Product Description
SALSA® MLPA® Probemix P479-A1 TCF12 - ERF

To be used with the MLPA General Protocol.

Version A1
New product.

Catalogue numbers:
- **P479-025R:** SALSA MLPA Probemix P479 TCF12 - ERF, 25 reactions.
- **P479-050R:** SALSA MLPA Probemix P479 TCF12 - ERF, 50 reactions.
- **P479-100R:** SALSA MLPA Probemix P479 TCF12 - ERF, 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 P479 TCF12 - ERF is a **research use only (RUO)** assay for the detection of deletions or duplications in the *TCF12* and *ERF* genes, which are associated with craniosynostosis.

Craniosynostosis is a clinically and genetically heterogeneous condition with a prevalence of ~1 in 2200 births. Several genes have been identified as a cause of craniosynostosis including *FGFR1*, *FGFR2*, *FGFR3*, *TWIST1*, *EFNB1*, *MSX2*, *ALX4*, *ZIC1*, *SMAD6* and, more recently, *TCF12* and *ERF*. Craniosynostosis is inherited in an autosomal dominant manner and is characterised by abnormal skull growth involving premature fusion of the cranial sutures. As a result, the growth velocity of the skull often cannot match that of the developing brain. This produces skull deformity and, in some cases, raises intracranial pressure, which must be treated promptly to avoid permanent neurodevelopmental disability. More information on *TCF12*-related and *ERF*-related craniosynostosis can be found in OMIM #615314 and #600775, respectively.

The *TCF12* gene spans ~371 kb of genomic DNA and is located on 15q21.3, 55 Mb from the p-telomere. The *TCF12* gene encodes a transcriptional regulator that belongs to the basic helix-loop-helix family. Approximately 10% of all craniosynostoses is caused by heterozygous point mutations in the *TCF12* gene (Sharma et al. 2013). Large rearrangements involving the complete *TCF12* gene have been described. Intragenic deletions and a duplication in the *TCF12* gene were reported in craniosynostosis patients (Goos et al. 2016).

The *ERF* gene spans ~8 kb of genomic DNA and is located on 19q13.2, 47 Mb from the p-telomere. The *ERF* gene encodes a transcriptional repressor that belongs to the ETS transcription factors family. No mutations or rearrangements involving the *ERF* gene have been reported so far. However, it has been shown that reduced dosage of *ERF* causes complex craniosynostosis in humans and mice (Twigg et al. 2013).

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 *TCF12* exon numbering used in this P479-A1 TCF12 - ERF product description is the exon numbering from the NG_033851.2 sequence. The *ERF* exon numbering used in this P479-A1 TCF12 - ERF product description is the exon numbering from the NG_042802.1 sequence. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the 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 P479-A1 TCF12 - ERF contains 40 MLPA probes with amplification products between 130 and 452 nucleotides (nt). This includes 25 probes for the *TCF12* gene (one probe for each exon and two probes for exons 4, 5, 6 and 8) and five probes for the *ERF* gene (one probe for each exon and one probe for intron 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 craniosynostosis. 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 number NA03184 from the Coriell Institute has been tested with this P479-A1 probemix at MRC Holland and can be used as a positive control sample to detect a heterozygous duplication of the *TCF12* gene. 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. 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 *TCF12* and *ERF* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P479 TCF12 - ERF.
- 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.

***TCF12* and *ERF* mutation databases**

https://databases.lovd.nl/shared/genes/TCF12 and https://databases.lovd.nl/shared/genes/ERF. 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 *TCF12* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P479-A1 TCF12 - ERF

