Product Description
SALSA® MLPA® Probemix P330-A4 PCDH19

To be used with the MLPA General Protocol.

Version A4
As compared to version A3, three reference probes have been replaced.
For complete product history see page 7.

Catalogue numbers:
- **P330-025R:** SALSA MLPA Probemix P330 PCDH19, 25 reactions.
- **P330-050R:** SALSA MLPA Probemix P330 PCDH19, 50 reactions.
- **P330-100R:** SALSA MLPA Probemix P330 PCDH19, 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 P330 PCDH19 is a **research use only (RUO)** assay for the detection of deletions or duplications in the protocadherin-19 gene (*PCDH19*), which is associated with early infantile epileptic encephalopathy-9 (EIEE9), better known as epilepsy female-restricted with mental retardation (EFMR).

EFMR is characterised by seizure with onset in infancy or early childhood and cognitive impairment. Additional features include delayed development of variable severity, autistic signs and psychosis. Despite an X-linked mode of inheritance, which usually affects male carriers, this disease is restricted to females. This is probably due to a mechanism called cellular interference (Niazi et al. 2019). *PCDH19* is subject to X-chromosome inactivation, which leads to a homogeneous population of cells negative for *PCDH19* in males. In heterozygous females, on the other hand, the presence of both *PCDH19*-positive and *PCDH19*-negative cells affects the communication between the cells and leads to EFMR.

The *PCDH19* gene (6 exons) spans ~119 kb of genomic DNA and is located on chromosome Xq22.1, ~99 Mb from the p-telomere.

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 *PCDH19* exon numbering used in this P330-A4 PCDH19 product description is the exon numbering from the LRG_843 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 P330-A4 PCDH19 contains 39 MLPA probes with amplification products between 130 and 483 nucleotides (nt). This includes 14 probes for the *PCDH19* gene, at least two probes for each exon, with the exception of exon 2 for which no probe has been included. Furthermore, this probemix contains seven probes upstream and eight probes downstream of the *PCDH19* gene. In addition, ten 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 epilepsy. 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. 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 or in or near the *PCDH19* 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.
- 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 *PCDH19* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P330 PCDH19.
- 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.

***PCDH19* mutation database**

https://databases.lovd.nl/shared/genes/PCDH19. 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 *PCDH19* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P330-A4 PCDH19

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*PCDH19*	Upstream	Downstream
64-105	Control fragments – see table in probemix content section for more information				
130 *	Reference probe 13917-L18702	Xq			
135 «	**PCDH19 probe** 13922-L15461		**Exon 1**		
142 ¬ «	**PCDH19 area probe** 13923-L15462			~9 kb	
148 ¬	**PCDH19 area probe** 13924-L15463				~17 kb
154	Reference probe 16067-L17406	Xq			
160	**PCDH19 probe** 13925-L15464		**Exon 4**		
167 ¬	**PCDH19 area probe** 13926-L15465			~68 kb	
175 ¬	**PCDH19 area probe** 13927-L29552				~366 kb
184	**PCDH19 probe** 13928-L15467		**Exon 6**		
190 ¬	**PCDH19 area probe** 13929-L15468			~85 kb	
201 «	**PCDH19 probe** 13930-L15469		**Exon 1**		
211 ¬	**PCDH19 area probe** 13931-L15470			~25 kb	
220	**PCDH19 probe** 13932-L15471		**Exon 5**		
226 *	Reference probe 07654-L07360	Xp			
238	**PCDH19 probe** 16390-L18804		**Exon 4**		
247	**PCDH19 probe** 13934-L15473		**Exon 6**		
258 ¬	**PCDH19 area probe** 13935-L18803				~119 kb
265 «	**PCDH19 probe** 13933-L18802		**Exon 3**		
274	Reference probe 12650-L13723	Xq			
283	**PCDH19 probe** 13937-L15476		**Exon 6**		
301 «	**PCDH19 probe** 13938-L15477		**Exon 1**		
310 ¬	**PCDH19 area probe** 13939-L15478			~25 kb	
319 ¬	**PCDH19 area probe** 13940-L15479				~119 kb
328	Reference probe 12605-L13689	Xq			
337 «	**PCDH19 probe** 13941-L15480		**Exon 3**		
346 ¬	**PCDH19 area probe** 13942-L15481				~366 kb
363 «	**PCDH19 probe** 13943-L15482		**Exon 1**		
373 ¬	**PCDH19 area probe** 13944-L15483				~846 kb
384	Reference probe 13750-L15237	Xp			
393	Reference probe 20061-L27180	Xq			
400	**PCDH19 probe** 13945-L15484		**Exon 5**		
409 ¬	**PCDH19 area probe** 13946-L15485			~85 kb	
419 ¬	**PCDH19 area probe** 13947-L15486				~17 kb
427	Reference probe 13207-L29553	Xp			
442 ¬	**PCDH19 area probe** 13948-L29496			~68 kb	
449	**PCDH19 probe** 13949-L29497		**Exon 6**		
456 ¬	**PCDH19 area probe** 15094-L29498				~846 kb
463 *	Reference probe 06475-L06001	Xp			
483	Reference probe 10764-L11368	Xq			

