Product Description
SALSA® MLPA® Probemix P015-F2 MECP2

To be used with the MLPA General Protocol.

Version F2
For complete product history see page 12.

Catalogue numbers:
- **P015-025R:** SALSA MLPA Probemix P015 MECP2, 25 reactions.
- **P015-050R:** SALSA MLPA Probemix P015 MECP2, 50 reactions.
- **P015-100R:** SALSA MLPA Probemix P015 MECP2, 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.

Intended purpose
The SALSA MLPA Probemix P015 MECP2 is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in the human *MECP2* gene, in order to confirm a potential cause for and clinical diagnosis of classic and atypical Rett syndrome, and of *MECP2* duplication syndrome. This assay can also be used for the detection of deletions or duplications in the human *NTNG1* and *CDKL5* genes, in order to confirm a potential cause for and clinical diagnosis of atypical Rett syndrome, and for the detection of deletions or duplications in the human *ARX* and *CDKL5* genes, in order to confirm a potential cause for and clinical diagnosis of early infantile epileptic encephalopathy (EIEE), and for molecular genetic testing of at-risk family members. This assay is for use with genomic DNA isolated from human peripheral whole blood specimens.

Copy number variations (CNVs) detected with P015 MECP2 should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *MECP2, CDKL5, ARX* and *NTNG1* genes are point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

Not all exons of the *CDKL5, ARX* and *NTNG1* genes are covered. The SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1 is available for the detection of deletions or duplications in other *CDKL5, ARX* and *NTNG1* exons.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1]Please note that this probemix is for In Vitro Diagnostic use (IVD) in the countries specified at the end of this product description. In all other countries, the product is for Research Use Only (RUO).
[2]To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

Clinical background

Rett syndrome (RTT) is a neurodevelopmental disorder affecting approximately 1:10,000–15,000 live female births. Classic RTT is characterized by a period of normal development during the first 6–18 months of life, followed by loss of already gained skills, such as speech and purposeful hand movement. Additional main features are acquired microcephaly, stereotypic hand movements, impaired locomotion and communication dysfunction (Hagberg et al. 1983). Patients, lacking one or more of the major features of RTT, are identified as atypical RTT cases which clustered into five distinct clinical subgroupings; congenital, early-onset seizure, preserved speech, late regression and forme fruste variants (Neul et al. 2010). The prevalence of atypical RTT is estimated at around 1 in 45,000 individuals (predominantly females).

X-linked methyl-CpG-binding protein 2 (*MECP2*) has been identified as the RTT gene, with mutations in *MECP2* found in 95–97% of classic RTT individuals (https://www.ncbi.nlm.nih.gov/books/NBK1497/). Approximately 3–5% of individuals who strictly meet clinical criteria for RTT do not have an identified mutation in *MECP2*, indicating that a mutation in this gene is not required to make the diagnosis of classic RTT. In contrast to classic RTT, mutations in *MECP2* have been identified in only 40% atypical RTT cases. Involvement of other genes in atypical RTT has been reported, such as *CDKL5* and *NTNG1* (Colak et al. 2011). Approximately 6.5-10% of patients with atypical RTT have large deletions in *CDKL5* (RTT database RettBASE). One report described a patient with atypical RTT who presented with early onset of epileptic seizures (not infantile spasms) and a *de novo* translocation disrupting the *NTNG1* gene (Borg et al. 2005). Please note that MLPA will not detect balanced translocations.

FOXG1 syndrome was previously described as a congenital variant of RTT. Both disorders are characterized by impaired development, intellectual disability, and problems with communication and language. However, RTT is diagnosed almost exclusively in females, while *FOXG1* syndrome affects both males and females. RTT also involves a period of apparently normal early development that does not occur in *FOXG1* syndrome. *FOXG1* syndrome is caused by mutations in *FOXG1* gene (Kortüm et al. 2011). The SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1 is available for the detection of deletions or duplications in *FOXG1* (Table 1).

While loss-of-function mutations in *MECP2* result in RTT, gain-of-function mutations are associated with *MECP2* duplication syndrome which occurs almost exclusively in males. *MECP2* duplication syndrome and RTT share overlapping clinical phenotypes including intellectual disability, motor deficits, epilepsy, hypotonia, and progressive spasticity (https://www.ncbi.nlm.nih.gov/books/NBK1284/).

