Product Description
SALSA® MLPA® Probemix P013-A2 ATRX

To be used with the MLPA General Protocol.

Version A2
For complete product history see page 7.

Catalogue numbers:
- **P013-025R:** SALSA MLPA Probemix P013 ATRX, 25 reactions.
- **P013-050R:** SALSA MLPA Probemix P013 ATRX, 50 reactions.
- **P013-100R:** SALSA MLPA Probemix P013 ATRX, 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 P013 ATRX is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ATRX* gene, which is associated with Alpha-thalassemia X-linked intellectual disability (ATRX) syndrome.

ATRX syndrome is characterized by distinctive craniofacial features - like microcephaly, telecanthus, widely spaced eyes, short nose -, genital anomalies, severe developmental delays, intellectual disability, hypotonia, skeletal anomalies, and alpha-thalassemia without molecular abnormalities of the alpha-globin gene complex. ATRX syndrome is inherited in an X-linked manner. The mother may be a carrier or the affected individual may have a de novo mutation. Female carriers have a 50% chance in each pregnancy of transmitting the *ATRX* pathogenic variant.

The *ATRX* gene encodes for the transcriptional regulator ATRX protein. The zinc finger domain functions as a transcription factor and the helicase domains function in the transcription process opening double-stranded DNA. In combination with other chromatin-associated proteins, the ATRX protein appears to play a role in chromatin remodelling, possibly silencing gene expression during development. The mutated ATRX protein downregulates the α-globin locus, resulting in thalassemia, and probably suppresses expression of other genes by disturbances in transcription and chromatin structure, leading to malformations and intellectual disability.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1449/.

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 *ATRX* exon numbering used in this P013-A2 ATRX product description is the exon numbering from the LRG_1153 sequence. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content
The SALSA MLPA Probemix P013-A2 ATRX contains 46 MLPA probes with amplification products between 130 and 490 nucleotides (nt). This includes 37 probes for the *ATRX* gene, with one probe for every exon and two probes for exons 9 and 35. In addition, nine reference probes are included that detect different locations on the X-chromosome. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains nine quality control fragments generating amplification products between 64 and 105nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MLPA technique
The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mrcholland.com).

MLPA technique validation
Internal validation of the MLPA technique using 16 DNA samples from healthy individuals of the same sex is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation ≤0.10 for all probes over the experiment.

Required specimens
Extracted DNA free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples
A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from different unrelated individuals who are from families without a history of ATRX syndrome. It is recommended to use samples of the same sex to facilitate interpretation. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

Positive control DNA samples

MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/) have diverse collections of biological resources which may be used as positive control DNA samples in your MLPA experiments. The quality of cell lines can change; therefore samples should be validated before use.

Data analysis

Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the FR of the probes can be used to interpret MLPA results:

Copy Number status: Male samples	Final ratio
Normal	0.80 < FR < 1.20
Deletion	FR = 0
Duplication	1.65 < FR < 2.25
Ambiguous copy number	All other values

Copy Number status: Female samples	Final ratio
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 2.15
Ambiguous copy number	All other values

Note: The term “dosage quotient”, used in older product description versions, has been replaced by “final ratio” to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.

- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *ATRX* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P013 ATRX.
- 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.

***ATRX* mutation database**

http://grenada.lumc.nl/LSDB_list/lsdbs/ATRX. We strongly encourage users to deposit positive results in one of these *ATRX* mutation databases. 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 *ATRX* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P013-A2 ATRX

