-1129.
- Wang Y et al. (2019). Genetic Screening in a Large Chinese Cohort of Childhood Onset Hypoparathyroidism by Next-Generation Sequencing Combined with TBX1-MLPA. *Journal Bone Miner Res*, 34(12), 2254-2263.

P463 Product history	
Version	*Modification*
A2	Five reference probes have been replaced and one probe length has been adjusted.
A1	First release.

Implemented changes in the product description
Version A2-01 — 16 July 2020 (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). - Ligation sites of the probes targeting the *HNF1B* gene updated according to new version of the NM_ reference sequence. *Version 01 – 08 August 2016 (55)* - Not applicable, new document.

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