Early infantile epileptic encephalopathy (EIEE) is a neurological disorder characterized by seizures. The disorder affects newborns, usually within the first three months of life (most often within the first 10 days) in the form of epileptic seizures. Most infants with the disorder show underdevelopment of part or all of the cerebral hemispheres or structural anomalies. The prevalence of EIEE is estimated at around 1 to 1.6 in 100,000 individuals. Mutations in more than 50 different genes are known to cause EIEE, including *ARX* and *CDKL5* (Bahi-Buisson et al. 2010, Kato et al. 2004). Due to the fact that *CDKL5* is located on the X chromosome, the prevalence of EIEE among women is four times higher than in men. However, the course is usually more severe among male patients.

Since there are multiple genes involved in the different syndromes and since these genes are covered in multiple probemixes, Table 1 is provided to give an overview.

Table 1: Overview of the genes and probemixes related to classic and atypical RTT, *MECP2* duplication syndrome, early infantile epileptic encephalopathy and *FOXG1* syndrome.

Condition	Genes	Probemix and coverage	Remarks
Classic Rett syndrome	*MECP2*	P015*: Each exon P245*: Exon 1, 3, 4	MECP2 probes in P245 are identical to P015 (different length).
***MECP2* duplication syndrome**	*MECP2*	P015*: Each exon P245*: Exon 1, 3, 4	MECP2 probes in P245 are identical to P015 (different length).
Atypical Rett syndrome	*MECP2*	P015*: Each exon P245*: Exon 1, 3, 4	MECP2 probes in P245 are identical to P015 (different length).
	CDKL5	P189*: Each exon P015*: Exons 3, 6, 9, 10	CDKL5 probes in P015 are identical to P189 (different length).
	NTNG1	P189*: Each exon P015*: Exons 2, 3, 5, 6	NTNG1 probes in P015 are identical to P189 except for exon 2 (different length).
Early infantile epileptic encephalopathy	*CDKL5*	P189*: Each exon P015*: Exons 3, 6, 9, 10	CDKL5 probes in P015 are identical to P189 (different length).
	ARX	P189*: Each exon P015*: Exons 1, 5 P106: Exons 1, 2, 4	ARX probes in P015 and P106 are identical to P189 (different length).
***FOXG1* syndrome**	*FOXG1* (single exon gene)	P189*: Exon 1 + upstream region P075: Exon 1 + upstream region P395: Exon 1 + upstream region	FOXG1 probes in P189 are identical to P075 and P395 (different length). One FOXG1 exon 1 probe is different between P075 and P395.

*IVD. All other for Research Use Only (RUO).

Gene structure
The *MECP2* gene spans 76 kilobases (kb) on chromosome Xq28 (reverse strand) and contains 4 exons. The *MECP2* LRG_764 is available at www.lrg-sequence.org and is identical to GenBank NG_007107.3.

The *CDKL5* gene spans 228 kb on chromosome Xp22.13 and contains 21 exons.

The *ARX* gene spans 12 kb on chromosome Xp21.3 and contains 5 exons.

The *NTNG1* gene spans 345 kb on chromosome 1p13.3 and contains 6 exons.

Transcript variants
For *MECP2,* multiple variants have been described. Transcript variant 1, also known as MECP2A, includes exon 2 (NM_004992.4; 10467 nt; coding sequence 213-1673, https://www.ncbi.nlm.nih.gov/gene/4204). This sequence is a reference standard in the NCBI RefSeq project. *MECP2* transcript variant 2 (NM_001110792.2) and transcript variants 3-7 are shorter variants compared to variant 1. Variants 3-7 all encode the same isoform 3.

For *CDKL5,* multiple variants have been described. Transcript variant 1 (NM_ 003159.3; 3428 nt; coding sequence 251-3343, https://www.ncbi.nlm.nih.gov/gene/6792) is a longer variant as compared to *CDKL5* transcript variant 2 (NM_001037343.2) and is a short variant as compared to transcript variant 3 (NM_001323289.2).

For *ARX,* one transcript variant has been described encoding the full length protein (NM_ 139058.3; 2893 nt; coding sequence 229-1917, https://www.ncbi.nlm.nih.gov/gene/170302). This sequence is a reference standard in the NCBI RefSeq project.

For *NTNG1,* multiple variants have been described. Transcript variant 3 (NM_014917.4; 6037 nt; coding sequence 638-1954, https://www.ncbi.nlm.nih.gov/gene/22854) contains a longer 5' UTR and lacks two exons in the 3' coding region, but maintains the reading frame, as compared to variant 1. The encoded isoform (G1c) is shorter than isoform a. This sequence is a reference standard in the NCBI RefSeq project.

