Product Description
SALSA® MLPA® Probemix P189-C2 CDKL5/ARX/FOXG1

To be used with the MLPA General Protocol.

Version C2
For complete product history see page 12.

Catalogue numbers:
- **P189-025R:** SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1, 25 reactions.
- **P189-050R:** SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1, 50 reactions.
- **P189-100R:** SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1, 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 P189 CDKL5/ARX/FOXG1 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 *CDKL5* and *FOXG1* genes, in order to confirm a potential cause for and clinical diagnosis of CDKL5 deficiency disorder and *FOXG1* syndrome, respectively. It can also be used for the detection of deletions and duplications in the human *ARX* and *NTNG1* genes, in order to confirm a potential cause for and clinical diagnosis of early infantile epileptic encephalopathy 1 (EIEE1) and atypical Rett syndrome, respectively. This assay is additionally intended for molecular genetic testing of at-risk family members, and is for use with genomic DNA isolated from human peripheral whole blood specimens.

Copy number variations (CNVs) detected with P189 CDKL5/ARX/FOXG1 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 *CDKL5*, *FOXG1*, *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.

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 (IVD) use 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
CDKL5 deficiency disorder (also known as early infantile epileptic encephalopathy 2) is a condition characterized by a broad range of clinical symptoms and severity. The primary symptoms include early-onset

epilepsy (starting within the first three months of life), generalized hypotonia, psychomotor development disorders, intellectual disability, and cortical vision disorders. CDKL5 deficiency disorder has several features in common with Rett syndrome and was previously described as the early-onset seizure variant of Rett syndrome (see below). However, as other signs and symptoms of CDKL5 deficiency disorder are distinct from those of Rett syndrome, the disorder is nowadays considered a separate clinical entity (Fehr et al. 2013).

CDKL5 deficiency disorder is an X-linked dominantly inherited disorder that is caused by mutations in the *CDKL5* gene (Kalscheuer et al. 2003; Scala et al. 2005; Weaving et al. 2004). The prevalence among women is four times higher than in men (Jakimiec et al. 2020), but the course of the disease is usually more severe in male patients. Most cases of CDKL5 deficiency disorder are the result of *de novo* mutations. It is estimated that ~6.5–10% of the *CDKL5* mutations are large deletions or duplications (RettBASE; RettSyndrome.org Variation Database). Mosaicism has been reported for *CDKL5* mutations with an overall frequency of 8.8% (Stosser et al. 2018). Large mosaic deletions have also been described (Bartnik et al. 2011; Boutry-Kryza et al. 2014; Mei et al. 2014), but the occurrence rate for mosaic copy number changes has not been determined.

FOXG1 syndrome is a condition characterized by impaired development and structural brain abnormalities. Affected infants are small at birth, and their heads grow more slowly than normal, leading to an unusually small head size (microcephaly) by early childhood. The condition is associated with a particular pattern of brain malformations and affects most aspects of development. Children with the condition typically have severe intellectual disability. *FOXG1* syndrome is an autosomal dominant condition that is caused by mutations in the *FOXG1* gene (Kortüm et al. 2011; Vegas et al. 2018; Wong et al. 2019). The percentage of *FOXG1* syndrome cases explained by large deletions or duplications of *FOXG1* varies depending on the phenotype examined, but has been estimated at ~11% (Vegas et al. 2018). *FOXG1* syndrome was previously described as the congenital variant of Rett syndrome (see below). However, whereas Rett syndrome is diagnosed almost exclusively in females, *FOXG1* syndrome affects both males and females. Because of these differences, *FOXG1* syndrome is nowadays considered a distinct disease entity.

Early infantile epileptic encephalopathy (EIEE; also known as developmental and epileptic encephalopathy) is a neurological disorder characterized by seizures. The disorder affects male and female 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. EIEE can be caused by mutations in more than 100 different genes. EIEE1 is an X-linked recessive disease that is caused by mutations in the *ARX* gene. Males with *ARX* mutations are often more severely affected than females, but female mutation carriers may also be affected (Kato et al. 2004; Wallerstein et al. 2008). Approximately 3% of identified *ARX* mutations are large deletions and duplications (Shoubridge et al. 2010).