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

* New in version A4.

« 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. *PCDH19* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
190 ¬	13929-L15468	Conserved region	85 kb upstream	GTGCAAGGTGGA-GAAGAGATTTAC	0.1 kb
409 ¬	13946-L15485	Conserved region	85 kb upstream	ATTTGGAGAGCT-TGGGACGTGGCT	16.7 kb
442 ¬	13948-L29496	Conserved region	68 kb upstream	CACGAGGGTCTA-ATCCTGCCAAGT	0.1 kb
167 ¬	13926-L15465	Conserved region	68 kb upstream	GGTGTGCTTGGA-AGAGCTTGCAGT	42.9 kb
211 ¬	13931-L15470	Conserved region	25 kb upstream	TAAAATGCTGCA-CTCTGCCATTTC	0.1 kb
310 ¬	13939-L15478	Conserved region	25 kb upstream	TTGAGAACTCAA-ATGAATTTCTCC	16.3 kb
142 ¬ «	13923-L15462	Conserved region	8 kb upstream	AGGCTTCCTGAT-GATCAGGGACTG	8.8 kb
		PCDH19	**NM_001184880.2**		
		start codon	*1677-1679 (Exon 1)*		
301 «	13938-L15477	Exon 1	213-214	TCATCCGAAATC-CTAAACCTCCTA	1.0 kb
201 «	13930-L15469	Exon 1	1273-1272 reverse	CCGGACAGTACT-TGAGGCGAAGGT	1.1 kb
363 «	13943-L15482	Exon 1	2367-2368	AGGTGACCGACT-CCAATGACAACA	1.4 kb
135 «	13922-L15461	Exon 1	3791-3792	AAGTGCAAGCGA-GACAACAAAGAG	3.6 kb
	No probe	Exon 2			
265 «	13933-L18802	Exon 3	7 nt before exon 3	TGCCTTTTCTGT-CACATAGAATTG	0.2 kb
337 «	13941-L15480	Exon 3	4180-4181	TTTCCTGAATGT-GGAGAACCAGAA	51.9 kb
238	16390-L18804	Exon 4	71 nt before exon 4 reverse	CTGCCCTACTTA-GAAGGAATTTTT	0.1 kb
160	13925-L15464	Exon 4	4343-4344	CGAGCCCATTTA-ATCAAGAGGTAT	8.6 kb
220	13932-L15471	Exon 5	4381-4382	CTTAGAGGGCAA-CAGCCTGAAGGA	0.1 kb
400	13945-L15484	Exon 5	4506-4505 *reverse*	AGGCATCTGAGA-TCCCATGGAGGT	45.2 kb
283	13937-L15476	Exon 6	4658-4659	ATCGCGCTGTCT-ATTGAAGCTACT	1.6 kb
449	13949-L29497	Exon 6	6233-6232 reverse	TTGCAGTCTAAT-TCTAATCATGAT	2.2 kb
247	13934-L15473	Exon 6	8384-8385	GCAGCAAGGACA-AAGGGCAGTTTA	0.8 kb
184	13928-L15467	Exon 6	9187-9188	CCATTACAGCAC-TGGTTAAGTAGA	17.2 kb
		stop codon	*5121-5123 (Exon 6)*		
419 ¬	13947-L15486	Conserved region	17 kb downstream	AGACACTTTTGC-GTATGAAATGCA	0.2 kb
148 ¬	13924-L15463	Conserved region	17 kb downstream	CACCTTCAGTGT-TCATTTCATCAA	101.7 kb
258 ¬	13935-L18803	Conserved region	119 kb downstream	ACTTTTAAAGGA-GTGAGTGTTTGG	0.2 kb
319 ¬	13940-L15479	Conserved region	119 kb downstream	CAGTGCCTGCTT-ATGGGAGGAGGG	247.4 kb
346 ¬	13942-L15481	Conserved region	366 kb downstream	ATGGATTTCATC-ATTTAGCATTGT	0.1 kb
175 ¬	13927-L29552	Conserved region	366 kb downstream	TAGGATTGTATC-AAGTTGACCAAG	479.6 kb
373 ¬	13944-L15483	Conserved region	846 kb downstream	GCTTTGAGCAAA-AGCTCTAACATT	0.1 kb
456 ¬	15094-L29498	Conserved region	846 kb downstream	GGAAATCAGGTT-ATGGTGAGGGCA	