Exon numbering
The *MECP2* exon numbering used in this P015-F2 MECP2 product description is the exon numbering from the LRG_764 sequence. The *MECP2* exon numbering has changed; the exon numbering used in previous versions of this product description can be found in between brackets in Table 3a. From description version F2-04 onwards, we have adopted the LRG sequence exon numbering. The *CDKL5* exon numbering used in this P015-F2 MECP2 product description is the exon numbering from the NG_008475.1 sequence. The *ARX* exon numbering used in this P015-F2 MECP2 product description is the exon numbering from the NG_008281.1 sequence. The *NTNG1* exon numbering used in this P015-F2 MECP2 product description is the exon numbering from the NG_042821.1 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 or 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 P015-F2 MECP2 contains 46 MLPA probes with amplification products between 130 and 467 nucleotides (nt). This includes 17 probes for the *MECP2* gene, covering every exon. Furthermore, several probes are present for genes in close proximity to *MECP2*. One of these probes detects the *VAMP7* gene and is located within the pseudo autosomal region 2 (*PAR2*). The P015-F2 probemix contains four *CDKL5* probes, two *ARX* probes, and four *NTNG1* probes. More probes for the *CDKL5*, *ARX* and *NTNG1* genes are present in the P189 CDKL5/ARX/FOXG1 probemix. 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.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 from human peripheral whole blood specimens, 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 RTT or early infantile epileptic encephalopathy. 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 numbers as described in the table below from the Coriell Institute have been tested with this P015-F2 probemix at MRC Holland and can be used as a positive control samples. 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 P015-F2	Expected copy number alteration
NA23599 (f)	Coriell Institute	Xq28	*MECP2* exon 3-4	Heterozygous deletion
NA23635 (f)	Coriell Institute	Xq28	*MECP2* exon 3 and partial exon 4	Heterozygous deletion
NA23648 (f)	Coriell Institute	Xq28	*MECP2* partial exon 4 deletion + *IRAK1*	Heterozygous deletion
NA23654 (f)	Coriell Institute	Xq28	*MECP2* exon 3 and partial exon 4	Heterozygous deletion
NA23733 (f)	Coriell Institute	Xq28	*FLNA, MECP2, IRAK1, L1CAM*	Heterozygous duplication
NA23734 (m)	Coriell Institute	Xq28	*FLNA, MECP2, IRAK1, L1CAM*	Duplication
NA23676 (f)	Coriell Institute	Xq28	*MECP2, IRAK1*	Heterozygous duplication

Performance characteristics

MECP2 deletions explain 5-8% of classic RTT cases. *MECP2* deletions explain 3% of atypical RTT cases (https://www.ncbi.nlm.nih.gov/books/NBK1497/). Duplications in *MECP2* explain 100% of *MECP2* Duplication Syndrome cases (https://www.ncbi.nlm.nih.gov/books/NBK1284/). Approximately 6.5-10% of patients with atypical RTT have large deletions in *CDKL5* (RTT database RettBASE). Approximately 8-16% of patients with early infantile epileptic encephalopathy have large deletions in *CDKL5* (Bahi-Buisson et al. 2008; Nemos et al. 2009; Mei et al. 2010). The frequency of *ARX* deletions or duplications is ~1% in early infantile epileptic encephalopathy cases (Guerrini et al. 2007). No deletions or duplications in the *NTNG1* gene have been described so far, however, the association between a mutation in *NTNG1* and atypical RTT is well established (Borg et al. 2005). The analytical sensitivity and specificity for the detection of deletions or duplications in the *MECP2*, *CDKL5*, *ARX*, and *NTNG1* genes is very high and can be considered >99% (based on a 2003-2020 literature review).

Analytical performance can be compromised by: Single nucleotide variants (SNV) or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

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 expected results for the probes detecting autosomal sequences are allele copy numbers of 2 (normal), 1 (heterozygous deletion), or 3 (heterozygous duplication). The same results can be expected for the X-chromosome-specific probes in female samples. For the X-chromosome-specific probes in male samples, expected copy numbers are 1 (normal), 0 (deletion) or 2 (duplication).

