Product Description
SALSA® MLPA® Probemix P075-B2 TCF4-FOXG1

To be used with the MLPA General Protocol.

Version B2
For complete product history see page 8.

Catalogue numbers:
- **P075-025R:** SALSA MLPA Probemix P075 TCF4-FOXG1, 25 reactions.
- **P075-050R:** SALSA MLPA Probemix P075 TCF4-FOXG1, 50 reactions.
- **P075-100R:** SALSA MLPA Probemix P075 TCF4-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.

General information
The SALSA MLPA Probemix P075 TCF4-FOXG1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *TCF4* and *FOXG1* genes, which are associated with Pitt-Hopkins syndrome (PTHS).

PTHS is a rare disorder characterized by severe intellectual disability, pervasive developmental delay, 'atypical' autistic characteristics, and hyperventilation. PTHS is caused by heterozygous hypomorphic or null mutation or deletion of the transcription factor 4 (*TCF4*; *E2-2*; *ITF2*) gene on human chromosome 18. The *TCF4* gene is also a risk factor with highly significant linkage to schizophrenia, presumably via overexpression of the *TCF4* gene product in the central nervous system.

Another disorder that has some phenotypical overlap with PTHS is the congenital variant of Rett syndrome. This syndrome occurs almost exclusively in females and is characterized by hypotonia and intellectual disability from the very first months of life. It has been reported that the main cause of the congenital variant of Rett syndrome is mutations in the *FOXG1* gene, which encodes a transcription factor of the forkhead family on chromosome 14.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK100240/.

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 *TCF4* and *FOXG1* exon numbering used in this P075-B2 TCF4-FOXG1 product description is the exon numbering from the NG_011716.2 and NG_009367.1 sequences, respectively. 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 P075-B2 TCF4-FOXG1 contains 50 MLPA probes with amplification products between 130 and 485 nucleotides (nt). This includes 35 probes for the *TCF4* gene covering all exons, with additional probes for exons 1, 4, 5, 6, 9, 11, and 20, and four intronic probes and four probes located upstream of *TCF4*, most of them detecting exons of other transcript variants. Furthermore, five probes for the *FOXG1* are included, two probes upstream of the gene and three probes targeting exon 1. 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 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 Pitt-Hopkins syndrome or Rett syndrome. 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

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 *FOXG1* 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 *TCF4* and *FOXG1* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P075 TCF4-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.

LOVD mutation database

https://databases.lovd.nl/shared/genes/. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (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 *TCF4* exons 13 and 15 but not exon 14) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P075-B2 TCF4-FOXG1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*TCF4*	*FOXG1*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 00797-L13645	5q		
138	**TCF4 probe** 13335-L14761		**Exon 4**	
143	**TCF4 probe** 13334-L26942		**Exon 14**	
148	**TCF4 probe** 13337-L14763		**Exon 18**	
154	Reference probe 08375-L08229	15q		
160	**TCF4 probe** 13336-L14762		**Exon 20**	
166	**TCF4 probe** 17729-L21843		**Upstream**	
172	Reference probe 11007-L11678	4q		
178	**TCF4 probe** 16847-L19641		**Exon 5**	
184	**TCF4 probe** 17345-L20919		**Exon 15**	
190 «	**FOXG1 probe** 13756-L15243			**Upstream**
196	**TCF4 probe** 13333-L14759		**Exon 6**	
202	**TCF4 probe** 13339-L14765		**Exon 8**	
208 «	**TCF4 probe** 12506-L13556		**Exon 1**	
214	**TCF4 probe** 19805-L15889		**Exon 4**	
220	**TCF4 probe** 13342-L15879		**Exon 16**	
226 «	**TCF4 probe** 17730-L21844		**Upstream**	
232	Reference probe 09641-L26943	17q		
238	**TCF4 probe** 13326-L14752		**Exon 10**	
247 «	**FOXG1 probe** 17346-L20920			**Exon 1**
256	**TCF4 probe** 13324-L14750		**Exon 6**	
264	Reference probe 09265-L10877	10q		
274 «	**FOXG1 probe** 13755-L15242			**Exon 1**
283	**TCF4 probe** 13346-L14772		**Exon 7**	
292	**TCF4 probe** 13325-L14751		**Exon 11**	
301	**TCF4 probe** 17731-L21845		**Upstream**	
310 «	**TCF4 probe** 13341-L14767		**Exon 1**	
316 Ж Ø «	**TCF4 probe** 19601-SP0845-L26944		**Intron 2**	
322	**TCF4 probe** 13345-L26945		**Exon 17**	
328	Reference probe 08881-L08937	7q		
335 «	**FOXG1 probe** 16850-L27389			**Upstream**
342 Ø	**TCF4 probe** 19602-L27390		**Intron 6**	
350 «	**TCF4 probe** 16851-L27392		**Exon 3**	
358	**TCF4 probe** 13327-L27395		**Exon 11**	
364	Reference probe 12656-L19936	16q		
372	**TCF4 probe** 16852-L19646		**Exon 19**	
379	**TCF4 probe** 19603-L26947		**Intron 5**	
386	**TCF4 probe** 13329-L26946		**Exon 12**	
391	**TCF4 probe** 12522-L13572		**Exon 20**	
401	**TCF4 probe** 13343-L14769		**Exon 5**	
409	**TCF4 probe** 13344-L14770		**Exon 9**	
418	Reference probe 06876-L05967	3p		
427 «	**TCF4 probe** 16853-L19647		**Exon 2**	
436	**TCF4 probe** 13340-L14766		**Exon 9**	
445	Reference probe 10667-L11249	6p		
454	**TCF4 probe** 19604-L26951		**Intron 8**	
461	**TCF4 probe** 13348-L26950		**Exon 13**	
468 «	**FOXG1 probe** 13754-L26949			**Exon 1**
476 Ж	**TCF4 probe** 17732-SP0544-L26948		**Upstream**	
485	Reference probe 13594-L22376	19p		