Rett syndrome is a neurodevelopmental disorder affecting approximately 1:10,000 live female births. Classic Rett syndrome 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 Rett syndrome are identified as atypical Rett syndrome cases, which are traditionally subdivided into three distinct clinical subgroups: congenital, early-onset seizure and preserved speech (Hagberg et al. 2002; Hagberg and Skjeldal 1994; Neul et al. 2010; Pini et al. 2016). Most cases of Rett syndrome are caused by *de novo* mutations in the *MECP2* gene (GeneReviews; https://www.ncbi.nlm.nih.gov/books/NBK1497/). However, there is one report that describes a patient with atypical Rett syndrome who presented with early onset of epileptic seizures (not infantile spasms) and a *de novo* translocation that disrupted the *NTNG1* gene on chromosome 1 (Borg et al. 2005). This balanced translocation will not be detected by MLPA as the *NTNG1* copy number is not altered. Deletions and duplications of *NTNG1* have not been described so far.

Since there are multiple genes involved in the above-described syndromes and since these genes are covered by two different probemixes, i.e. SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1 and SALSA MLPA

Probemix P015 MECP2, the table below provides an overview of conditions and genes covered by SALSA MLPA Probemix P015-F2 MECP2 and SALSA MLPA Probemix P189-C2 CDKL5/ARX/FOXG1.

Condition	Genes	Probemix and coverage	Remarks
Classic Rett syndrome	*MECP2* (4 exons)	P015-F2: Each exon	-
***MECP2* duplication syndrome**	*MECP2* (4 exons)	P015-F2: Each exon	-
Atypical Rett syndrome	*MECP2* (4 exons)	P015-F2: Each exon	-
	NTNG1 (6 exons)	**P189-C2**: Each exon P015-F2: Exons 2, 3, 5, 6	Exon 3, 5 and 6 probes in P015 have the same ligation site as probes in P189.
CDKL5 deficiency disorder	*CDKL5* (21 exons)	**P189-C2**: Each exon P015-F2: Exons 3, 6, 9, 10	Probes in P015 have the same ligation sites as probes in P189.
Early infantile epileptic encephalopathy 1	*ARX* (5 exons)	**P189-C2**: Each exon P015-F2: Exons 1, 5	Probes in P015 have the same ligation sites as probes in P189.
***FOXG1* syndrome**	*FOXG1* (1 exon)	**P189-C2**: Exon 1 and upstream region	-

Gene structure
The *CDKL5* gene spans ~228 kilobases (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 ~342 kb on chromosome 1p13.3 and contains 6 exons.

The *FOXG1* gene spans ~3.5 kb of genomic DNA on chromosome 14q12 and contains 1 exon.

Transcript variants
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 1 nt longer than transcript variant 2 (NM_001037343.2) and shorter than 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) is a reference standard in the NCBI RefSeq project. It contains a longer 5' UTR and lacks two exons in the 3' coding region, but maintains the reading frame, as compared to variant 1 (NM_001113226.3). The encoded isoform (G1c) is shorter than isoform G1a.

For *FOXG1*, one transcript variant has been described encoding the full length protein (NM_005249.5; 3491 nt; coding sequence 494-1963; https://www.ncbi.nlm.nih.gov/gene/2290). This sequence is a reference standard in the NCBI RefSeq project.