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 when **reference samples of the same sex** have been used:

Copy number status		**Final ratio (FR)**
Autosomal sequences and X chromosome sequences in females	**X chromosome sequences in males**	
Normal	Normal	0.80 < FR < 1.20
Homozygous deletion	Deletion	FR = 0
Heterozygous deletion		0.40 < FR < 0.65
Heterozygous duplication		1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	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 (Mei et al. 2014, Zhang et al. 2019). 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 *MECP2* and *ARX* genes. 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.
- Deletion of a probe's recognition sequence on the X-chromosome will lead to a complete absence of the corresponding probe amplification product in males, whereas female heterozygotes are recognizable by a 35-50% reduction in relative peak height.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *MECP2*, *CDKL5*, *ARX*, and *NTNG1* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P015 MECP2.
- 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.
- Not all exons of the *CDKL5*, *ARX* and *NTNG1* genes are covered in the P015 MECP2 probemix.

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.

Mutation databases

RettBASE: For *MECP2* and *CDKL5* mutations; http://mecp2.chw.edu.au/mecp2/mecp2_home.php and http://mecp2.chw.edu.au/cdkl5/cdkl5_home.php. We strongly encourage users to deposit positive results in the RettBASE.

Leiden Open Variation Database (LOVD): For *ARX* and *NTNG1* mutations; https://databases.lovd.nl/shared/genes/ARX and https://databases.lovd.nl/shared/genes/NTNG1. We strongly encourage users to deposit positive results in the 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 SNVs and unusual results (e.g., a duplication of *MECP2* exons 1 and 3 but not exon 2) to MRC Holland: info@mrcholland.com.

Table 2. SALSA MLPA P015-F2 MECP2 probemix

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]				
		Reference	*MECP2* Xq28	*CDKL5* Xp22.13	*ARX* Xp21.3	*NTNG1* 1p13.3
64-105	Control fragments – see table in probemix content section for more information					
130	Reference probe 00797-L21056	5q31				
137 « ¬	**GDI1 probe** 16875-L19669		Xq28			
144	**CDKL5 probe** 06466-L06567			**Exon 10**		
149	**NTNG1 probe** 18447-L24143					**Exon 2**
155 ~	**MECP2 probe** 18442-L24243		**Exon 4**			
161 «	**ARX probe** 18440-L24338				**Exon 1**	
166	Reference probe 08222-L24146	10q26				
172 « ¬	**SLC6A8 probe** 01879-L24147		Xq28			
178 « ¬	**IRAK1 probe** 10835-L24148		Xq28			
184 «	**MECP2 probe** 10836-L24339		**Exon 1**			
190	**CDKL5 probe** 06458-L23617			**Exon 3**		
196	**MECP2 probe** 10839-L23618		**Exon 3**			
202 «	**MECP2 probe** 03409-L16570		**Exon 1**			
208	Reference probe 09865-L08705	13q32				
214 «	**MECP2 probe** 03770-L13387		**Upstream**			
221 ¬	**F8 probe** 06288-L05892		Xq28			
229 ~	**MECP2 probe** 18441-L12494		**Exon 4**			
235 «	**ARX probe** 06455-L21229				**Exon 5**	
241 ¬	**L1CAM probe** 07051-L14553		Xq28			
247	Reference probe 05959-L05376	7p11				
254 «	**MECP2 probe** 03768-L23833		**Exon 2**			
260 « ±	**MECP2 probe** 01769-L23834		**Exon 4**			
266	Reference probe 10728-L22588	6p12				
274 ~	**MECP2 probe** 01768-L13824		**Exon 4**			
280	Reference probe 16434-L24149	18q21				
286	**CDKL5 probe** 06462-L24150			**Exon 6**		
292 ~	**MECP2 probe** 18444-L24151		**Exon 4**			
299	Reference probe 16621-L24152	1q32				
305 «	**MECP2 probe** 02002-L24153		**Exon 1**			
312	**NTNG1 probe** 06487-L24154					**Exon 5**
319 « ¬	**DKC1 probe** 18761-L24340		Xq28			
338	Reference probe 09776-L24156	15q21				
346 ~	**MECP2 probe** 01347-L24157		**Exon 4**			
356	**MECP2 probe** 10841-L24158		**Exon 4**			
365	**MECP2 probe** 01348-L24159		**Exon 3**			
373 «	**MECP2 probe** 01349-L24160		**Exon 2**			
384 ¬	**IDH3G probe** 01887-L23933		Xq28			
391	**MECP2 probe** 14737-L24161		**Exon 3**			
400	**CDKL5 probe** 06465-L05991			**Exon 9**		
409	Reference probe 17462-L21218	12p13				
418 «	**MECP2 probe** 18446-L23620		**Exon 4**			
427 ±	**NTNG1 probe** 06488-L23934					**Exon 6**
438	**NTNG1 probe** 06483-L24162					**Exon 3**
447 « ¬ ±	**FLNA probe** 04138-L24163		Xq28			
457 ¬	**VAMP7 probe** 01094-L24164		Xq28-**PAR2**			
467	Reference probe 02674-L24165	11q22				

[a] See section Exon numbering on page 4 for more information.

