Product Description
SALSA® MLPA® Probemix P266-B2 CLCNKB

To be used with the MLPA General Protocol.

Version B2
For complete product history see page 7.

Catalogue numbers:
- **P266-025R:** SALSA MLPA Probemix P266 CLCNKB, 25 reactions.
- **P266-050R:** SALSA MLPA Probemix P266 CLCNKB, 50 reactions.
- **P266-100R:** SALSA MLPA Probemix P266 CLCNKB, 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 P266 CLCNKB is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CLCNKB* and *CLCNKA* genes, which are associated with Bartter syndrome.

Bartter syndrome refers to a group of disorders in which salt reabsorption in the thick ascending loop of Henle is impaired by approximately 70%. The phenotype of this syndrome is characterised by short stature, hyperactive renin-angiotensin system, lack of effect of angiotensin on blood pressure, renal potassium wasting, increased renal prostaglandin production, and occasionally hypomagnesemia. At present, four types of Bartter syndrome have been identified:
- Antenatal Bartter syndrome type 1 and 2 are caused by loss-of-function mutations in the *SLC12A1* gene and *KCNJ1* gene, respectively.
- Bartter syndrome type 3 is due to mutations in the kidney chloride channel B (*CLCNKB*) gene.
- Bartter syndrome type 4 (or infantile Bartter syndrome with sensorineural deafness) results from mutations of the *BSND* gene (type 4A) or by simultaneous mutations in both the *CLCNKA* and *CLCNKB* genes (type 4B).

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 *CLCNKB* and *CLCNKA* exon numbering used in this P266-B2 CLCNKB product description is the exon numbering from the NG_013079.1 and NG_009359.1 sequences, respectively. 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 P266-B2 CLCNKB contains 29 MLPA probes with amplification products between 136 and 409 nucleotides (nt). This includes 14 probes covering 14 out of 20 exons of the *CLCNKB* gene and two probes for the *CLCNKA* gene, one for exon 5 and one for exon 10. Furthermore, two flanking probes located upstream of *CLCNKA* have been included to aid determination of the extent of a deletion/duplication. In addition, 11 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.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 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 Bartter syndrome. 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. 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 for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
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 *CLCNKB* and *CLCNKA* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P266 CLCNKB.
- 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.

LOVD mutation database

https://databases.lovd.nl/shared/genes. 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 *CLCNKB* exons 13 and 15 but not exon 14) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P266-B2 CLCNKB

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*CLCNKB*	*CLCNKA*
64-105	Control fragments – see table in probemix content section for more information			
136	Reference probe 07990-L07771	7q		
142	**CLCNKB probe** 10834-L12513		**Exon 2**	
148	**CLCNKB probe** 08559-L08560		**Exon 10**	
154	Reference probe 07972-L07753	17q		
166	**CLCNKB probe** 08563-L08564		**Exon 15**	
171	**CLCNKB probe** 08558-L08559		**Exon 8**	
177	Reference probe 06074-L06374	22q		
184 ¬	**CASP9 probe** 02880-L11959			**Upstream**
190	**CLCNKA probe** 08551-L21236			**Exon 5**
196	**CLCNKB probe** 08566-L08567		**Exon 19**	
211	Reference probe 05977-L05402	20p		
220	**CLCNKB probe** 08562-L11960		**Exon 14**	
229	**CLCNKB probe** 08553-L08554		**Exon 1**	
238	Reference probe 05799-L05246	15q		
247	Reference probe 06747-L06351	8q		
256	**CLCNKB probe** 08561-L11961		**Exon 13**	
264	**CLCNKB probe** 08564-L08565		**Exon 17**	
274	Reference probe 21213-L29588	9p		
288	Reference probe 21389-L04248	5p		
301	**CLCNKB probe** 08556-L11234		**Exon 5**	
310	**CLCNKB probe** 14862-L16584		**Exon 11**	
319	Reference probe 06440-L05966	3p		
328	**CLCNKA probe** 08552-L08553			**Exon 10**
346	Reference probe 05273-L04655	2p		
360 ¬«	**PRDM2 probe** 04702-L21354			**Upstream**
369	**CLCNKB probe** 08555-L08556		**Exon 3**	
377	**CLCNKB probe** 08557-L10728		**Exon 6**	
400 «	**CLCNKB probe** 08565-L11962		**Exon 18**	
409	Reference probe 09497-L09754	11q		

[a] See section Exon numbering on page 1 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.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