Exon numbering
The exon numbering used in this P189-C2 CDKL5/ARX/FOXG1 product description is the exon numbering from NG_008475.1 for *CDKL5*, from NG_008281.1 for *ARX*, from NG_042821.1 for *NTNG1*, and from NG_009367.1 for *FOXG1*. 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 P189-C2 CDKL5/ARX/FOXG1 contains 52 MLPA probes with amplification products between 130 and 500 nucleotides (nt). This includes 24 probes for the *CDKL5* gene, five probes for the *ARX* gene, 12 probes for the *NTNG1* gene and two probes for the *FOXG1* gene, covering all exons of these genes. In addition, nine 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 developmental delay or epilepsy. Importantly, all reference samples need to be of the same sex (all male, or all female) for correct data analysis. In addition, it is recommended to use a set of reference samples of the same sex as patient samples, for ease of 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 from the Coriell Institute described in the table below have been tested with this P189-C2 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 ID number	Gender	Source	Expected copy number variation
NA23710	female	Coriell Institute	Heterozygous deletion of *CDKL5* intron 16 – exon 18
NA22765	male	Coriell Institute	Heterozygous deletion of *FOXG1*
NA01750	male	Coriell Institute	Heterozygous duplication of *FOXG1*

Performance characteristics

Approximately 6.5–10% of the patients with CDKL5 deficiency disorder have large deletions or duplications in the *CDKL5* gene (RettBASE; RettSyndrome.org Variation Database). The percentage of *FOXG1* syndrome cases explained by large deletions or duplications of *FOXG1* varies depending on the phenotype examined, but has been estimated at ~11% (Vegas et al. 2018). Approximately 3% of the *ARX* mutations identified are large deletions or duplications (Shoubridge et al. 2010). No deletions or duplications in the *NTNG1* gene have been described so far. However, a translocation disrupting the *NTNG1* gene has been described as the genetic cause in a patient with atypical Rett syndrome (Borg et al. 2005). The analytical sensitivity and specificity for the detection of deletions or duplications in the *CDKL5*, *FOXG1*, *ARX* and *NTNG1* genes is very high and can be considered >99% (based on a 2006-2021 literature review).

Analytical performance can be compromised by: single nucleotide variants (SNVs) 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. 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 *CDKL5, NTNG1, ARX* and *FOXG1* 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 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.

P189 specific note:

- The use of fixed cut-off values for the FR of the probes as mentioned in the table above may not allow detection of mosaic deletions or duplications. Mosaic *CDKL5* deletions have been reported in CDKL5 deficiency disorder (Bartnik et al. 2011; Boutry-Kryza et al. 2014; Mei et al. 2014). In order to detect mosaic samples, the experiment has to have little variation and the final ratios should be significantly different from the reference samples (see Coffalyser.Net Reference Manual, Appendix I – Normalisation and result interpretation). Mosaic samples may not be detected if the percentage of cells that have the deletion or duplication is low.

Limitations of the procedure

- In most populations, the major cause of genetic defects in *CDKL5*, *ARX* and *FOXG1* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1.
- 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.

