Product Description
SALSA® MLPA® Probemix P475-A1 FOXP1-FOXP2

To be used with the MLPA General Protocol.

Version A1
For complete product history see page 8.

Catalogue numbers:
- **P475-025R:** SALSA MLPA Probemix P475 FOXP1-FOXP2, 25 reactions.
- **P475-050R:** SALSA MLPA Probemix P475 FOXP1-FOXP2, 50 reactions.
- **P475-100R:** SALSA MLPA Probemix P475 FOXP1-FOXP2, 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 P475 FOXP1-FOXP2 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *FOXP1* and *FOXP2* genes, which are associated with intellectual disability and speech and language disorder.

Forkhead box (FOX) proteins are a family of transcription factors defined by a forkhead DNA-binding domain, which are involved in a range of functions. Of the FOXP protein subfamily, which includes FOXP1-4, *FOXP1* and *FOXP2* have been indicated in cognitive disorders (Bacon & Rappold 2012). Mutations in *FOXP1* are associated with intellectual disability and may also cause language impairment as well as autistic features (OMIM 613670). *FOXP2* mutations are known to cause a severe speech and language disorder (OMIM 602081); autistic features have been reported in some cases.

The *FOXP1* gene (21 exons) spans ~629 kb of genomic DNA and is located on 3p14.1, 71 Mb from the p-telomere. The *FOXP2* gene (18 exons) spans ~276 kb of genomic DNA and is located on 7q31.1, 114 Mb from the p-telomere.

This SALSA MLPA probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Gene structure and transcript variants:
Entrez Gene shows transcript variants of each gene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering
The *FOXP1* exon numbering used in this P475-A1 FOXP1-FOXP2 product description is the exon numbering from the NG_028243.1 sequence. The *FOXP2* exon numbering used in this P475-A1 FOXP1-FOXP2 product description is the exon numbering from the NG_007491.3 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 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 P475-A1 FOXP1-FOXP2 contains 54 MLPA probes with amplification products between 124 and 509 nucleotides (nt). This includes 26 probes for the *FOXP1* gene and 20 probes for the *FOXP2* gene, one probe for each exon of these genes and two probes for *FOXP1* exons 3-7 and *FOXP2* exons 3 and 4. In addition, eight reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

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

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

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

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

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

Reference samples
A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from different unrelated individuals who are from families without a history of intellectual disability or speech or language impairment. 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 NA01059 and NA12519 from the Coriell Institute have been tested with this P475-A1 probemix at MRC Holland and can be used as positive control samples to detect a heterozygous deletion and a homozygous duplication of *FOXP2*, respectively. 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 for autosomal chromosomes or pseudo-autosomal regions:

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

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

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the *FOXP1* gene. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun

the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *FOXP1* and *FOXP2* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P475 FOXP1-FOXP2.
- 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.

***FOXP1* and *FOXP2* mutation databases**

https://databases.lovd.nl/shared/genes/FOXP1 and https://databases.lovd.nl/shared/genes/FOXP2. We strongly encourage users to deposit positive results in the Leiden Open Variation Database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results (e.g., a duplication of *FOXP1* exons 12 and 14 but not exon 13) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P475-A1 FOXP1-FOXP2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*FOXP1*	*FOXP2*
64-105	Control fragments – see table in probemix content section for more information			
124	Reference probe 19616-L26275	4p		
130 ◎	**FOXP1** probe 21303-L29709		**Exon 4**	
136	**FOXP2** probe 21304-L29710			**Exon 9**
142	**FOXP1** probe 21305-L29711		**Exon 10**	
148 ◎	**FOXP1** probe 21306-L29712		**Exon 2**	
154	**FOXP2** probe 21307-L29713			**Exon 3**
160 ◎	**FOXP1** probe 21308-L29714		**Exon 5**	
166	**FOXP1** probe 21309-L29715		**Exon 14**	
172	Reference probe 21193-L29851	6p		
178	**FOXP1** probe 21310-L29716		**Exon 20**	
184	**FOXP2** probe 21311-L29717			**Exon 13**
190	**FOXP2** probe 21312-L29718			**Exon 3**
195	**FOXP2** probe 21313-L29719			**Exon 6**
201	**FOXP2** probe 21314-L30057			**Exon 2**
208	**FOXP1** probe 21315-L29721		**Exon 19**	
214	**FOXP1** probe 21316-L29722		**Exon 7**	
220	Reference probe 21057-L30157	10q		
226	**FOXP1** probe 21317-L29723		**Exon 12**	
232	**FOXP1** probe 21585-L29737		**Exon 15**	
238	**FOXP2** probe 21319-L29725			**Exon 10**
244 ◎	**FOXP1** probe 21320-L29726		**Exon 4**	
251 ◎	**FOXP1** probe 21321-L29727		**Exon 5**	
256	**FOXP1** probe 21322-L30058		**Exon 18**	
263	Reference probe 20981-L30218	5q		
267	**FOXP1** probe 21323-L29729		**Exon 16**	
274	**FOXP2** probe 21324-L29730			**Exon 15**
280	**FOXP1** probe 21325-L29731		**Exon 13**	
286	**FOXP2** probe 21326-L30059			**Exon 12**
292	**FOXP1** probe 21327-L29733		**Exon 17**	
300 ◎	**FOXP2** probe 21328-L29734			**Exon 1**
305	**FOXP2** probe 21329-L30060			**Exon 4**
313	Reference probe 18735-L30158	2q		
320	**FOXP2** probe 21330-L29736			**Exon 14**
328	**FOXP1** probe 21584-L29724		**Exon 6**	
337	**FOXP2** probe 21529-L29738			**Exon 16**
346 ◎	**FOXP1** probe 21333-L29739		**Exon 1**	
355	**FOXP1** probe 21334-L29740		**Exon 11**	
364	**FOXP2** probe 21335-L29741			**Exon 5**
373	**FOXP2** probe 21336-L29742			**Exon 7**
383	Reference probe 21008-L29226	8q		
391 «	**FOXP1** probe 21337-L29743		**Exon 9**	
400	**FOXP1** probe 18497-L24542		**Exon 21**	
409	**FOXP1** probe 21338-L29744		**Exon 6**	
418	**FOXP2** probe 21339-L29745			**Exon 4**
427	**FOXP2** probe 21340-L29746			**Exon 17**
436 ◎	**FOXP1** probe 21341-L29747		**Exon 3**	
445	Reference probe 16286-L18578	13q		
454	**FOXP2** probe 21342-L29748			**Exon 8**
463 «	**FOXP1** probe 21343-L29749		**Exon 8**	
472	**FOXP2** probe 21344-L29750			**Exon 18**