± SNP rs267608346 could influence the 260 nt MECP2 probe signal. SNP rs201741317 could influence the 427 nt NTNG1 probe signal. SNP rs782037353 could influence the 447 nt FLNA probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

« 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.

~ Probe located within, or close to, a hotspot region of the *MECP2* gene in which small deletions occur frequently (Laccone et al. 2004, Huppke et al. 2005). In addition to small deletions, many SNPs are also present in this region, making this probe more prone to false positive deletion results.

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.

Table 3. P015-F2 probes arranged according to chromosomal location

Table 3a. *MECP2* / Xq28

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_004992.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		q-telomere			*~90.0 kb*
457 ¬	01094-L24164	*VAMP7* (**PAR2 region**)		TGTGGGAAAAGT-GTTTCCATTCTG	957.0 kb
221 ¬	06288-L05892	*F8*		TTCAGGGAGTCT-GGCCAAGGAAAA	218.1 kb
319 « ¬	18761-L24340	*DKC1*		CATGATGTGCTT-GATGCTCAGTGG	331.9 kb
137 « ¬	16875-L19669	*GDI1*		CCTGACCATGGA-CGAGGAATACGA	79.7 kb
447 « ¬ ±	04138-L24163	*FLNA*		TGACGGCACGTA-TACAGTGGCCTA	221.7 kb
		MECP2			
		start codon	*213-215 (exon 2)*		
214 «	03770-L13387	Upstream	1.1 kb before exon 1	GCAAGAATGTTA-GTTTGCTGTCTG	0.7 kb
305 «	02002-L24153	Exon 1	394 nt before exon 1, reverse	GGGACGCCTGTT-TGCGCTGCTCTG	0.1 kb
202 «	03409-L16570	Exon 1	331 nt before exon 1	CATTAATCCTTA-ACATTCAAATTC	0.4 kb
184 «	10836-L24339	Exon 1	38-39	GGGCTGTGGTAA-AAGCCGTCCGGA	5.3 kb
254 «	03768-L23833	Exon 2	44 nt before exon 2	GAAAAAGGTCGT-GCAGCTCAATGG	0.1 kb
373 «	01349-L24160	Exon 2	174-175	GACTCCCCAGAA-TACACCTTGCTT	59.6 kb
196	10839-L23618	Exon 3	47 nt before exon 3	ACTTGTTCTGCA-GACTGGCATGTT	0.2 kb
365	01348-L24159	Exon 3	371-372	GCCCACCACTCT-GCTGAGCCCGCA	0.2 kb
391	14737-L24161	Exon 3	568-569	CTCTGCTGGGAA-GTATGATGTGTA	0.7 kb
356	10841-L24158	Exon 4 (4b)	32 nt before exon 4	AGAGCCTCTAAT-TGTTCCTTGTGT	0.3 kb
229 ~	18441-L12494	Exon 4 (4b)	882-883	TCCTTGTCAAGA-TGCCTTTTCAAA	0.3 kb
346 ~	01347-L24157	Exon 4 (4b)	1229-1230	CTGAAGACCTGT-AAGAGCCCTGGG	0.2 kb
155 ~	18442-L24243	Exon 4 (4b)	1465-1466	AGAGGAGAAGAT-GCCCAGAGGAGG	0.1 kb
274 ~	01768-L13824	Exon 4 (4b)	1608-1609	TTTCATCCTCCA-TGCCAAGGCCAA	0.1 kb
292 ~	18444-L24151	Exon 4 (4b)	1668-1669	CCGAGAGAGTTA-GCTGACTTTACA	2.2 kb
418 «	18446-L23620	Exon 4 (4b)	3857-3858	TGCTGCCATGAA-CTGTCAAGTGTG	3.2 kb
260 « ±	01769-L23834	Exon 4 (4b)	7022-7023	CAGTAACACATA-GACTGTGCGCAT	5.9 kb
		stop codon	*1671-1673 (exon 4)*		
178 « ¬	10835-L24148	*IRAK1*		TTTATGAAGCTT-TTCCAGGCTCCC	154.4 kb
241 ¬	07051-L14553	*L1CAM*		CAGCGGGTGAAA-ACTACAGTGTCG	70.3 kb
384 ¬	01887-L23933	*IDH3G*		TCCCCGAAACTT-CGCACCCCGTCG	99.8 kb
172 « ¬ #	01879-L24147	*SLC6A8*		ACCCCGCTGGTC-TGCATGGTAAGG	-