Mutation databases

RettBASE for *CDKL5* and *FOXG1* mutations: http://mecp2.chw.edu.au/cdkl5/cdkl5_home.php and http://mecp2.chw.edu.au/foxg1/foxg1_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 *CDKL5* exons 8 and 10 but not exon 9) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P189-C2 CDKL5/ARX/FOXG1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]				
		Reference	*CDKL5* Xp22.13	*ARX* Xp21.3	*NTNG1* 1p13.3	*FOXG1* 14q12
64-105	Control fragments – see table in probemix content section for more information					
130	Reference probe 00797-L13645	5q				
136	**CDKL5 probe** 06456-L24567		**Exon 1**			
144	**CDKL5 probe** 06466-L06567		**Exon 10**			
149 ± «	**NTNG1 probe** 06478-L06568				**Exon 1**	
154	**CDKL5 probe** 13667-L15127		**Exon 1**			
161	**CDKL5 probe** 06457-L06570		**Exon 2**			
166	Reference probe 07724-L07434	7p				
172	**CDKL5 probe** 06467-L06571		**Exon 11**			
178	**NTNG1 probe** 06484-L06010				**Exon 4**	
185	**CDKL5 probe** 06458-L05984		**Exon 3**			
191	Reference probe 06057-L06042	4p				
196 « +	**ARX probe** 18790-L24442			**Exon 2**		
202 «	**NTNG1 probe** 06479-L06005				**Exon 1**	
208 ±	**NTNG1 probe** 06486-L06572				**Exon 5**	
215	Reference probe 08570-L08571	17q				
222 « +	**ARX probe** 02898-L04200			**Exon 4**		
227	**CDKL5 probe** 06459-L23728		**Exon 4**			
233 « +	**ARX probe** 13669-L23729			**Exon 1**		
239	**CDKL5 probe** 06474-L23730		**Exon 18**			
246	**CDKL5 probe** 06461-L23731		**Exon 5**			
252	**CDKL5 probe** 06470-L16143		**Exon 14**			
257 «	**NTNG1 probe** 06480-L21520				**Exon 2**	
265 «	**ARX probe** 21262-L29842			**Exon 5**		
274	**NTNG1 probe** 06485-L29843				**Exon 4**	
280	Reference probe 12494-L29844	1q				
286	**CDKL5 probe** 21263-L29845		**Intron 16**			
292	**CDKL5 probe** 06462-L29846		**Exon 6**			
298	**CDKL5 probe** 06476-L26217		**Exon 20**			
305	**NTNG1 probe** 06487-L29847				**Exon 5**	
310 «	**NTNG1 probe** 06481-L06007				**Exon 2**	
319	**CDKL5 probe** 06463-L05989		**Exon 7**			
326	**CDKL5 probe** 06471-L05997		**Exon 15**			
333 «	**FOXG1 probe** 16850-L19644					**Upstream**
340	**CDKL5 probe** 06882-L21233		**Exon 12**			
348	Reference probe 16441-L18894	18q				
355 « +	**ARX probe** 13670-L21234			**Exon 3**		
364	**NTNG1 probe** 06489-L06015				**Exon 6**	
373	**CDKL5 probe** 06464-L05990		**Exon 8**			
383	**CDKL5 probe** 06472-L05998		**Exon 16**			
391 ±	**NTNG1 probe** 06482-L06008					**Exon 3**
400	**CDKL5 probe** 06465-L05991		**Exon 9**			
409	Reference probe 09497-L09754	11q				
418	**CDKL5 probe** 06473-L05999		**Exon 17**			
427	**NTNG1 probe** 06488-L06014				**Exon 6**	
436	**NTNG1 probe** 06483-L06009				**Exon 3**	
445	**CDKL5 probe** 06469-L05995		**Exon 13**			
452	**CDKL5 probe** 21264-L05986		**Exon 4**			
463	**CDKL5 probe** 06475-L06001		**Exon 19**			
472 «	**FOXG1 probe** 17292-L15242					**Exon 1**
481	**CDKL5 probe** 17286-L20734		**Exon 21**			
490	Reference probe 09772-L21655	15q				
500	Reference probe 17001-L22947	20q				

[a] See section Exon numbering on page 3 for more information.

± SNP rs147336854 could influence the 149 nt NTNG1 probe signal, SNP rs2587905 could influence the 208 nt NTNG1 probe signal, SNP rs144330931 could influence the 391 nt NTNG1 probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by the affected probe(s).
« 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.
+ The presence of salt in DNA samples can result in incomplete denaturation of CpG islands, which may result in false positive results: apparent deletions of this probe should be handled with care. Usually Coffalyser.Net issues a sample denaturation warning when the 88 nt and/or 96 nt D-fragments are too low. The 196 nt, 222 nt, 233 nt and 355 nt ARX probes target extremely GC-rich chromosomal areas, and are affected by salt concentrations that not yet affect the control D- fragments, thus without Coffalyser.Net issuing a warning. False positive results are more likely when DNA has been extracted by the Qiagen EZ1, M48 or M96 systems, as these leave a higher salt concentration in the sample. High salt concentrations can also be due to evaporation (dried out samples; SpeedVac concentration or other related technique).

