Product Description
SALSA® MLPA® Probemix P445-A3 KDM6A

To be used with the MLPA General Protocol.

Version A3
As compared to version A2, four reference probes have been replaced and one target probe has been changed in length (not in sequence detected). For complete product history see page 7.

Catalogue numbers:
- **P445-025R:** SALSA MLPA Probemix P445 KDM6A, 25 reactions.
- **P445-050R:** SALSA MLPA Probemix P445 KDM6A, 50 reactions.
- **P445-100R:** SALSA MLPA Probemix P445 KDM6A, 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 P445 KDM6A is a **research use only (RUO)** assay for the detection of deletions or duplications in the *KDM6A* gene, which is associated with Kabuki syndrome.

Kabuki syndrome is a congenital mental retardation syndrome with additional features including postnatal dwarfism and distinctive facial features. Type 1 Kabuki syndrome (KS1) has been linked to mutations in the *MLL2* gene (also known as *KMT2D*), while Type 2 Kabuki syndrome (KS2) has been associated with the X-chromosomal *KDM6A* gene, also known as *UTX* and *KABUK2*. *KDM6A* encodes a histone demethylase that interacts with *MLL2*.

The *KDM6A* gene (30 exons) spans ~240 kb of genomic DNA and is located on Xp11.3, about 45 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK62111/.

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 *KDM6A* exon numbering used in this P445-A3 KDM6A product description is the exon numbering from the LRG_616 sequence. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content
The SALSA MLPA Probemix P445-A3 KDM6A contains 41 MLPA probes with amplification products between 130 and 463 nucleotides (nt). This includes 32 probes for the *KDM6A* gene, one probe for each exon (with the exception of exon 14) and two probes for exon 3, 4 and 19. In addition, nine reference probes are included that detect locations on the X-chromosome. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.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.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal 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.mrcholland.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 different unrelated individuals who are from families without a history of Kabuki 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 (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. Sample ID number NA10636 (female) from the Coriell Institute has been tested with this P445-A3 probemix at MRC Holland and can be used as a positive control sample to detect a heterozygous duplication of the *KDM6A* 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.mrcholland.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 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:

Copy Number status: Male samples	Final ratio
Normal	0.80 < FR < 1.20
Deletion	FR = 0
Duplication	1.65 < FR < 2.25
Ambiguous copy number	All other values

Copy Number status: Female samples	Final ratio
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous 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.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *KDM6A* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P445 KDM6A.
- 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.

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.

***KDM6A* mutation database**

https://databases.lovd.nl/shared/genes/KDM6A. We strongly encourage users to deposit positive results in the Leiden Open Variation 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 SNVs and unusual results (e.g., a duplication of *KDM6A* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P445-A3 KDM6A

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*KDM6A*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 13917-L02320	Xq	
142	**KDM6A probe** 19373-L25780		**Exon 19**
148	**KDM6A probe** 19374-L26405		**Exon 6**
154	**KDM6A probe** 19375-L25782		**Exon 21**
162	**KDM6A probe** 19376-L26504		**Exon 17**
166	Reference probe 07099-L26081	Xp	
172	**KDM6A probe** 19377-L25784		**Exon 25**
177	**KDM6A probe** 19378-L25785		**Exon 20**
184	Reference probe 05592-L04518	Xq	
190	**KDM6A probe** 19379-L25786		**Exon 29**
196	**KDM6A probe** 19380-L25787		**Exon 27**
203 ¥	**KDM6A probe** 19381-L32656		**Exon 15**
209	**KDM6A probe** 19382-L26082		**Exon 1**
220	**KDM6A probe** 19383-L25790		**Exon 28**
227	**KDM6A probe** 19384-L25791		**Exon 16**
233	**KDM6A probe** 19385-L25792		**Exon 4**
238 *	Reference probe 03120-L02560	Xq	
244	**KDM6A probe** 19386-L26505		**Exon 24**
250	**KDM6A probe** 19387-L26506		**Exon 7**
256	**KDM6A probe** 19388-L25795		**Exon 30**
265	**KDM6A probe** 19389-L25796		**Exon 10**
274	**KDM6A probe** 19390-L25797		**Exon 26**
292	**KDM6A probe** 19391-L25798		**Exon 5**
301 *	Reference probe 12603-L13687	Xq	
310	**KDM6A probe** 19392-L25799		**Exon 3**
319	**KDM6A probe** 19393-L25800		**Exon 9**
328	Reference probe 15358-L17192	Xq	
337	**KDM6A probe** 19394-L25801		**Exon 18**
346	**KDM6A probe** 19395-L25802		**Exon 4**
355	**KDM6A probe** 19396-L25803		**Exon 11**
364	**KDM6A probe** 19398-L25805		**Exon 22**
373 Ø	**KDM6A probe** 19399-L25806		**Exon 8**
383	Reference probe 06472-L05998	Xp	
391	**KDM6A probe** 19400-L25807		**Exon 2**
400	**KDM6A probe** 19401-L25808		**Exon 12**
408*	Reference probe 19792-L26598	Xp	
418	**KDM6A probe** 19402-L25809		**Exon 23**
426	**KDM6A probe** 19548-L25804		**Exon 13**
436 Ø	**KDM6A probe** 19403-L25810		**Exon 3**
445	**KDM6A probe** 19404-L25811		**Exon 19**
463 *	Reference probe 14798-L16507	Xp	

[a] See section Exon numbering on page 1 for more information.

* New in version A3.
¥ Changed in version A3. Minor alteration, no change in sequence detected.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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.