Table 3b. *CDKL5* / Xp22.13

Length (nt)	SALSA MLPA probe	CDKL5 exon[a]	Ligation site NM_003159.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*251-253 (exon 2)*		
190	06458-L23617	Exon 3	41 nt before exon 3	GAGCTTTGTAGT-TTGTATGCGTGC	69.1 kb
286	06462-L24150	Exon 6	570-571	GCCAAATGGAGT-TCCACCTGAGAA	8.2 kb
400	06465-L05991	Exon 9	910-911	ATTGACCAACTT-TTTACTATTCAG	7.3 kb
144	06466-L06567	Exon 10	1023-1024	TCCTCAGTCCTT-GGAAAGAAGATA	-
			3341-3343 (exon 21)		

Table 3c. *ARX* / Xp21.3

Length (nt)	SALSA MLPA probe	ARX exon[a]	Ligation site NM_139058.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*229-231 (exon 1)*		
161 «	18440-L24338	Exon 1	115-116	AGATCGCAATAA-TATCCGTTATAA	11.2 kb
235 «	06455-L21229	Exon 5	1981-1982	CAGCACCACTCA-AGACCAAATGGA	-
			1915-1917 (exon 5)		

Table 3d. *NTNG1* / 1p13.3

Length (nt)	SALSA MLPA probe	NTNG1 exon[a]	Ligation site NM_014917.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*638-640 (exon 2)*		
149	18447-L24143	Exon 2	408-409	ACAAGTATGTTA-GGCTTCCACCAA	176.5 kb
438	06483-L24162	Exon 3	1436-1437	GGATAAGGCTGT-TAAGACCAGCCG	83.1 kb
312	06487-L24154	Exon 5	227 nt after exon 5	TATGACTTTTCT-GACTACTCTTAA	72.7 kb
427 ±	06488-L23934	Exon 6	1737-1738	GAATGTCTGCGA-CAACGAGCTCCT	-
			1952-1954 (exon 6)		

[a] See section Exon numbering on page 4 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 within, or close to, a hotspot region of the *MECP2* gene in which small deletions occur frequently (Laccone et al. 2004, Huppke et al. 2005). In addition to small deletions, many SNPs are also present in this region, making this probe more prone to false positive deletion results.

± SNP rs267608346 could influence the 260 nt MECP2 probe signal. SNP rs201741317 could influence the 427 nt NTNG1 probe signal. SNP rs782037353 could influence the 447 nt FLNA probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

« 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

P189 CDKL5/ARX/FOXG1	Contains probes for the *CDKL5*, *NTNG1*, *ARX* and *FOXG1* genes.
P075 TCF4-FOXG1	Contains probes for the *TCF4* and *FOXG1* genes (Pitt-Hopkins syndrome).
P336 UBE3A	For the detection of small rearrangements in the *UBE3A* gene, involved in Angelman syndrome.
P395 MEFC2-FOXG1	Contains probes for the *MEFC2* and *FOXG1* genes (mental retardation).
P106 X-linked ID	Contains probes for various genes that cause X-linked intellectual disability when defect.
ME028 Prader Willi / Angelman syndrome	For the detection of both copy number as well as methylation changes of the Prader Willi / Angelman region.
P245 Microdeletion Syndromes-1A	Includes probes for *MECP2*.
P137 SCN1A	Includes probes for *SCN1A* (Dravet syndrome).