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*ATRX*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 13917-L18702	Xq23	
135	**ATRX probe** 15549-L17404		**Exon 1**
142	**ATRX probe** 15550-L17405		**Exon 32**
148	Reference probe 06110-L05565	Xq25	
154	**ATRX probe** 16067-L17406		**Exon 28**
160	Reference probe 16675-L20191	Xp11	
171	**ATRX probe** 15554-L17409		**Exon 8**
178	**ATRX probe** 15555-L18327		**Exon 6**
185	**ATRX probe** 15556-L17411		**Exon 29**
190	Reference probe 12595-L13679	Xq12	
196	**ATRX probe** 15557-L17412		**Exon 22**
202	**ATRX probe** 15558-L17413		**Exon 20**
207	**ATRX probe** 20262-L18328		**Exon 33**
214	**ATRX probe** 15560-L18329		**Exon 9**
220	Reference probe 18221-L25392	Xp21	
226	**ATRX probe** 15561-L18330		**Exon 23**
232	**ATRX probe** 15562-L17417		**Exon 12**
238	**ATRX probe** 15563-L17418		**Exon 25**
244	**ATRX probe** 15564-L17419		**Exon 31**
250	**ATRX probe** 15552-L18326		**Exon 7**
256	**ATRX probe** 15565-L17420		**Exon 10**
263	**ATRX probe** 15553-L17408		**Exon 18**
269	**ATRX probe** 15566-L18331		**Exon 21**
275	**ATRX probe** 15567-L18332		**Exon 16**
283	**ATRX probe** 15568-L17423		**Exon 9**
301	**ATRX probe** 15569-L17424		**Exon 19**
312	**ATRX probe** 15570-L17425		**Exon 30**
319	**ATRX probe** 15571-L17426		**Exon 13**
328	**ATRX probe** 15572-L18333		**Exon 4**
337	Reference probe 06911-L06491	Xq22	
346	**ATRX probe** 15573-L17428		**Exon 24**
355	**ATRX probe** 15574-L18334		**Exon 11**
364	**ATRX probe** 15575-L17430		**Exon 2**
372	**ATRX probe** 15576-L17431		**Exon 15**
383	Reference probe 06472-L05998	Xp22	
392	**ATRX probe** 15577-L17432		**Exon 35**
401	**ATRX probe** 15578-L17433		**Exon 3**
409	**ATRX probe** 15579-L18335		**Exon 14**
418	**ATRX probe** 15580-L17435		**Exon 26**
427	Reference probe 13117-L14336	Xp11	
436	**ATRX probe** 15581-L17436		**Exon 35**
445	**ATRX probe** 15582-L18336		**Exon 17**
454	**ATRX probe** 15583-L17438		**Exon 27**
471	**ATRX probe** 15584-L17439		**Exon 34**
481	**ATRX probe** 15585-L17440		**Exon 5**
490	Reference probe 13918-L15455	Xp11	

[a] See section Exon numbering on page 2 for more information.