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 2. P189-C2 probes arranged according to chromosomal location

Table 2a. *CDKL5* (Xp22.13) and *ARX* (Xp21.3)

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		CDKL5	**NM_003159.3**		
		start codon	*251-253 (Exon 2)*		
154	13667-L15127	Exon 1	26-27	CTGGGGAAGGTA-AAGCGGCGACGG	0.1 kb
136	06456-L24567	Exon 1	80 nt after exon 1	CTTCTCCTGAGA-GTGGAGACCCAC	81.2 kb
161	06457-L06570	Exon 2	169-170	CTTCAGACGGTT-TTGGATCTTACT	3.8 kb
185	06458-L05984	Exon 3	41 nt before exon 3	GAGCTTTGTAGT-TTGTATGCGTGC	53.7 kb
227	06459-L23728	Exon 4	13 nt before exon 4	CCTTCTGCTTCT-TTTCCCTTGCAG	0.2 kb
452	21264-L05986	Exon 4	98 nt after exon 4	TGGGATCTAGCT-GGTCAGACACTG	10.8 kb
246	06461-L23731	Exon 5	485-486	TGAAGGAAGCAT-TTCGTCGGAGGG	4.4 kb
292	06462-L29846	Exon 6	570-571	GCCAAATGGAGT-TCCACCTGAGAA	2.0 kb
319	06463-L05989	Exon 7	686-685 reverse	TAGGACATCATT-GTGGCTGATTAA	2.4 kb
373	06464-L05990	Exon 8	748-749	GGCAATAATGCT-AATTACACAGAG	3.8 kb
400	06465-L05991	Exon 9	910-911	ATTGACCAACTT-TTTACTATTCAG	7.3 kb
144	06466-L06567	Exon 10	1023-1024	TCCTCAGTCCTT-GGAAAGAAGATA	3.1 kb
172	06467-L06571	Exon 11	1123-1124	ACAGAACAGTGT-TTGAATCACCCT	5.7 kb
340	06882-L21233	Exon 12	1565-1566	AGTCAAACAGCA-GATCTCAGCAGA	4.6 kb
445	06469-L05995	Exon 13	2243-2244	GATCTTCGGTCA-AAGAGACCTCCA	0.6 kb
252	06470-L16143	Exon 14	2311-2312	GGAGTGTATCAT-GACCCACACTCT	3.7 kb
326	06471-L05997	Exon 15	2475-2476	TCTACCATCAGA-GAGCAGTTCTGG	6.7 kb
383	06472-L05998	Exon 16	2565-2564 reverse	TCTCTTTTTCCT-TGAGTTGCTCTG	4.0 kb
286	21263-L29845	Intron 16	1225 nt before exon 17	AACGGGGAGAAT-CCAAGCATCAAG	1.3 kb
418	06473-L05999	Exon 17	2673-2674	GCAGAAATCCAT-TCATTCTGCTAG	3.3 kb
239	06474-L23730	Exon 18	2861-2862	CCAAAAATTCCT-TCTCAGAAATTC	17.6 kb
463	06475-L06001	Exon 19	2991-2992	CAGAAGACAGAG-ACACCATTCTGG	4.4 kb
298	06476-L26217	Exon 20	2 nt before exon 20 reverse	AAGTATTCTCCT-GGAAAATATTCA	3.2 kb
481	17286-L20734	Exon 21	3399-3400	CTCATGGAAGAA-CCAATTAACACC	6.4 **M**b to *ARX* Exon 5
		stop codon	*3341-3343 (Exon 21)*		
		ARX	**NM_139058.3**		
		stop codon	*1915-1917 (Exon 5)*		
265 «	21262-L29842	Exon 5	1981-1982	CAGCACCACTCA-AGACCAAATGGA	2.5 kb
222 « +	02898-L04200	Exon 4	1652-1651 reverse	GGCTGATGAAAG-CTGGGTGTCGGA	2.9 kb