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*FOXP1*	*FOXP2*
484	**FOXP1** probe 21345-L29751		**Exon 7**	
493	**FOXP2** probe 21346-L29752			**Exon 11**
500 ๑	**FOXP1** probe 21347-L29753		**Exon 3**	
509	Reference probe 18539-L30161	17q		

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

« 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.
๑ The significance of *FOXP1* exon 1-5, and *FOXP2* exon 1 deletions is not clear as these exons are non-coding and alternative transcript variants using other transcription start sites are known.

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. P475-A1 probes arranged according to chromosomal location

Table 2a. *FOXP1* gene

Length (nt)	SALSA MLPA probe	*FOXP1* exon[a]	Ligation site NM_032682.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
346 ๑	21333-L29739	Exon 1	10 nt after exon 1	GGGTGCGTGAAG-CGGAGAGGGGAC	2.2 kb
148 ๑	21306-L29712	Exon 2	5 nt before exon 2	TTTCTTGTCGAC-TGCAGAACCCGA	88.2 kb
436 ๑	21341-L29747	Exon 3	292-291 *reverse*	CAAATCAGAGAA-GGGATCGAAGCC	0.1 kb
500 ๑	21347-L29753	Exon 3	348-intron 3	CTAGCCAAAAGG-GTGAGTGAATGA	134.2 kb
244 ๑	21320-L29726	Exon 4	373-374	GTTGCAGTCCTG-TGGCATTATGCA	0.1 kb
130 ๑	21303-L29709	Exon 4	30 nt after exon 4	TGATGCTGTATA-CCACCTAAAAAT	59.2 kb
251 ๑	21321-L29727	Exon 5	52 nt before exon 5	CCTTGGCAGAGG-TGGTTGCTACTT	0.1 kb
160 ๑	21308-L29714	Exon 5	469-470	TTTTGAGGTGAC-TATAACTGAAGA	101.5 kb
		start codon	*516-518 (Exon 6)*		
409	21338-L29744	Exon 6	542-541 *reverse*	CCGTTACTTTTT-GTCTCAGTCCCA	0.1 kb
328	21584-L29724	Exon 6	651-652	CGGCCGTGGACA-TCGGGGCAGCTG	85.7 kb
484	21345-L29751	Exon 7	773-774	TCTCCCAAGAGG-AATGACAAACAA	0.1 kb
214	21316-L29722	Exon 7	97 nt after exon 7	GCAAATCATGTA-AGAAAATCTTCT	58.8 kb
463 «	21343-L29749	Exon 8	924-923 *reverse*	CTGTTGAAGCAT-GAGGGCCTGCTG	1.1 kb
391 «	21337-L29743	Exon 9	993-994	TTCAACTTTTAC-AACAACAACATG	5.5 kb
142	21305-L29711	Exon 10	1052-1053	ACCCAGCAGTTG-GCTTTTCAGCAG	5.7 kb
355	21334-L29740	Exon 11	1312-1313	TGCACCTTCCAA-GACCTCCTTAAT	25.8 kb
226	21317-L29723	Exon 12	1463-1464	TGTGAAGCAGTG-TGCGAAGATTTC	14.6 kb
280	21325-L29731	Exon 13	1539-1540	CAGCCCAATGTA-GAGTACAAATGC	13.0 kb
166	21309-L29715	Exon 14	1631-1632	CTGCATGTGAAG-TCTACAGAACCC	10.1 kb
232	21585-L29737	Exon 15	1786-1787	CCCCTCTGTCAT-CACAACCACCAG	0.3 kb
267	21323-L29729	Exon 16	1942-1943	TTTAATTAGGCA-GGTAAGTAAATA	0.7 kb
292	21327-L29733	Exon 17	2041-2040 *reverse*	AGCTTACCTTCC-ACGTGGCCGCGT	4.3 kb
256	21322-L30058	Exon 18	2072-2071 *reverse*	AAACACTTGTGA-AGACTAAGATTA	1.9 kb
208	21315-L29721	Exon 19	2235-2236	ATGCAGCTTTAC-AGGTAAGATTAA	4.8 kb
178	21310-L29716	Exon 20	2381-2382	GACAGCAGTCCA-GGCAGATCTCCT	6.6 kb
400	18497-L24542	Exon 21	2518-2519	CAGAGATTACGA-AGATGAACCAGT	
		stop codon	*2547-2549 (Exon 21)*		