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. P266-B2 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
360 ¬«	04702-L21354	*PRDM2*		TTGACCTTCCCT-CCACTCTTACAG	1.8 **Mb**
184 ¬	02880-L11959	*CASP9*		GGTCGAGAAGAT-TGTGAACATCTT	0.5 **Mb**
190	08551-L21236	*CLCNKA* Exon 5	NM_004070.4; 518-517, reverse	TTGCCCAGGAAC-AGGGTGCTGCCG	1.8 kb
328	08552-L08553	*CLCNKA* Exon 10	NM_004070.4; 906-905, reverse	GACGCCGCAGAT-GCCACTGCGGGG	15.9 kb
		CLCNKB	NM_000085.5		
		start codon	*107-109 (Exon 2)*		
229	08553-L08554	Exon 1	16 nt after exon 1	GGGGTCGCTGCA-AGATGCTGGGGC	0.6 kb
142 #	10834-L12513	Exon 2	28 nt before exon 2	AGCAGCTCACCG-CGGTCCCTCCCT	1.2 kb
369	08555-L08556	Exon 3	321-322	CTTGGCTGTTGA-GAGTGTGGTCCG	2.3 kb
301 #	08556-L11234	Exon 5	482-483	GAATCCCGGAGG-TGAAGACCATGT	0.4 kb
377 #	08557-L10728	Exon 6	634-635	TCTGTGATGATG-GCTGCCTACCTG	0.8 kb
171 #	08558-L08559	Exon 8	806-805, reverse	CCAGTAATCCCA-GACAGAGAAGTG	0.7 kb
148	08559-L08560	Exon 10	1041-1042	CTTCATCAGGAA-CAATAGGTTCAG	0.7 kb
310	14862-L16584	Exon 11	1133-1134	CCTACCCACCCA-GCGCCGGCCGCT	1.0 kb
256 #	08561-L11961	Exon 13	1401-1402	CATCTTTGTCTA-TGGTGAGTCTGG	0.2 kb
220	08562-L11960	Exon 14	1445-1446	CTCTCTCTTTTA-TCTTCCCTGAGG	0.5 kb
166 #	08563-L08564	Exon 15	1577-1576, reverse	CTGGCCGGTCAC-CTCGAAGGCCAG	3.2 kb
264 #	08564-L08565	Exon 17	1885-1886	CTGGTGGGCATA-GTGCGAAGGGCC	0.3 kb
400 «	08565-L11962	Exon 18	2007-2008	AGTGACCCTGAA-GCTGTCCCCAGA	0.7 kb
196 #	08566-L08567	Exon 19	2041-2040, reverse	TCAAAGAGGTTG-TGTGCCTGGATG	
		stop codon	*2168-2170 (Exon 20)*		

[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.

« 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.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.
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. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Related SALSA MLPA probemixes

P136 Gitelman Contains probes for the *SLC12A3* gene involved in Gitelman 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 P266 CLCNKB

- Andrini O et al. (2014). CLCNKB mutations causing mild Bartter syndrome profoundly alter the pH and Ca2+ dependence of ClC-Kb channels. Pflugers Arch. 466:1713-23.
- Castaño AG et al. (2017). Poor phenotype-genotype association in a large series of patients with Type III Bartter syndrome. *PloS one*, 12(3), e0173581.
- Han Y et al. (2019). Thirteen novel CLCNKB variants and genotype/phenotype association study in 42 Chinese patients with Bartter syndrome type 3. *Endocrine*, 1-11.
- Matsunoshita N et al. (2015). Differential diagnosis of Bartter syndrome, Gitelman syndrome, and pseudo-Bartter/Gitelman syndrome based on clinical characteristics. Genet Med. 18(2), 180-188.
- Nozu K et al. (2018). Detection of copy number variations by pair analysis using next-generation sequencing data in inherited kidney diseases. *Clin Exp Nephrol*, 22(4), 881-888.
- Okhubo K et al. (2014). A novel mutation of CLCNKB in a Japanese patient of Gitelman-like phenotype with diuretic insensitivity to thiazide administration. Meta Gene. 2:342-8.
- Sahbani D et al. (2020). Functional Study of Novel Bartter's Syndrome Mutations in ClC-Kb and Rescue by the Accessory Subunit Barttin Toward Personalized Medicine. *Front Pharmacol*, 11, 327.
- Seys, E et al. (2017). Clinical and genetic spectrum of Bartter syndrome type 3. *J Am Soc Nephrol*, 28(8), 2540-2552.
- de Vos B et al. (2018). A novel succinate dehydrogenase subunit B germline variant associated with head and neck paraganglioma in a Dutch kindred: A family-based study. *Clin Otolaryngol*, 43(3), 841-845.

P266 product history	
Version	*Modification*
B2	One reference probe has been replaced and one probe length has been adjusted.
B1	One target probe for *CLCNKB* exon 11 and one reference probe have been replaced, four reference probes have been removed and QDX2 fragments have been added.
A1	First release.

Implemented changes in the product description

Version B2-02 – 15 February 20200 (04P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the *CLCNKB* and *CLCNKA* genes updated according to new version of the NM_ reference sequence.

Version B2-01 – 22 March 2018 (01P)
- Product description restructured and adapted to a new template.
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

Version 07 – 26 November 2015 (55)
- Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).
- Ligation sites of the CLCNKB probes adjusted to the NM_sequence.

Version 06 (48)
- Electropherogram picture of old buffer (introduced in Dec. 2012) removed.

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