Product Description
SALSA® MLPA® Probemix P029-C1 WBS

To be used with the MLPA General Protocol.

Version C1
For complete product history see page 7.

Catalogue numbers
- **P029-025R:** SALSA MLPA Probemix P029 WBS, 25 reactions.
- **P029-050R:** SALSA MLPA Probemix P029 WBS, 50 reactions.
- **P029-100R:** SALSA MLPA Probemix P029 WBS, 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 P029 WBS is a **research use only (RUO)** assay for the detection of deletions or duplications in the WBS critical region (WBSCR) on the 7q11.23 chromosomal region, which is associated with Williams-Beuren syndrome (WBS).

Williams-Beuren Syndrome (WBS) is an autosomal dominant disorder characterised by cardiovascular disease, distinctive facial features, connective tissue abnormalities, intellectual disability, a specific cognitive profile, unique personality characteristics, growth abnormalities and endocrine abnormalities. WBS is estimated to occur at a frequency of approximately 1 in 7500 live births (Morris 1999).

Deletions of the WBSCR on 7q11.23, including the *ELN* gene, are the main cause of WBS. Besides deletions of the WBS region, some duplications have also been described, giving rise to the Williams-Beuren duplication syndrome (OMIM 609757). The commonly deleted or duplicated chromosomal region has a size of 1.55 Mb (90-95%) or 1.84 Mb (5-10%) and is flanked by highly homologous DNA sequences. However, smaller deletions within the WBSCR, with a variable phenotype, have also been described.

More information is available at http://www.ncbi.nlm.nih.gov/books/NBK1249/

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 exon numbering used in this P029-C1 WBS product description is the exon numbering from the NG_sequences:

- NG_012028.1 (*FZD9*)
- NG_023281.1 (*TBL2*)
- NG_013360.1 (*STX1A*)
- NG_009261.1 (*ELN*)
- NG_008129.1 (*LIMK1*)
- NG_008102.1 (*RFC2*)
- NG_011830.1 (*CLIP2*)
- NG_008930.1 (*POR*)
- NG_008995.1 (*HSPB1*)

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 NG 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 P029-C1 WBS contains 37 MLPA probes with amplification products between 132 and 427 nucleotides (nt). This includes 28 probes for the 7q11.23 chromosomal region. In addition, 9 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 WBS. 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) has a diverse collections of biological resources which may be used as positive control DNA samples in your MLPA experiments. Sample ID number NA13464 from the Coriell Institute has been tested with this P029-C1 probemix at MRC Holland and can be used as a positive control sample(s) to detect a deletion of the .The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Chromosomal position of copy number alteration	Altered target genes in P029-C1	Expected copy number alteration
NA13464	Coriell Institute	7q11.23	*FZD9, TBL2, STX1A, ELN, LIMK1, RFC2, CLIP2.*	Heterozygous deletion

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

- 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.

Database of genomic variation and phenotype in humans using Ensembl resources (DECIPHER): https://decipher.sanger.ac.uk/. We strongly encourage users to deposit positive results in the DECIPHER 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 *ELN* exons 3 and 6 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P029-C1 WBS