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*TCF12*	*ERF*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 00797-L00463	5q		
136	**TCF12** probe 21700-L30358		**Exon 8**	
142 ◎	**ERF** probe 21701-L30359			**Exon 1**
148	Reference probe 20517-L28107	1q		
160	**TCF12** probe 21702-L30360		**Exon 7**	
167 Ø	**ERF** probe 21703-L30361			Intron 1
172	**TCF12** probe 21704-L30362		**Exon 8**	
178	Reference probe 05354-L04733	11p		
184	**TCF12** probe 21705-L30363		**Exon 10**	
190	**TCF12** probe 21706-L30730		**Exon 13**	
196	**TCF12** probe 21707-L30365		**Exon 4**	
202	**TCF12** probe 21708-L30366		**Exon 6**	
208	**TCF12** probe 21709-L30367		**Exon 4**	
214	Reference probe 21316-L29722	3p		
220	**TCF12** probe 21710-L30368		**Exon 14**	
230	**TCF12** probe 21711-L30369		**Exon 17**	
238	**ERF** probe 21712-L30370			**Exon 2**
247	**TCF12** probe 21928-L30731		**Exon 20**	
256	**TCF12** probe 21714-L30372		**Exon 15**	
265	**TCF12** probe 21715-L30373		**Exon 3**	
274	**TCF12** probe 21716-L30374		**Exon 16**	
283	**TCF12** probe 21717-L30375		**Exon 18**	
291	Reference probe 19968-L27030	4p		
298	Reference probe 17265-L26123	6q		
308	**TCF12** probe 21718-L30376		**Exon 2**	
319	**TCF12** probe 21719-L30377		**Exon 12**	
329	**ERF** probe 21720-L30378			**Exon 3**
346	**TCF12** probe 21721-L30379		**Exon 5**	
355	Reference probe 19319-L28443	7q		
364 ◎	**ERF** probe 21722-L30380			**Exon 4**
372	Reference probe 05953-L28763	2p		
382 ◎	**TCF12** probe 21929-L30732		**Exon 1**	
390	**TCF12** probe 21723-L30381		**Exon 6**	
400	**TCF12** probe 21724-L30382		**Exon 9**	
409	**TCF12** probe 21725-L30383		**Exon 19**	
418	**TCF12** probe 21726-L30384		**Exon 5**	
427	**TCF12** probe 21727-L30385		**Exon 11**	
436	Reference probe 12576-L17703	20p		
445 ◎	**TCF12** probe 21728-L30386		**Exon 21**	
452	Reference probe 19636-L26295	10p		

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

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.

◎ The significance of deletions of these sequences is not clear as they target non-coding parts of the gene.

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. P479-A1 probes arranged according to chromosomal location

Table 2a. *TCF12* gene

Length (nt)	SALSA MLPA probe	*TCF12* exon[a]	Ligation site NM_207037.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
382 ◎	21929-L30732	Exon 1	163-164	TGCGGGGCAAAG-TGAACCGAGCCG	1.1 kb
		start codon	*286-288 (Exon 2)*		
308	21718-L30376	Exon 2	302-303	TCCCCAGCAACA-ACGCATGGCCGC	1.1 kb
265	21715-L30373	Exon 3	392-391 *reverse*	TAGTTGGTCTAG-TTTTCCCACTAT	142.7 kb
208	21709-L30367	Exon 4	468-469	ACATCTTGGGGA-ACAAGTGGTCAA	0.1 kb
196	21707-L30365	Exon 4	35 nt after exon 4	ATTAAAGCTGTA-ATTTGGCAGACT	28.0 kb
346	21721-L30379	Exon 5	558-559	GACAGTCGATTA-GGAGCCCATGAA	0.1 kb
418	21726-L30384	Exon 5	10 nt after exon 5	GGGTAAGTTGGT-AATTCTCTGCAA	74.5 kb
390	21723-L30381	Exon 6	651-650 *reverse*	AATCCAGTATCT-CTGCTGTACAGG	0.1 kb
202	21708-L30366	Exon 6	58 nt after exon 6	TAAATAAAGGCT-CTATTAATAGAT	25.7 kb
160	21702-L30360	Exon 7	754-753 *reverse*	AGAGAATGAATA-GTATGCTGTCCC	5.4 kb
136	21700-L30358	Exon 8	106 nt before exon 8 *reverse*	CTTGTTTTCCAA-TATTTGGGAGTA	0.2 kb
172	21704-L30362	Exon 8	66 nt after exon 8	CGTATTACCTTG-CTACAAGGGTAC	33.3 kb
400	21724-L30382	Exon 9	912-913	CGTGAATCTCCT-AGTTATCCATCT	1.2 kb
184	21705-L30363	Exon 10	1071-1072	CACATGTCTCAA-TCCAGTAGTTAT	0.4 kb
427	21727-L30385	Exon 11	1147-1148	CAACAGACATAA-ACACGAGTCTTC	1.4 kb
319	21719-L30377	Exon 12	1313-1312 *reverse*	TCACAGATGCCA-AAGCCTTTCCAA	9.4 kb
190	21706-L30730	Exon 13	1396-1395 *reverse*	AAGCCTACCTGT-GAGAGGTGAAGG	7.8 kb
220	21710-L30368	Exon 14	1405-1406	TAACAGGTACCA-GTCAGTGGCCAA	1.1 kb
256	21714-L30372	Exon 15	1494-1495	GTTGAGCAGCAA-CTTCACGAGCAT	0.9 kb
274	21716-L30374	Exon 16	1664-1663 *reverse*	GGGATGGTCCCA-ATAAACTATGTA	8.8 kb
230	21711-L30369	Exon 17	1796-1797	CAATGGCAATCA-TTCAGTCCTGTC	1.0 kb
283	21717-L30375	Exon 18	1886-1887	GCAAAGTCAGTC-TGGAACTGTTGT	10.1 kb
409	21725-L30383	Exon 19	10 nt after exon 19 *reverse*	CATCTCGGCTGA-ACCTACTTACCT	9.2 kb
247	21928-L30731	Exon 20	2274-2275	GAGAGGAACCTT-AACCCCAAAGCA	4.5 kb
		stop codon	*2404-2406 (Exon 20)*		
445 ◎	21728-L30386	Exon 21	3205-3206	TGTGACCATAGC-CTAGCTAGCATT	