[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.

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

- P137 SCN1A: Contains probes for the *SCN1A* gene, which is associated with Dravet syndrome and familial hemiplegic migraine 3.
- P138 SLC2A1 STXBP1: Contains probes for the *SLC2A1* and *STXBP1* genes, which are associated with Glucose transporter type 1 deficiency syndrome, *STXBP1* encephalopathy with epilepsy and Ohtahara syndrome, respectively.
- P197 KCNQ3: Contains probes for the *KCNQ3*, *CHRNA4*, *EPM2A*, *NHLRC1*, and *CHRNB2* genes, which are associated with epilepsy.
- P408 ADLTE LGI1: Contains probes for the *ADAM22*, *ADGRV1*, *KCNA1*, *KCNA4*, *KCNAB1*, *LGI1* and *PDYN* genes, which are associated with autosomal dominant lateral temporal epilepsy (ADLTE).

References

- Niazi R et al. (2019). A mutation update for the PCDH19 gene causing early-onset epilepsy in females with an unusual expression pattern. *Hum Mutat*. 40:243-257.
- 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 P330 PCDH19

- Gursoy S et al. (2020). Identification of PCDH19 gene mutations/deletions in patients with early onset epilepsy. *Ann Indian Acad Neurol.* 23:206-10.
- Van Harssel JJT et al. (2013). Clinical and genetic aspects of PCDH19-related epilepsy syndromes and the possible role of PCDH19 mutations in males with autism spectrum disorders. *Neurogenetics.* 14:23-34.
- Hoffman Zacharskaa D et al. (2016). Can the p.Thr1174Ser mutation in SCN1A gene shape genetic background in epileptic encephalopathies? *J Genet Syndr Gene Ther.* 7:1000290.
- Liu A et al (2017). The clinical spectrum of female epilepsy patients with PCDH19 mutations in a Chinese population. *Clin Genet.* 91:54-62.
- Liu A et al. (2019). Mosaicism and incomplete penetrance of PCDH19 mutations. J Med Genet. 56:81-8.
- Royer-Bertrand B et al. (2021). CNV Detection from Exome Sequencing Data in Routine Diagnostics of Rare Genetic Disorders: Opportunities and Limitations. *Genes (Basel)*. 12:1427.
- Terracciano A et al. (2016). PCDH19-related epilepsy in two mosaic male patients. *Epilepsia.* 57:e51-5.

P330 product history	
Version	*Modification*
A4	Three reference probes have been replaced.
A3	Two reference probes have been added and two replaced, in addition several lengths have been adjusted.
A2	One reference probe has been removed.
A1	First release.

Implemented changes in the product description
Version A4-02 – 08 December 2021 (04P) - Product description rewritten and adapted to a new template. - Small changes of probe lengths in Table 1 and 2 and the Probemix content section in order to better reflect the true lengths of the amplification products. - Ligation site corrected for probe 16390-L18804 in Table 2. - New publication added to the list of selected publications. Version A4-01 — 22 April 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). Version 04 – 31 August 2017 (55) - Warning added in Table 1, 142 nt probe 13923-L15462.

More information: www.mrcholland.com; www.mrcholland.eu	
	MRC Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, The Netherlands
E-mail	info@mrcholland.com (information & technical questions) order@mrcholland.com (orders)
Phone	+31 888 657 200