Table 2b. *FOXP2* gene

Length (nt)	SALSA MLPA probe	*FOXP2* exon[a]	Ligation site NM_148898.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
300 ◎	21328-L29734	Exon 1	340-341	CTGGGACGTGAT-CGGGCAGAGGTG	11.4 kb
		start codon	*557-559 (Exon 2)*		
201	21314-L30057	Exon 2	585-586	GACAGAGACAAT-AAGCAACAGTTC	108.1 kb
190	21312-L29718	Exon 3	767-768	AGCAGCAAACAA-GTGGATTGAAAT	0.1 kb
154	21307-L29713	Exon 3	34 nt after exon 3 *reverse*	AACATCTTTTAA-AATAAGACCCCA	36.1 kb
418	21339-L29745	Exon 4	847-848	TTATGTATCTGT-GGCCACTCTTCT	0.1 kb
305	21329-L30060	Exon 4	28 nt after exon 4	AATTAGAAATTC-AGCTACTAGGCA	57.7 kb
364	21335-L29741	Exon 5	972-973	ACAAGTCCTGTC-TCCTCAGCAGCT	1.2 kb
195	21313-L29719	Exon 6	1036-1037	CAGCAACAACTA-CAAGAGTTTTAC	1.9 kb
373	21336-L29742	Exon 7	1359-1360	GGGACTCATCTC-CATTCCACCTGG	10.8 kb
454	21342-L29748	Exon 8	1452-1453	GAAAGAAGTGAC-TGGAGTTCACAG	2.3 kb
136	21304-L29710	Exon 9	1663-1662 *reverse*	CAAACTCCATGG-CCATAGAGAGTG	7.6 kb
238	21319-L29725	Exon 10	1811-1812	AGTTAGAAATAC-AGGTTTGTTAAA	1.6 kb
493	21346-L29752	Exon 11	1825-1826	CTTTCTAAAGAA-CGCGAACGTCTT	4.3 kb
286	21326-L30059	Exon 12	2033-2034	TCACCCCAGCCA-GTGTGCCCAATG	1.2 kb
184	21311-L29717	Exon 13	2169-2168 *reverse*	TTACCTGCCTTA-TGAGAGTTGCAT	0.2 kb
320	21330-L29736	Exon 14	2191-2192	ATCATGGAGTCA-TCTGACAGGCAG	2.6 kb
274	21324-L29730	Exon 15	2392-2393	AGGTCACAAAAG-ATAACAGGGTAT	1.3 kb
337	21529-L29738	Exon 16	2462-2463	CTCTTAATGCCA-GTTTGCAGGTAA	0.9 kb
427	21340-L29746	Exon 17	2583-2582 *reverse*	TGCTGTCAATGT-GATCCAGAGAAC	25.5 kb
		stop codon	*2777-2779 (Exon 18)*		
472	21344-L29750	Exon 18	2784-2785	GGAATGAGAACT-GACTTGTGAAAC	

[a] See section Exon numbering on page 1 for more information.
[b] Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
◎ The significance of *FOXP1* exon 1-5, and *FOXP2* exon 1 deletions is not clear as these exons are non-coding and alternative transcript variants using other transcription start sites are known.

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

- P343 Autism-1: contains probes for genes implicated in autism, such as *UBE3A*, *GABRB3* and *SHANK3*.

References

- Bacon C and Rappold GA. (2012). The distinct and overlapping phenotypic spectra of FOXP1 and FOXP2 in cognitive disorders. *Hum Genet*. 131:1687-1698.
- 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.

P475 product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-01 – 09 June 2021 (04P) - Product description rewritten and adapted to a new template. - Removed a remark on probe variability from Table 1 and Table 2. - Ligation sites of the probes targeting the *FOXP1* and *FOXP2* genes updated according to new versions of the NM_ reference sequences. Version 01 – 20 September 2017 (55) - Not applicable, new document.

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