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*ELN*	7q11.23
64-105	Control fragments – see table in probemix content section for more information			
132	Reference probe 00797-L21698	5q31		
142 ¬	***HSPB1* probe** 09538-L09961			Exon 1
148 ¬	***HSPB1* probe** 09540-L09963			Exon 3
154	Reference probe 14924-L16657	6q22		
160 «	***ELN* probe** 17573-L21523		Exon 4	
166 *	Reference probe 10904-L11573	9q34		
178 ¬	***POR* probe** 13768-L21866			Exon 11
183 ¥ «	***STX1A* probe** 20987-L21869			Exon 3
192	***ELN* probe** 17575-L21525		Exon 16	
197	Reference probe 05525-L04953	20q13		
208 ¬	***HSPB1* probe** 09539-L09962			Exon 2
214	***LIMK1* probe** 17576-L21868			Exon 13
227 ¥	***LIMK1* probe** 08235-L29891			Exon 9
234	***TBL2* probe** 17578-L21872			Exon 2
240 ¥ «	***FZD9* probe** 21191-L29979			Exon 1
247	***CLIP2* probe** 17580-L21530			Exon 10
254 *	Reference probe 05223-L26271	2q32		
261	***ELN* probe** 17579-L21529		Exon 9	
274 «	***STX1A* probe** 17582-L21880			Exon 7
281 «	***LIMK1* probe** 17584-L21534			Exon 2
287	Reference probe 03926-L20793	15q21		
293	***ELN* probe** 17583-L21881		Exon 26	
308	***ELN* probe** 16350-L21883		Exon 27	
315 *	Reference probe 20425-L27907	18q11		
323	***ELN* probe** 17585-L21884		Exon 3	
330	***RFC2* probe** 17586-L21885			Exon 10
337	***ELN* probe** 01334-L21886		Exon 6	
346 «	***ELN* probe** 01335-L00879		Exon 33	
355 ¬	***POR* probe** 12692-L13770			Exon 12
364	Reference probe 06489-L06015	1p13		
373	***ELN* probe** 01336-L21887		Exon 20	
382 «	***TBL2* probe** 17587-L21537			Exon 7
390 «	***LIMK1* probe** 01337-L21889			Exon 4
398	***CLIP2* probe** 17588-L21888			Exon 4
407	***RFC2* probe** 17589-L21890			Exon 3
418	***CLIP2* probe** 01339-L00885			Exon 14
427	Reference probe 07817-L07547	3p22		

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

* New in version C1.
¥ Changed in version C1. 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.
¬ 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. P029-C1 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site *ELN*	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
Start of common chromosome 7q11.23 deletion region					
240 «	21191-L29979	*FZD9* exon 1		TTATTCCAGTCA-CAGCTTCCAGAG	134.9 kb
382 «	17587-L21537	*TBL2* exon 7		TGAGCTGTGATT-TTCTGTGTTGCA	3.6 kb
234	17578-L21872	*TBL2* exon 2		AGTAACTGGAGA-ACAACATGGCCC	129.3 kb
274 «	17582-L21880	*STX1A* exon 7		ACCCCACTCGCT-TGCAGCCGGCAG	4.9 kb
183 «	20987-L21869	*STX1A* exon 3		GAATCTCCTCCA-CCTGCGGACCAA	327.8 kb
		ELN	**NM_000501.4**		
		start codon	*16-18 (exon 1)*		
323	17585-L21884	Exon 3	24 nt before exon 3	CCGATGATCTCT-CTTTCTCTTTCT	1.2 kb
160 «	17573-L21523	Exon 4	205-204 reverse	CTTACCTGGCTT-AAGAGGTTTGCC	5.0 kb
337	01334-L21886	Exon 6	306-307	GGTGGAGTGGCT-GACGCTGCTGCA	1.2 kb
261	17579-L21529	Exon 9	22 nt before exon 9	TGCTGCTAGTAA-CTTTGCTTTCTT	8.0 kb
192	17575-L21525	Exon 16	889-890	CAATTCCTGGAA-TTGGAGGCATCG	4.5 kb
373	01336-L21887	Exon 20	1236-1237	TTTCCCGGCTTT-GGTGTCGGAGTC	4.8 kb
293	17583-L21881	Exon 26	17 nt after exon 26	TGCACCCCACAA-CCACTTGTGGCT	2.0 kb
308	16350-L21883	Exon 27	39 nt before exon 27	GAGACCCATCGT-TCAGAAATGGAA	5.6 kb
346 «	01335-L00879	Exon 33	2226-2227	ACCTCATCAACG-TTGGTGCTACTG	17.1 kb
		stop codon	*2188-2190 (exon 33)*		
281 «	17584-L21534	*LIMK1* exon 2		CCAGTCCGCGTT-CAGGGCCTGGAG	11.3 kb
390 «	01337-L21889	*LIMK1* exon 4		TGTGGGACCTTT-ATCGGTGACGGG	10.8 kb
227	08235-L29891	*LIMK1* exon 9		TGATGGTGATGA-AGGAGCTGATCC	8.0 kb
214	17576-L21868	*LIMK1* exon 13		TGGACGAGAAGA-CTCAGCCTGAGG	119.8 kb
330	17586-L21885	*RFC2* exon 10		TGCCACAAGTGA-GCAAGAATCTAG	14.0 kb
407	17589-L21890	*RFC2* exon 3		TGTCTCATCCCA-CTGTCCGCTGAA	104.2 kb
398	17588-L21888	*CLIP2* exon 4		GAGTCTCCCTGT-GTGCCGACAGGT	22.9 kb
247	17580-L21530	*CLIP2* exon 10		AACAGGAGGTCG-AGAGTTTGCGGG	20.4 kb
418	01339-L00885	*CLIP2* exon 14		GGAGGCCAATCG-TCACTCCCCAGG	2.0 **M**b
End of common chromosome 7q11.23 deletion region					
178 ¬	13768-L21866	*POR* exon 11		CACCCATTCCCG-TGCCCTACGTCC	0.4 kb
355 ¬	12692-L13770	*POR* exon 12		CCTCATCCTCCA-AGGTGAGGGCCG	317.5 kb
142 ¬	09538-L09961	*HSPB1* exon 1		CTGACTCTGCTC-TGGACGTCTGCT	1.2 kb
208 ¬	09539-L09962	*HSPB1* exon 2		CTTCACGCGGAA-ATACACGTGAGT	0.3 kb
148 ¬ #	09540-L09963	*HSPB1* exon 3		GCGGCAGTCTCA-TCGGATTTTGCA	