[a] See section Exon numbering on page 2 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.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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. P075-B2 probes arranged according to chromosomal location

Table 2a. *TCF4*

Length (nt)	SALSA MLPA probe	*TCF4* exon[a]	Ligation site NM_001083962.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*176-178 (Exon 2)*		
476 Ж	17732-SP0544-L26948	Upstream	NM_001243226.3; 97-98 and 130-131	TAGCACAGGTGC--33 nt spanning oligo-CAAATTACTCAA	0.1 kb
301	17731-L21845	Upstream	NM_001243226.3; 235-236	GCTACTCGAGCT-TCTCCAGGAGGC	4.0 kb
166	17729-L21843	Upstream	NM_001243226.3; 315 nt before exon 2	CTAAGGCACTCA-CCTACTCTCAGA	41.9 kb
226 «	17730-L21844	Upstream	NM_001243227.2; 46-45, reverse	TCCCTGAAAGAT-ACATTGTAATCC	1.2 kb
208 «	12506-L13556	Exon 1	338 nt before exon 1	CCGAGGGATGCA-ACGGGCAAAAAC	0.3 kb
310 «	13341-L14767	Exon 1	35 nt before exon 1, reverse	CTCTTAACACCA-ACTCTCTTCTCC	1.2 kb
427 «	16853-L19647	Exon 2	226-225, reverse	TCCAGTAAATCA-CTCAGCTCTTTG	1.1 kb
316 Ж Ø «	19601-SP0845-L26944	Intron 2	698 nt and 668 nt before exon 3	CCCTAGGCAGGC--30 nt spanning oligo-CTTTCTCCATTC	0.7 kb
350 «	16851-L27392	Exon 3	305-306	TGGCAAGTGGAC-ATTTTACTGGCT	121.0 kb
214	19805-L15889	Exon 4	147 nt before exon 4, reverse	AGTGGCTTCTGA-CCCATCTACTTA	0.2 kb
138	13335-L14761	Exon 4	14 nt after exon 4, reverse	TGGGAGAAAAGA-TTAGATATACTT	2.8 kb
401	13343-L14769	Exon 5	130 nt before exon 5	GATTCCTTCTAG-TGAAGTTCCAGG	0.2 kb
178	16847-L19641	Exon 5	446-447	CACATGACAATC-TCTCTCCACCTT	38.8 kb
379	19603-L26947	Intron 5	18.8 kb before exon 6 (NM_001243233.2; 20-21)	GCCACAACAGTT-TATTCATCCACA	18.7 kb
196	13333-L14759	Exon 6	100 nt before exon 6	CCTACTTTACGT-ATGTAAACATCG	0.1 kb
256	13324-L14750	Exon 6	512-513	ACTCATCTTATG-GGAGAGAATCAA	1.3 kb
342 Ø	19602-L27390	Intron 6	1.3 kb after exon 6	ACAGTGCTTGGT-TAAGAGCTCCTG	51.2 kb
283	13346-L14772	Exon 7	636-635, reverse	TTCGGGGATTAT-TGCTAGAATACT	0.5 kb
202	13339-L14765	Exon 8	703-702, reverse	AAACCTGGAGGA-ACTTTTCGAACT	28.7 kb
454	19604-L26951	Intron 8	28.7 kb after exon 8 (NM_001243234.2; 119-120)	ACTGCGCATACA-CAATCCCGGGCA	42.1 kb
436	13340-L14766	Exon 9	740-741	ATGCTCCATCAG-CAAGCACTGCCG	0.1 kb
409	13344-L14770	Exon 9	8 nt after exon 9	CAAGGTAAGATG-CTGCTGCTTCTG	3.9 kb
238	13326-L14752	Exon 10	919-920	TCTTCTCATATT-CCACAGTCCAGC	5.8 kb
292	13325-L14751	Exon 11	1020-1021	TCCGATGTCCAC-TTTCCATCGTAG	0.1 kb
358	13327-L27395	Exon 11	18 nt after exon 11	CACAGAAATGCC-AATTCTGATACC	8.3 kb
386	13329-L26946	Exon 12	1162-1161, reverse	TTCCTCACCGAA-GCAAGTGCTTTC	1.6 kb
461	13348-L26950	Exon 13	17 nt after exon 13	GTATTTCAAATC-CCATTTCATCAT	2.5 kb
143	13334-L26942	Exon 14	1247-1248	TAATATCAGCAG-GCACAGCTGTTT	2.8 kb