Table 2b. *ERF* gene

Length (nt)	SALSA MLPA probe	*ERF* exon[a]	Ligation site NM_006494.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
142 ◎	21701-L30359	Exon 1	281 nt before exon 1	TGCCGAAGACTT-AAGTTAGCTGCA	3.3 kb
		start codon	*130-132 (Exon 1)*		
167 Ø	21703-L30361	Intron 1	1511 nt before exon 2	GTTGAACACCTA-GATTCCCAAGAG	1.9 kb
238	21712-L30370	Exon 2	189 nt after exon 2	AGGAGAGATTAC-AGTCACCAGTGG	0.3 kb
329	21720-L30378	Exon 3	470-471	ACTGGTGCTGGT-CAATTACCCATT	1.7 kb
		stop codon	*1774-1776 (Exon 4)*		
364 ◎	21722-L30380	Exon 4	2115-2116	AGGAACTTTTCT-ATTACAATCGCT	

[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.

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.
◎ The significance of deletions of these sequences is not clear as they target non-coding parts of the gene.

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

- P054 FOXL2-TWIST1: Contains probes for the *TWIST1* gene, involved in craniosynostosis.
- P080 Craniofacial: Contains probes for multiple genes involved in craniofacial disorders: *FGFR1*, *FGFR2*, *FGFR3*, *TWIST1*, *MSX2*, *ALX1*, *ALX3*, *ALX4*, *EFNB1* and *RUNX2*.
- P133 Kallmann-2: Contains probes for the *FGFR1* gene, involved in craniosynostosis.
- P267 Dandy-Walker Malformation: Contains probes for the *ZIC1* gene, involved in craniosynostosis.

References

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- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat*. 28:205.
- Sharma VP et al. (2013). Mutations in TCF12, encoding a basic helix-loop-helix partner of TWIST1, are a frequent cause of coronal craniosynostosis. *Nat Genet*. 45:304-7.
- Twigg SRF et al. (2013). Reduced dosage of ERF causes complex craniosynostosis in humans and mice and links ERK1/2 signaling to regulation of osteogenesis. *Nat Genet*. 45:308-13.
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P479 product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-03 – 11 August 2021 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *TCF12* and *ERF* genes updated according to new versions of the NM_ reference sequences. *Version A1-02 – 04 May 2020 (01P)* - Length of the reference probe 17265-L26123 changed from 299 nt to 298 nt to better reflect the true probe lengths. *Version A1-01 – 01 March 2018 (01P)* - Not applicable, new document.

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