References

- Borg I et al. (2005). Disruption of Netrin G1 by a balanced chromosome translocation in a girl with Rett syndrome. *Eur J Hum Genet*. 13(8):921-7.
- Bahi-Buisson N et al. (2008). Key clinical features to identify girls with CDKL5 mutations. *Brain*. 131(10): 2647–2661.
- Bahi-Buisson N et al. (2010). Epileptic encephalopathy in a girl with an interstitial deletion of Xp22 comprising promoter and exon 1 of the CDKL5 gene. *Am J Med Genet B Neuropsychiatr Genet*. 153B(1): 202-207.
- Colak D et al. (2011). Genomic and transcriptomic analyses distinguish classic Rett and Rett-like syndrome and reveals shared altered pathways. *Genom.* 97(1): 19-28.
- Guerrini R et al. (2007). Expansion of the first PolyA tract of ARX causes infantile spasms and status dystonicus. *Neurol*. 69: 427–433.
- Hagberg B et al. (1983). A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: Report of 35 cases. *Ann Neurol.* 14(4): 471-479.
- Huppke P et al. (2005). Molecular diagnosis of Rett syndrome. *J Child Neurol*. 20(9):732-6.
- Kato M et al. (2004). Mutations of ARX Are Associated with Striking Pleiotropy and Consistent Genotype-Phenotype Correlation. *Hum Mutat*. 23(2): 147-159.
- Kortüm F et al. (2011). The core FOXG1 syndrome phenotype consists of postnatal microcephaly, severe mental retardation, absent language, dyskinesia, and corpus callosum hypogenesis. *J Med Genet*. 48: 396–406.
- Laccone F et al. (2004). Large deletions of the MECP2 gene detected by gene dosage analysis in patients with Rett syndrome. *Hum Mutat*. 23(3):234-44.
- Mei D et al. (2010). Xp22.3 genomic deletions involving the CDKL5 gene in girls with early onset epileptic encephalopathy. *Epilep*. 51(4): 647-654.
- Mei D et al. (2014). Optimizing the molecular diagnosis of CDKL5 gene-related epileptic encephalopathy in boys. *Epilep.* 55(11): 1748-1753.
- Nemos C et al. (2009). Mutational spectrum of CDKL5 in early-onset encephalopathies: a study of a large collection of French patients and review of the literature. *Clin Genet*. 76(4): 357-371.
- Neul JL et al. (2010). Rett Syndrome: Revised Diagnostic Criteria and Nomenclature. *Ann Neurol.* 68(6): 944–950.
- 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.

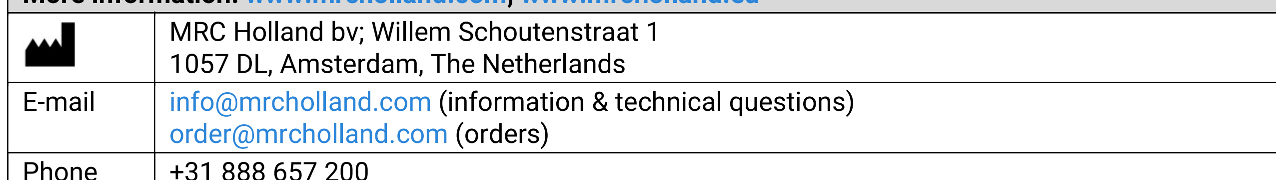
- Zhang Q et al. (2019). Genomic mosaicism in the pathogenesis and inheritance of a Rett syndrome cohort. *Genet Med*. 21(6): 1330-1338.

Selected publications using SALSA MLPA Probemix P015 MECP2

- Aradhya et al. (2012). Exon-level array CGH in a large clinical cohort demonstrates increased sensitivity of diagnostic testing for Mendelian disorders. *Genet Med*, 14(6), 594-603.
- Archer et al. (2006). Gross rearrangements of the MECP2 gene are found in both classical and atypical Rett syndrome patients. *J Med Genet*, 43(5), 451-456.
- Bartholdi et al. (2006). Clinical profiles of four patients with Rett syndrome carrying a novel exon 1 mutation or genomic rearrangement in the MECP2 gene. *Clin Genet*, 69(4), 319-326.
- Bartnik et al. (2011). Early-onset seizures due to mosaic exonic deletions of CDKL5 in a male and two females. *Genet Med*, 13(5), 447-452.
- Bijlsma et al. (2012). Xq28 duplications including MECP2 in five females: Expanding the phenotype to severe mental retardation. *Eur J Med Genet*, 55, 404-413.
- Boutry-Kryza at al. (2014). Complex mosaic CDKL5 deletion with two distinct mutant alleles in a 4-year-old girl. *Am J Med Genet A*, 164A(8), 2025-2028.
- Echenne et al. (2009). Neurologic aspects of MECP2 gene duplication in male patients. *Pediatr Neurol*, 41(3), 187-191.
- Erlandson et al. (2003). Multiplex ligation-dependent probe amplification (MLPA) detects large deletions in the MECP2 gene of Swedish Rett syndrome patients. *Genet Test*, 7(4), 329-332.
- Fieremans et al. (2014). De novo MECP2 duplications in two females with intellectual disability and unfavorable complete skewed X-inactivation. *Hum Genet*, 133(11), 1359-1367.
- Friez et al. (2006). Recurrent infections, hypotonia, and mental retardation caused by duplication of MECP2 and adjacent region in Xq28. *Pediatr*, 118(6), e1687-1695.
- Liang et al. (2011). CDKL5 alterations lead to early epileptic encephalopathy in both genders. *Epilep,* 52(10), 1835-1842.
- Mayo et al. (2011). De novo interstitial triplication of MECP2 in a girl with neurodevelopmental disorder and random X chromosome inactivation. *Cytogenet Genome Res*, 135(2), 93-101.
- Ostrander et al. (2018). Whole-genome analysis for effective clinical diagnosis and gene discovery in early infantile epileptic encephalopathy. *NPJ Genom Med*, 3, 22.
- Sharaf-Eldin et al. (2020). Mutation spectrum in the gene encoding methyl-CpG-binding protein 2 in Egyptian patients with Rett syndrome. *Meta Gene*, 24, 100620.