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. *ATRX* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ATRX* exon[a]	Ligation site NM_000489.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*216-218 (Exon 1)*		
135	15549-L17404	Exon 1	130-131	ACTAAGACTCCA-GTGCATTTCTAT	68.9 kb
364	15575-L17430	Exon 2	274-275	GAAGCTTCATGA-CTTCCTTGCACA	18.7 kb
401	15578-L17433	Exon 3	80 nt after exon 3	CTTCTGGACACA-TGTATGTGTCGA	0.8 kb
328	15572-L18333	Exon 4	410-409, reverse	TCTGAAGAGCTA-GTTCCCTAGATG	1.0 kb
481	15585-L17440	Exon 5	536-537	AATGAAGATGCG-TCTAATGAAAAT	2.7 kb
178	15555-L18327	Exon 6	631-632	TGAAGACAAAGA-TGATTTTAAAGG	5.0 kb
250	15552-L18326	Exon 7	729-730	TTGTGAGCTGCA-CTGCTTGTGGAC	3.9 kb
171	15554-L17409	Exon 8	817-816, reverse	TGTAATACTTAA-AGCAATTCTATT	0.6 kb
283	15568-L17423	Exon 9	1039-1040	GCCTTTGTTGGA-CTTGGTCACTGC	2.7 kb
214	15560-L18329	Exon 9	3695-3696	GATGCTGAGGAA-AGTTCTGAAGAT	5.5 kb
256	15565-L17420	Exon 10	3994-3995	CACAGTGGATGA-TGATGATGACGA	11.6 kb
355	15574-L18334	Exon 11	4102-4103	AGATGATGAGCC-AGAAGAAGGGAA	1.1 kb
232	15562-L17417	Exon 12	4163-4164	CATTTAGAAGCA-AAAAATCAAGTC	6.9 kb
319	15571-L17426	Exon 13	4397-4396, reverse	TCTTCGGATTCA-CTAACTTCTTCA	2.4 kb
409	15579-L18335	Exon 14	2 nt before exon 14, reverse	TTTGCAGACCTG-ACGAAAATTTAA	2.0 kb
372	15576-L17431	Exon 15	4634-4635	TCCAAGTCTCCT-GGAAAAGGCAGA	16.2 kb
275	15567-L18332	Exon 16	4800-4801	ATGCTTCACCCA-CCAAGTGTCCAA	1.4 kb
445	15582-L18336	Exon 17	4996-4997	CATTCTTGCCCA-CTGTATGGGCCT	1.0 kb
263	15553-L17408	Exon 18	5153-5152, reverse	TTCTCATCATCT-TTTAATCCCTCT	0.2 kb
301	15569-L17424	Exon 19	5211-5212	CTCAGGAGAGAA-GCTACATGCTGC	12.9 kb
202	15558-L17413	Exon 20	5456-5455, reverse	AGTGGTGTTCCT-GTTAAAATAATC	1.6 kb
269	15566-L18331	Exon 21	5607-5608	TGGTAGATGTCA-GAGTGATGAAAA	2.2 kb
196	15557-L17412	Exon 22	5755-5756	GTGCAAGCTCTA-TCAGTACTACTT	16.1 kb
226	15561-L18330	Exon 23	5855-5854, reverse	GTCCATATTCTA-CTTAACATCTGA	0.8 kb
346	15573-L17428	Exon 24	5966-5967	GATTCTGATGAA-ACCTCCATGAGT	0.3 kb
238	15563-L17418	Exon 25	6084-6085	AAGTGATTAAGG-TCTGGAATTCAA	5.7 kb
418	15580-L17435	Exon 26	6220-6221	TCCAGACTGGTA-CAAAGATTTTGT	3.9 kb
454	15583-L17438	Exon 27	6358-6359	CATATCTCTGGA-CTTGATTGAAGA	15.6 kb
154	16067-L17406	Exon 28	6497-6498	ACTGCACAGTCA-AGGAAGAAGTGG	15.6 kb
185	15556-L17411	Exon 29	6648-6649	CATCTTATGACA-TCCAGAGTATAT	1.1 kb
312	15570-L17425	Exon 30	6779-6780	CTGTCTTTTCGA-GTTGTTGATCAG	34.2 kb
244	15564-L17419	Exon 31	6934-6935	ACTTGCAGAGCT-CCTTCAGATACA	1.0 kb
142	15550-L17405	Exon 32	7098-7099	TACCAACTGGGA-CCAATTTACCCC	0.9 kb
207	20262-L18328	Exon 33	7247-7248	AGTGTGACAGCA-GTGAGGATTCAA	0.6 kb
471	15584-L17439	Exon 34	7350-7351	GTAGACAAGCCA-GCCAGGAGCTTG	12.8 kb
436	15581-L17436	Exon 35	7959-7960	CAAGTCCTTCCA-TAATAGTAACAA	2.8 kb
392	15577-L17432	Exon 35	10722-10723	AAATGAGCTGAA-TATTTTGTAGCC	
		stop codon	*7692-7694 (Exon 35)*		

[a] See section Exon numbering on page 2 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Related SALSA MLPA probemixes

P140 HBA	Contains probes for the 16p HBA region, involved in alpha-thalassemia.
P106 X-linked ID	Contains probes for genes involved in X-linked intellectual disability.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

Selected publications using SALSA MLPA Probemix P013 ATRX

- Clay MR et al. (2019). DNA methylation profiling reveals prognostically significant groups in pediatric adrenocortical tumors: a report from the International Pediatric Adrenocortical Tumor Registry. *JCO Precis Oncol*, 3, 1-21.
- Fishbein L et al. (2015). Whole-exome sequencing identifies somatic ATRX mutations in pheochromocytomas and paragangliomas. *Nat Commun*. 6:6140.
- Latysheva A et al. (2019). Dynamic susceptibility contrast and diffusion MR imaging identify oligodendroglioma as defined by the 2016 WHO classification for brain tumors: histogram analysis approach. *Neuroradiology*, 61(5), 545-555.
- Pinto EM et al. (2017). Identification of clinical and biologic correlates associated with outcome in children with adrenocortical tumors without germline TP53 mutations: a St Jude Adrenocortical Tumor Registry and Children's Oncology Group Study. *J Clin Oncol*. 35(35):3956-3963.
- Wentz E et al. (2014). A case report of two male siblings with autism and duplication of Xq13-q21, a region including three genes predisposing for autism. *Eur Child Adolesc Psychiatry*. 23:329-336.

P013 product history	
Version	*Modification*
A2	Three reference probes have been replaced and the control fragments adjusted (QDX2).
A1	First release.

Implemented changes in the product description
Version A2-03 – 20 November 2024 (04P) - The term 'mental retardation' is considered outdated and was updated to 'intellectual disability' where appropriate. *Version A2-02 – 11 November 2021 (04P)* - Product description rewritten and adapted to a new template.

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