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
355 « +	13670-L21234	Exon 3	222 nt after exon 3 reverse	GACTCCTTCCTT-GGGCTCCCAAAC	3.0 kb
196 « +	18790-L24442	Exon 2	1168-1167 reverse	GCTGCCCGCAGA-GAGGCACACGCT	2.8 kb
233 « +	13669-L23729	Exon 1	115-116	AGATCGCAATAA-TATCCGTTATAA	
		start codon	*229-231 (Exon 1)*		

Table 2b. *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 ± «	06478-L06568	Exon 1	17 nt after exon 1	ATAAGGTTTGCT-ATCCTTCCACTT	0.1 kb
202 «	06479-L06005	Exon 1	156 nt after exon 1	TCACTCTTTAAT-TGGCACTGGCCC	7.8 kb
257 «	06480-L21520	Exon 2	247-248	TTGAGGATCACT-CAGGGTTATCGG	0.5 kb
310 «	06481-L06007	Exon 2	736-737	TATGATTTGTGT-AAGACTCAGATT	175.8 kb
391 ±	06482-L06008	Exon 3	1048-1049	ATCACTCTGTCT-TGGAGCAAAACC	0.4 kb
436	06483-L06009	Exon 3	1436-1437	GGATAAGGCTGT-TAAGACCAGCCG	70.3 kb
178	06484-L06010	Exon 4	1551-1552	CCATGCCACTGT-ATGTGTGTATGA	0.2 kb
274	06485-L29843	Exon 4	27 nt after exon 4	TGGTAACAGCAT-ATTCTGTGCACC	12.1 kb
208 ±	06486-L06572	Exon 5	246 nt before exon 5	ATTAACTTCCCA-ACTCTGATGAGC	0.5 kb
305	06487-L29847	Exon 5	227 nt after exon 5	TATGACTTTTCT-GACTACTCTTAA	72.7 kb
427	06488-L06014	Exon 6	1737-1738	GAATGTCTGCGA-CAACGAGCTCCT	0.7 kb
364	06489-L06015	Exon 6	2410-2411	ACAAGCTGCCAT-ATTGGCCTGCTT	
		stop codon	*1952-1954 (Exon 6)*		

Table 2c. *FOXG1* (14q12)

Length (nt)	SALSA MLPA probe	*FOXG1* exon[a]	Ligation site NM_005249.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*494-496 (Exon 1)*		
333 «	16850-L19644	Upstream	559 nt before exon 1	AAATGCCAGACA-CTGGCCTGCAAG	2.5 kb
472 «	17292-L15242	Exon 1	1907-1906 reverse	GAAATAATCAGA-CAGTCCCCCAGA	
		stop codon	*1961-1963 (Exon 1)*		

[a] See section Exon numbering on page 3 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.

± SNP rs147336854 could influence the 149 nt NTNG1 probe signal, SNP rs2587905 could influence the 208 nt NTNG1 probe signal, SNP rs144330931 could influence the 391 nt NTNG1 probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by the affected probe(s).
« 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.
+ The presence of salt in DNA samples can result in incomplete denaturation of CpG islands, which may result in false positive results: apparent deletions of this probe should be handled with care. Usually Coffalyser.Net issues a sample denaturation warning when the 88 nt and/or 96 nt D-fragments are too low. The 196 nt, 222 nt, 233 nt and 355 nt ARX probes target extremely GC-rich chromosomal areas, and are affected by salt concentrations that not yet affect the control D- fragments, thus without Coffalyser.Net issuing a warning. False positive results are more likely when DNA has been extracted by the Qiagen EZ1, M48 or M96 systems, as these leave a higher salt concentration in the sample. High salt concentrations can also be due to evaporation (dried out samples; SpeedVac concentration or other related technique).