Length (nt)	SALSA MLPA probe	*TCF4* exon[a]	Ligation site NM_001083962.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
184	17345-L20919	Exon 15	1420-1419, reverse	TGCATGTCCCCA-TGACCACCAGGC	20.0 kb
220	13342-L15879	Exon 16	1609-1610	CCACAGCTTCCT-GTCCAGTCTGCG	1.9 kb
322	13345-L26945	Exon 17	60 nt before exon 17	GCAGCCTTGCAA-TCTGGTGTGCAG	3.7 kb
148	13337-L14763	Exon 18	1827-1828	GTCCCACAGCAA-TAATGACGATGA	0.8 kb
372	16852-L19646	Exon 19	2156-2157	CACACCCTGGAA-TGGGAGACGCAT	0.5 kb
160	13336-L14762	Exon 20	2467-2468	ACAGGCTGAGAC-ACAGCCCAGAGA	0.6 kb
391	12522-L13572	Exon 20	3028-3029	CCTGTAGTGCCA-ACTCTGCTTCCA	
		stop codon	*2189-2191 (Exon 19)*		

Table 2b. *FOXG1*

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)*		
335 «	16850-L27389	Upstream	559 nt before exon 1	AAATGCCAGACA-CTGGCCTGCAAG	0.2 kb
190 «	13756-L15243	Upstream	349 nt before exon 1	GAGGAAGCCGGA-AATGTGAGCTAT	1.9 kb
247 «	17346-L20920	Exon 1	1508-1509	CCAGCCACCCCA-TGCCCTACAGCT	0.4 kb
274 «	13755-L15242	Exon 1	1907-1906, reverse	GAAATAATCAGA-CAGTCCCCCAGA	0.2 kb
468 «	13754-L26949	Exon 1	2101-2102	TCTAGGGTTGTT-TATTATTCTAAC	
		stop codon	*1961-1963 (Exon 1)*		

[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.

« 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.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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

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

Related products

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
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P075 product history	
Version	*Modification*
B2	Two reference probes have been replaced and one probe length has been adjusted.
B1	One *FOXG1* probe is removed.
A1	First release.

Implemented changes in the product description
Version B2-04 – 27 May 2026 (04P) - Section related products replaced with link to the website. *Version B2-03 – 25 November 2024 (04P)* - The term 'mental retardation' is considered outdated and was updated to 'intellectual disability' where appropriate. *Version B2-02 – 10 November 2021 (04P)* - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *TCF4* gene updated according to new version of the NM_ sequences. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

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