Product Description
SALSA® MLPA® Probemix P054-C3 FOXL2-TWIST1

To be used with the MLPA General Protocol.

Version C3
As compared to version C2, four reference probes have been replaced. For complete product history see page 8.

Catalogue numbers:
- **P054-025R:** SALSA MLPA Probemix P054 FOXL2-TWIST1, 25 reactions.
- **P054-050R:** SALSA MLPA Probemix P054 FOXL2-TWIST1, 50 reactions.
- **P054-100R:** SALSA MLPA Probemix P054 FOXL2-TWIST1, 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 P054 FOXL2-TWIST1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *TWIST1*, *FOXL2*, *FOXC1*, *FOXC2*, *ATR*, *PITX2*, *PISRT1* and *GPR143* (former *OA1*) genes, which are associated with ophthalmogenetic anomalies.

The protein encoded by the *TWIST1* gene may affect the transcription of fibroblast growth factor receptors (FGFRs), a gene family implicated in craniosynostosis. It is suggested that TWIST1 proteins also regulate cytokine signalling. The *TWIST1* gene is located on chromosome 7p21.1, contains two exons and spans only ~2.2 kb. Mutations in the *TWIST1* gene are the major cause of Saethre-Chotzen syndrome (SCS; OMIM 101400). It has been estimated that 23% of SCS patients have a deletion of one copy of the *TWIST1* gene. The majority of patients with a *TWIST1* deletion are also developmentally delayed, presumably due to haploinsufficiency of nearby genes. The P054-C3 FOXL2-TWIST1 probemix contains two probes for the *TWISTNB* (*POLR1F*) gene, located at a distance of ~580 kb from *TWIST1* in a relatively gene-poor region. There is no clear evidence yet, however, that a deletion of *TWISTNB* is the cause of the developmental delay. More information about SCS is available at https://www.ncbi.nlm.nih.gov/books/NBK1189/.

The *FOXL2* gene encodes a forkhead transcription factor. Mutations in *FOXL2* can cause blepharophimosis syndrome (BPES; OMIM 110100), an autosomal dominant syndrome characterised by eyelid malformation, sometimes associated with premature ovarian insufficiency (BPES type I), and sometimes not (BPES type II). The *FOXL2* gene is located on chromosome 3q22.3 and consists of a single 2.7 kb exon. Several *FOXL2* deletions have been identified. The deletions can span several Mb of chromosomal DNA and may extend to the *ATR* gene located 3.6 Mb telomeric of *FOXL2* (D'haene et al. 2010). Three probes for *ATR* are included in this probemix. The non-protein-coding *PISRT1* gene is located on 3q23 and consists of a single 0.5 kb exon. *PISRT1* is located 0.3 Mb telomeric from *FOXL2* and shares a common transcriptional regulatory region with *FOXL2*. More information about BPES is available at https://www.ncbi.nlm.nih.gov/books/NBK1441/.

Other genes detected by this probemix are *PITX2* (4q25, Axenfeld-Rieger syndrome; OMIM 180500), *GPR143* (Xp22.2; ocular albinism type I) and the forkhead transcription factors *FOXC1* (6p25.3) and *FOXC2* (16q24.1).