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

P015 MECP2	Contains probes for the *MECP2*, *CDKL5*, *ARX* and *NTNG1* genes.
P075 TCF4-FOXG1	Contains three additional probes for the *FOXG1* gene. The ligation sites of the FOXG1 probes are identical to those in P395 MECF2-FOXG1, with the exception of one of the exon 1 probes.
P106 X-linked ID	Contains three probes for the *ARX* gene. The ligation sites of these probes are identical to probes included in P189 CDKL5/ARX/FOXG1.
P137 SCN1A	Contains probes for the *SCN1A* gene. Mutations in *SCN1A* have been described in two females who fulfil the diagnostic criteria for classic RTT (Henriksen et al. 2018).
P245 Microdeletion Syndromes-1A	Contains three probes for the *MECP2* gene. The ligation sites of these probes are identical to probes included in P015 MECP2.
P395 MEFC2-FOXG1	Contains three additional probes for the *FOXG1* gene. The ligation sites of the FOXG1 probes are identical to those in P075 TCF4-FOXG1, with the exception of one of the exon 1 probes.

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P189 product history	
Version	*Modification*
C2	Several probes have been adjusted in length, but no change in sequence detected. The name of the probemix has been changed from “CDKL5 / Atypical Rett syndrome” to “CDKL5/ARX/FOXG1”.
C1	One additional probe for *CDKL5,* two probes for *FOXG1* and one reference probe have been added. One probe for *CDKL5*, one probe for *ARX* and five reference probes have been replaced.

B2	The 88 and 96 nt control fragments have been replaced (QDX2).
B1	An extra probe for *CDKL5* exon 1 has been added and four probes for *ARX* have been replaced. In addition, four reference probes have been replaced and two additional reference probes have been added. All reference probes now detect autosomal chromosomes.
A2	Extra control fragments at 88, 96, 100 and 105 nt have been added.
A1	First release.

Implemented changes in the product description

Version C2-05 – 16 November 2023 (04P)
- In section Reference samples, clarification added that all reference samples need to be the same sex for correct data analysis.
- Warning for hypersensitivity to salt added for the probes at 196 nt, 222 nt, 233 nt and 355 nt (ARX) to Table 1 and Table 2.
- Updated Rett syndrome clinical subgroups information and related references.
- Selected publications using SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1 updated.
- Various minor textual or layout changes.
- Tables were renumbered.

Version C2-04 – 21 March 2023 (04P)
- Intended purpose adjusted; mutations in *CDKL5* are associated with CDKL5 deficiency disorder.
- Clinical background section updated; section completely rewritten and information about CDKL5 deficiency disorder added.
- Two additional positive control samples added to the Positive control DNA samples section.
- Performance characteristics section updated; percentage of cases explained by deletions or duplications adjusted and information about CDKL5 deficiency disorder added.
- Note about mosaicism added to the Interpretation of results section, P189 specific note.
- Small changes of probe lengths in Table 2 and 3 in order to better reflect the true lengths of the amplification products.
- References to SALSA MLPA Probemix P259 RPS6KA3 and SALSA MLPA Probemix P374 Microdeletion Syndromes 8 removed throughout the document as these probemixes were discontinued in July 2021 and October 2021, respectively.
- Information about related SALSA MLPA probemixes updated.
- Selected publications using SALSA MLPA Probemix P189 CDKL5/ARX/FOXG1 updated.
- P189 product history adjusted; modifications in B1 version clarified.
- Various minor textual and layout changes.

Version C2-03 – 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.
- Ligation sites of the probes targeting the *CDKL5*, *ARX*, *FOXG1* and *NTNG1* genes updated according to new version of the NM_ reference sequence.
- Warning added to Table 2 and Table 3c for SNP rs2587905 that could influence the 208 nt NTNG1 probe signal and SNP rs144330931 that could influence the 391 nt NTNG1 probe signal.
- Added P137, P245 and P374 probemixes 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 C2-02 – 11 May 2020 (04)
Israel added as country with IVD status.

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