[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.

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.
« 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.

Complete probe sequences are available at www.mrcholland.com.

Related SALSA MLPA probemixes

- P245 Microdeletion Syndromes-1A: Contains a few probes for the *ELN* gene.
- P064 Microdeletion Syndromes-1B: Contains a few probes for the *ELN* gene.

References

- Morris CA (1999) [Updated 2017 Mar 23]. Williams Syndrome. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. GeneReviews®. Seattle (WA): University of Washington, Seattle; 1993-2020. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1249/
- 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 P029 WBS

- Dutra RL et al. (2015) Rare genomic rearrangement in a boy with Williams-Beuren syndrome associated to XYY syndrome and intriguing behavior. *Am J Med Genet Part A*. 167A:3197–203.
- Honjo, RS et al. (2015) Williams-Beuren Syndrome: A Clinical Study Of 55 Brazilian Patients And The Diagnostic Use Of MLPA. *BioMed Research International*. 1-6.
- Sireteanu, A et al. (2014) Detection Of Chromosomal Imbalances Using Combined MLPA Kits In Patients With Syndromic Intellectual Disability. *Romanian Review of Laboratory Medicine*. 22:157-164.
- Dutra RL et al. (2012) Copy number variation in Williams-Beuren syndrome: suitable diagnostic strategy for developing countries. *BMC Res Notes*. 5:13.
- Beunders C et al. (2010) A triplication of the Williams–Beuren syndrome region in a patient with mental retardation, a severe expressive language delay, behavioural problems and dysmorphisms. *J Med Genet*. 47:271-5.
- Van Hagen JM et al. (2007) Comparing two diagnostic laboratory tests for Williams syndrome: fluorescent in situ hybridization versus multiplex ligation-dependent probe amplification. *Genet Test*. 11:321-7.
- Depienne C et al. (2007) Autism, language delay and mental retardation in a patient with 7q11 duplication. *J Med Genet*. 44:452-8.

P029 product history	
Version	*Modification*
C1	Two target probes have been removed, three reference probes have been replaced and three probe lengths have been adjusted.
B1	The probemix has been completely redesigned.
A1	First release.

Implemented changes in the product description
Version C1-01 – 02 December 2020 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *ELN* 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 16 – 29 May 2017 (55) - 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). - Various minor textual changes. - Related products updated.

- Two references added. - Warnings added in Table 1 and 2, 183 nt probe 20987-L21869 and 382 nt probe 17587-L21537. Version 15 – 10 January 2017 (55) - Warning added in Table 1, 274 nt probe 17582-L21880 and 346 nt probe 01335-L00879. Version 14 – 04 December 2015 (55) - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). - New reference added on page 2. - Figure 2 removed.

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