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 *TWIST1*, *FOXL2*, *FOXC1*, *FOXC2*, *ATR*, *PITX2*, *PISRT1* and *GPR143* exon numbering used in this P054-C3 FOXL2-TWIST1 product description is the exon numbering from the NG_008114.2, LRG_1295, LRG_1245, LRG_1292, LRG_1403, NG_007120.1, NR_027070.1 and NG_009074.1 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 P054-C3 FOXL2-TWIST1 contains 45 MLPA probes with amplification products between 130 and 490 nucleotides (nt). This includes six probes for the *TWIST1* gene (three probes for exon 1, two probes for exon 2, and one probe for the upstream region of the gene), nine probes for the *GPR143* (*OA1*) gene (one probe for each exon), two probes for the *PITX2* gene, three probes for the *ATR* gene, five probes for the *PISRT1* gene, three probes for the *FOXL2* gene (all targeting exon 1), and two probes each for the *FOXC1* and *FOXC2* genes (all targeting exon 1 of the respective gene). 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 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 ophthalmogenetic anomalies. 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 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 *TWIST1*, *FOXL2*, *FOXC1* and *FOXC2* 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 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 *TWIST1*, *FOXL2*, *FOXC1*, *FOXC2*, *ATR*, *PITX2* and *GPR143* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P054 FOXL2-TWIST1.
- 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 *GPR143* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P054-C3 FOXL2-TWIST1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]				
		Reference	*TWIST1*	*FOXL2*	*GPR143*	Other
64-105	Control fragments – see table in probemix content section for more information					
130	Reference probe 09988-L10447	7q22				
136 *	Reference probe 21700-L30358	15q21				
148 «	**FOXC1 probe** 02561-L02029					6p25.3
157	**GPR143 probe** 02974-L02405				**Exon 6**	
166	**PITX2 probe** 02978-L02409					4q25
172	**PISRT1 probe** 19186-L25212			Telomeric		
178	**GPR143 probe** 02972-L16214				**Exon 3**	
184 «	**FOXL2 probe** 01943-L01489			**Exon 1**		
190 *	Reference probe 22509-L31658	14q32				
196 «	**TWIST1 probe** 02079-L15512		**Exon 2**			
203	**PITX2 probe** 19187-L30295					4q25
211 «	**FOXL2 probe** 01944-L01490			**Exon 1**		
220	**GPR143 probe** 02975-L02406				**Exon 9**	
229 ¬	**TWISTNB probe** 02147-L01963		Centromeric			
238 «	**FOXL2 probe** 01945-L01491			**Exon 1**		
244	Reference probe 11436-L12165	1q41				
250 «	**TWIST1 probe** 04916-L16215		**Upstream**			
256	**ATR probe** 02557-L02020			Telomeric		
265	**GPR143 probe** 02565-L02026				**Exon 2**	
274 *	Reference probe 22235-L31350	22q13				
279 «	**TWIST1 probe** 02080-L16218		**Exon 1**			
286	**GPR143 probe** 03134-L16217				**Exon 1**	
295 ¬	**FERD3L probe** 19188-L25214		Centromeric			
301	**ATR probe** 02558-L02021			Telomeric		
310 *	Reference probe 22061-L31023	13q14				
319 «	**TWIST1 probe** 01969-L02364		**Exon 1**			
329	**PISRT1 probe** 19190-L25216			Telomeric		
337	Reference probe 21099-L29544	11p15				
346 «	**TWIST1 probe** 04915-L04301		**Exon 1**			
355 «	**FOXC2 probe** 02563-L02024					16q24.1
362 Ж	**GPR143 probe** 19192-SP0762-L25218				**Exon 4**	
373	**ATR probe** 02560-L02023			Telomeric		
382 «	**FOXC1 probe** 02562-L02030					6p25.3
391	Reference probe 04837-L04221	5p13				
400	**PISRT1 probe** 19193-L25219			Telomeric		
408 «	**FOXC2 probe** 20729-L30296					16q24.1
418	**GPR143 probe** 03135-L02404				**Exon 5**	
427	**GPR143 probe** 14491-L16219				**Exon 7**	
436	Reference probe 05426-L04836	17q21				
445 «	**TWIST1 probe** 19194-L25220		**Exon 2**			
454	**GPR143 probe** 19195-L25221				**Exon 8**	
463	**PISRT1 probe** 19196-L25222			Telomeric		
472 ¬	**TWISTNB probe** 19197-L25223		Centromeric			
481	**PISRT1 probe** 19198-L25224			Telomeric		
490	Reference probe 18601-L23958	2q33				

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

* New in version C3.
« 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.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

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. P054-C3 probes arranged according to chromosomal location

Table 2a. *TWIST1*

Length (nt)	SALSA MLPA probe	*TWIST1* exon[a]	Ligation site NM_000474.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
472 ¬	19197-L25223	*TWISTNB* Exon 1	240-241 (NM_001002926.2)	GGCATTCGAGAA-CAGCTTGATGCG	10.2 kb
229 ¬	02147-L01963	*TWISTNB* Exon 4	769-770 (NM_001002926.2)	AGCTAGCAGATG-ATGCAGATGACA	553.3 kb
295 ¬	19188-L25214	*FERD3L* Exon 1	114-115 (NM_152898.2)	ACTTCGTCGCAG-ACCTGTCCCTGG	26.0 kb
		start codon	*316-318 (Exon 1)*		
250 «	04916-L16215	Upstream	1683 nt before exon 1	GCTGGAGAAATA-ACACTCGCCCTC	1.7 kb
346 «	04915-L04301	Exon 1	17 nt before exon 1	AAGAGCCTCCAA-GTCTGCAGCTCT	0.5 kb
279 «	02080-L16218	Exon 1	532-531, reverse	CTTGCCGCGCTT-GCCCTGGGCCGG	0.3 kb
319 «	01969-L02364	Exon 1	823-824	ACGAGCTGGACT-CCAAGATGGCAA	0.8 kb
445 «	19194-L25220	Exon 2	1135-1134, reverse	ACTGTCCACGGG-CCTGTCTCGCTT	0.2 kb
196 «	02079-L15512	Exon 2	1300-1301	TCGTGCCAATCA-GCCACTGAAAGG	
		stop codon	*922-924 (Exon 1)*		