P015 product history	
Version	*Modification*
F2	Two reference probes have been replaced, the 118 nt Y fragment has been removed.
F1	Four MECP2 probes (exons 3 and 4) and two additional reference probes have been included. One ARX, one NTNG1 and several reference probes have been replaced. One CDKL5 and one IRAK1 probe have been removed. The 88 and 96 nt denaturation control probes have been replaced (QDX2).
E1	One *MECP2* exon 3 probe has been removed.
D2	Several new MECP2 probes have been included and extra probes up- and downstream of *MECP2* were added. In addition, some probes for the *CDKL5*, *ARX* and *NTNG1* genes are included. New reference probes have been included.
D1	Test Lot.
C2	Extra control fragments at 88, 96, 100 and 105 nt have been added.
B1	One extra probe for *MECP2* exon 1, three extra *MECP2* exon 4 probes and extra reference probes have been included.
A1	First release.

Implemented changes in the product description
Version F2-05 – 25 August 2021 (04P) - References to SALSA MLPA Probemix P259 RPS6KA3 removed throughout the document as this probemix was discontinued in July 2021. - References to SALSA MLPA Probemix P374 Microdeletion Syndromes 8 removed throughout the document as this probemix will be discontinued in October 2021. - Small changes of probe lengths in Table 2 and 3 in order to better reflect the true lengths of the amplification products. - Minor textual and layout changes throughout the document.
Version F2-04 – 16 March 2021 (04P) - Product description rewritten and adapted to a new template. - Updated the intended purpose and changed X-linked intellectual disability syndrome into early infantile epileptic encephalopathy. - Various minor textual or layout changes. - Positive DNA sample information is updated; six new samples added and NA23676 result is adjusted. - Ligation sites of the probes targeting the *MECP2*, *CDKL5*, *ARX* and *NTNG1* genes updated according to new version of the NM_ reference sequence. - Warning added to Table 2 and Table 3a for 319 nt probe DKC1 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. - Warning added to Table 2 and Table 3 for SNP rs201741317 that could influence the 427 nt NTNG1 probe signal and SNP rs782037353 that could influence the 448 nt FLNA probe signal. - Exon numbering of the *MECP2* gene has been changed. - Added P137 SCN1A probemix to the Related SALSA MLPA probemixes list. - Added references to the Reference list and updated the Selected publications list. - UK has been added to the list of countries in Europe that accept the CE mark.
Version F2-03 – 18 May 2020 (04) - Israel added as country with IVD status.
Version F2-02 – 30 July 2018 (04) - Warning added to Table 3 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. - Selected publications were updated using P015.
Version F2-01 – 01 March 2018 (04) - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 2 and Table 3). - Table 1 is included to give an overview of the included genes in different probemixes related to (atypical) Rett syndrome.
Version 15 – 27 January 2017 (55) - Probe number (182nt MECP2 probe 10836-L24339) adjusted as it was incorrect displayed in previous product description version.
Version 14 – 15 November 2015 (55) - Lot number added, new pictures included. - CDKL5 exon numbering adjusted. - Various minor textual changes.



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IVD	EUROPE* CE ISRAEL
RUO	ALL OTHER COUNTRIES

*comprising EU (candidate) member states, members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.