Table 2. *KDM6A* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*KDM6A* exon[a]	Ligation site NM_001291415.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*365-367 (Exon 1)*		
209	19382-L26082	Exon 1	26 nt after exon 1	CACTCGCCCGGT-CGGCTCCGGACG	0.2 kb
391	19400-L25807	Exon 2	4 nt before exon 2	GTGTCTGTCTCC-ACAGCCGCCTCT	87.4 kb
310 #	19392-L25799	Exon 3	646-645 *reverse*	TGACAAAAGAAA-TCAGACTCCACT	0.5 kb
436 Ø	19403-L25810	Exon 3	396 nt after exon 3	AATCTGATCGTT-AAGTAGCCATGT	12.7 kb
233	19385-L25792	Exon 4	153 nt before exon 4	TACGTTTCTGAA-CTGAGGGGGGCC	0.2 kb
346	19395-L25802	Exon 4	734-735	ACAGTTTACAGT-CTGACTACTGGA	36.3 kb
292	19391-L25798	Exon 5	805-806	AATGCATTTCAG-TGGTAAGTTGAC	9.7 kb
148	19374-L26405	Exon 6	913-912 *reverse*	AAACTAGACTCA-TAGTCTGTGTTC	14.2 kb
250	19387-L26506	Exon 7	38 nt before exon 7 *reverse*	TGCAGTCAATTT-AGAGTCACACAA	2.2 kb
373 Ø	19399-L25806	Exon 8	553 nt before exon 8	ATGCCTGATGTC-ACAACAAATGTA	14.8 kb
319	19393-L25800	Exon 9	141 nt after exon 9	AGCATATTTGTA-TCCTCTGCCCAG	2.1 kb
265	19389-L25796	Exon 10	114 nt after exon 10	TTTTCTCTTCTA-AATACATTATTA	4.7 kb
355	19396-L25803	Exon 11	273 nt before exon 11	ATTATAATATTG-AAGTGATATGTC	0.6 kb
400 #	19401-L25808	Exon 12	1414-1413 *reverse*	GCTGCAGCATGG-CCATGGTCCAAT	0.5 kb
426	19548-L25804	Exon 13	157 nt before exon 13	AGGTTTATATTC-CGGTTACCCTGT	1.3 kb
	no probe	*Exon 14*			
203	19381-L32656	Exon 15	220 nt before exon 15	TTTGAGGGTGGG-CCTTTTTGGGTG	1.4 kb
227	19384-L25791	Exon 16	108 nt before exon 16	CAGTGTGTTTGA-CCAGATAGTGGT	1.3 kb
162	19376-L26504	Exon 17	26 nt after exon 17 *reverse*	ATGTGAAAAGCC-AACAAGGAAGCT	5.8 kb
337	19394-L25801	Exon 18	2500-2501	GAACGACCTCTC-TCTTCCACTGGG	7.2 kb
445	19404-L25811	Exon 19	35 nt after exon 19	AAATAAAACAGT-GTTTGCAACTAC	0.2 kb
142	19373-L25780	Exon 19	233 nt after exon 19 *reverse*	AATTAGGGCAAT-AAAAAATGTTTC	1.3 kb
177	19378-L25785	Exon 20	9 nt before exon 20 *reverse*	CAACTGTAAAAT-TAAAATATGTGG	0.6 kb
154	19375-L25782	Exon 21	133 nt before exon 21	GGTTGTGCAGAG-GCCCTAGTTTTT	3.5 kb
364	19398-L25805	Exon 22	7 nt before exon 22 *reverse*	TCTTCCTATAGA-TTAAGCAAAAAG	0.2 kb
418	19402-L25809	Exon 23	3790-3791	AATATTGACCTA-TCTGATGACAAA	0.8 kb
244	19386-L26505	Exon 24	3944-3945	TTCCAGGGAGCA-GAACACCAGGTA	2.4 kb
172	19377-L25784	Exon 25	47 nt after exon 25	TTAGAAAGCAGT-AATTGTAAACGA	4.0 kb
274	19390-L25797	Exon 26	96 nt after exon 26	TCACTAAAAGTT-GTAAGAAGAATG	0.8 kb
196	19380-L25787	Exon 27	4319-4320	GTGTGAAGTCAA-TAGTACCCATGG	16.6 kb
220	19383-L25790	Exon 28	4411-4412	TATTGTCTTCTA-AGAACTCTGAAG	2.8 kb
190	19379-L25786	Exon 29	4672-4673	GAGGACCTGATG-CAAGTCTATGAC	1.1 kb
256	19388-L25795	Exon 30	20 nt before exon 30	ATCAAAATAACC-TACCTTACTTTT	
		stop codon	*4724-4726 (Exon 30)*		

[a] See section Exon numbering on page 1 for more information.
[b] Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.
This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

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

- P389 MLL2: Contains probes for the *MLL2* gene, involved in Kabuki syndrome type 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 P445 KDM6A

- Greif PA et al. (2018). Evolution of cytogenetically normal acute myeloid leukemia during therapy and relapse: an exome sequencing study of 50 patients. *Clin Cancer Res*. 24:1716-1726.
- Paděrová J et al. (2016). Molecular genetic analysis in 14 Czech Kabuki syndrome patients is confirming the utility of phenotypic scoring. *Clin Genet*. 90:230-237.
- So PL et al. (2020). Clinical and molecular characterization study of Chinese Kabuki syndrome in Hong Kong. *Am J Med Genet A*. 185:675-686.

P445 product history	
Version	*Modification*
A3	Four reference probes have been replaced and one target probe has been changed in length (not in sequence detected).
A2	One reference probe has been removed from the probemix.
A1	First release.

Implemented changes in the product description
Version A3-01 – 14 April 2021 (04P) - 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 *KDM6A* gene updated according to new version of the NM_ reference sequence. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. *Version 03 (55) – 10 May 2017* - 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).

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