Table 2b. *GPR143* (*OA1*)

Length (nt)	SALSA MLPA probe	*GPR143* exon[a]	Ligation site NM_000273.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*31-33 (Exon 1)*		
286	03134-L16217	Exon 1	97-98	AGCTCGTGCTGA-GCTTCCAGCCGC	5.0 kb
265	02565-L02026	Exon 2	359-360	CCACACGGAAAT-TTGGCCTGCTGC	1.4 kb
178	02972-L16214	Exon 3	434-435	CTGGTGGCTGTT-TTGCTATGCAGT	10.8 kb
362 Ж	19192-SP0762-L25218	Exon 4	511-512; 535-536	ATCACATCATGG-24 nt spanning oligo-GTGTGGAGGGAG	2.5 kb
418	03135-L02404	Exon 5	649-650	TGCTGGTTCTCG-TGGCGAACCCCA	2.4 kb
157	02974-L02405	Exon 6	715-716	AAGGAAGACAAG-GCATTTACACGG	2.3 kb
427	14491-L16219	Exon 7	871-870, reverse	GACAGGTTTCAA-AGAACCTCCATT	1.7 kb
454	19195-L25221	Exon 8	924-925	CAGGGAATCCTG-AATCCAGCCCAG	13.9 kb
220	02975-L02406	Exon 9	1205-1206	CTGCAACAAAAA-TGAGGGTGACCC	
		stop codon	*1243-1245 (Exon 9)*		

Table 2c. *PITX2*

Length (nt)	SALSA MLPA probe	*PITX2* exon[a]	Ligation site NM_153427.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*1590-1592 (Exon 3)*		
203	19187-L30295	Intron 3	1102 nt before exon 4 (606-607 in NM_000325.6)	TAGAGCGTCGCG-CTCCCTCGTCCA	4.4 kb
166	02978-L02409	Exon 5	2480-2481	CTGGGAATTATG-CTAGAAGGTCGT	
		stop codon	*2403-2405 (Exon 5)*		

Table 2d. *ATR*, *PISRT1* and *FOXL2*

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		ATR	**NM_001184.4**		
		start codon	*30-32 (Exon 1)*		
256	02557-L02020	Exon 4	1065-1066	TAAAAGCAGCTT-TGTGCCATTTAC	38.2 kb
301	02558-L02021	Exon 22	4055-4056	CTTTTGAAAGGT-TGCCAAGATGCA	74.5 kb
373	02560-L02023	Exon 47	7814-7815	GTTCTTGACATT-GAGCAGCGACTA	3212.7 kb
		stop codon	*7962-7964 (Exon 47)*		
		PISRT1	**NR_027070.1**		
481	19198-L25224	Upstream	3336 nt before exon 1	AACTAATTTAAA-GAAGATTTGGGA	2.0 kb
463	19196-L25222	Upstream	1285 nt before exon 1	ACTCAGACACCA-AGGATCCTTGAG	1.2 kb
400	19193-L25219	Exon 1	109 nt before exon 1	GGCGATCAGATC-CTTTGGGTCTGC	0.3 kb
172	19186-L25212	Exon 1	214-215	ATAAGAATAATG-GCAAACACTGAT	2.7 kb
329	19190-L25216	Downstream	2372 nt after exon 1	CCCGTTTGCGGT-TTCTCTGGGACT	284.2 kb
		FOXL2	**NM_023067.4**		
		start codon	*416-418 (Exon 1)*		
184 «	01943-L01489	Exon 1	692-693	CGTTCTACGAGA-AGAATAAGAAGG	0.3 kb
211 «	01944-L01490	Exon 1	1016-1017	CTGGCTTCCTCA-ACAACTCGTGGC	0.5 kb
238 «	01945-L01491	Exon 1	1493-1494	TGCATTGCTCTT-ACTGGGACCACG	
		stop codon	*1544-1546 (Exon 1)*		

Table 2e. *FOXC1*

Length (nt)	SALSA MLPA probe	*FOXC1* exon[a]	Ligation site NM_001453.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*532-534 (Exon 1)*		
148 «	02561-L02029	Exon 1	1456-1457	GCCAGGGCTTCA-GCGTGGACAACA	0.6 kb
382 «	02562-L02030	Exon 1	2063-2062, reverse	TCTGTGACTCGA-ACATCTCCCGCA	
		stop codon	*2191-2193 (Exon 1)*		

Table 2f. *FOXC2*

Length (nt)	SALSA MLPA probe	*FOXC2* exon[a]	Ligation site NM_005251.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*508-510 (Exon 1)*		
355 «	02563-L02024	Exon 1	576-577	AGCGAGCAGAAT-TACTACCGGGCT	0.8 kb
408 «	20729-L30296	Exon 1	1330-1331	GCGTGGAGAACA-TCATGACCCTGC	
		stop codon	*2011-2013 (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.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

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.

References

- D'haene B et al. (2010). FOXL2 copy number changes in the molecular pathogenesis of BPES: unique cohort of 17 deletions. *Hum Mutat*. 31:E1332-E1347.
- 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 P054 FOXL2-TWIST1

- Aravidis C et al. (2014). A familial case of Muenke syndrome. Diverse expressivity of the FGFR3 Pro252Arg mutation–case report and review of the literature. *J Matern Fetal Neonatal Med*. 27:1502-1506.
- Bunyan DJ and Thomas NS (2019). Screening of a large cohort of blepharophimosis, ptosis, and epicanthus inversus syndrome patients reveals a very strong paternal inheritance bias and a wide spectrum of novel FOXL2 mutations. *Eur J Med Genet*. 62:103668.
- Castets S et al. (2020). Hypopituitarism in Patients with Blepharophimosis and FOXL2 Mutations. *Horm Res Paediatr*. 93:30-39.
- Hernández-Martínez N et al. (2018). Molecular characterization of Axenfeld-Rieger spectrum and other anterior segment dysgeneses in a sample of Mexican patients. *Ophthalmic Genet*. 39:728-734.
- Krepelova A et al. (2016). Analysis of FOXL2 detects three novel mutations and an atypical phenotype of blepharophimosis-ptosis-epicanthus inversus syndrome. *Clin Exp Ophthalmol*. 44:757-762.
- Lang E et al. (2020). Exome sequencing in a Swiss childhood glaucoma cohort reveals CYP1B1 and FOXC1 variants as most frequent causes. *Transl Vis Sci Technol*. 9:47.
- Siggs OM et al. (2019). Prevalence of FOXC1 variants in individuals with a suspected diagnosis of primary congenital glaucoma. *JAMA Ophthalmol*. 137:348-355.
- Souzeau, E et al. (2017). Glaucoma spectrum and age-related prevalence of individuals with FOXC1 and PITX2 variants. *Eur J Hum Genet*. 25:839-847.
- Wang X et al. (2018). Mutation survey of candidate genes and genotype–phenotype analysis in 20 Southeastern Chinese patients with Axenfeld–Rieger syndrome. *Curr Eye Res*. 43:1334-1341.

P054 product history	
Version	*Modification*
C3	Four reference probes have been replaced.
C2	Two reference probes have been added and two have been replaced. In addition, two probe lengths have been adjusted.
C1	Several target probes have been altered and probes for the *PISRT1* region have been added.
B2	Control fragments have been adjusted (QDX2).
B1	One *GPR143* probe has been added and one *ATR* and one *TWIST1* probe have been removed. The number of reference probes has been increased from three to eight. Finally, four extra control fragments have been included.
A1	First release.

Implemented changes in the product description
Version C3-01 – 21 July 2021 (04P) - Product description rewritten and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Ligation sites of the probes targeting the *TWIST1*, *GPR143*, *ATR*, *FOXL2*, *FOXC1* and *FOXC2* genes updated according to new version of the NM_ reference sequence. - Partial sequence of 472 nt probe 19197-L25223 and distances to next probe corrected in Table 2a. *Version 16 – 09 August 2017 (55)* - Product description adapted to a new product version (version number changed, lot number added, changes in Table 1 and Table 2, new picture included). - New references added on page 2. - Exon numbering of the *PITX2* gene has been changed. - Various minor textual and layout changes. *Version 15 – 10 January 2017 (55)* - Warning added in Table 1, 355 nt probe 02563-L02024 and 407 nt probe 02564-L02025. *Version 14 (53)* - Product description adapted to a new product version (version number changed, lot number added, changes in Table 1 and Table 2, new picture included).

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