Product Description
SALSA® MLPA® Probemix P153-B2 EYA1

To be used with the MLPA General Protocol.

Version B2
For complete product history see page 7.

Catalogue numbers:
- **P153-025R:** SALSA MLPA Probemix P153 EYA1, 25 reactions.
- **P153-050R:** SALSA MLPA Probemix P153 EYA1, 50 reactions.
- **P153-100R:** SALSA MLPA Probemix P153 EYA1, 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 P153 EYA1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *EYA1* gene, which is associated with Branchio-oto-renal dysplasia (BOR) syndrome.

BOR syndrome, an autosomal dominant disorder, affects an estimated 2% of profoundly deaf children. Besides deafness, it is also characterized by renal, head, and neck abnormalities. Defects in the *EYA1* gene are the cause of this syndrome. Complex genomic rearrangements account for a considerable fraction of all identified mutations in *EYA1* (Chang et al. 2004; Vervoort et al. 2002).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1380/.

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 *EYA1* exon numbering used in this P153-B2 EYA1 product description is the exon numbering from the NG_011735.3 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 P153-B2 EYA1 contains 28 MLPA probes with amplification products between 130 and 346 nucleotides (nt). This includes 18 probes for the *EYA1* gene, one probe for each exon. 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 branchio-oto-renal dysplasia 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 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 *EYA1* gene are small (point) mutations, none of which will not be detected by using SALSA MLPA Probemix P153 EYA1.
- 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.

***EYA1* mutation database**

https://databases.lovd.nl/shared/genes/EYA1. 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 *EYA1* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P153-B2 EYA1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*EYA1*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 18709-L24593	5q	
136	**EYA1 probe** 16901-L19771		**Exon 13**
143	Reference probe 13872-L27818	7p	
148	Reference probe 21005-L29223	13q	
154	**EYA1 probe** 04977-L28846		**Exon 3**
161	Reference probe 09787-L20010	15q	
171	**EYA1 probe** 04978-L28848		**Exon 4**
178	**EYA1 probe** 05666-L28847		**Exon 10**
189	**EYA1 probe** 16900-L19770		**Exon 8**
196	**EYA1 probe** 04985-L23260		**Exon 11**
202	Reference probe 12424-L13425	22q	
211	**EYA1 probe** 05348-L04365		**Exon 5**
220	**EYA1 probe** 16902-L19772		**Exon 16**
226	**EYA1 probe** 20828-L29016		**Exon 6**
234	**EYA1 probe** 20829-L28844		**Exon 9**
240	**EYA1 probe** 04981-L29017		**Exon 7**
247	**EYA1 probe** 20830-L29018		**Exon 14**
256	**EYA1 probe** 20831-L28721		**Exon 17**
267	Reference probe 17834-L22900	6q	
279	**EYA1 probe** 04988-L20303		**Exon 15**
286	Reference probe 07737-L21372	20q	
300	**EYA1 probe** 04976-L28726		**Exon 2**
307	**EYA1 probe** 20726-L29019		**Exon 12**
313	Reference probe 06580-L24038	2q	
322	**EYA1 probe** 04990-L28720		**Exon 18**
331	Reference probe 02833-L18063	21q	
337	**EYA1 probe** 16899-L19769		**Exon 1**
346	Reference probe 18927-L24522	1p	

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

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

Table 2. *EYA1* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*EYA1* exon[a]	Ligation site NM_000503.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*478-480 (Exon 3)*		
337	16899-L19769	Exon 1	376-377	CTAACCCAGAAA-ATTCGAAGGAAC	5.2 kb
300	04976-L28726	Exon 2	435-436	GTGCAAACATCT-CAAGCCAGTTCA	1.6 kb
154	04977-L28846	Exon 3	503-504	TCTAACCAGCCC-GCATAGCCGTCT	20.7 kb
171	04978-L28848	Exon 4	627-628	ATGAGCAGCAGT-GAAACAGCTTCA	11.9 kb
211	05348-L04365	Exon 5	706-707	GTTTCAGCCCAC-GACCAACTCACC	0.5 kb
226	20828-L29016	Exon 6	1 nt after exon 6	TTCCTCATATGG-TGAGTAACCTGC	4.1 kb
240	04981-L29017	Exon 7	985-986	ATGGCACAAGCT-TCAGTACCCCTC	17.9 kb
189	16900-L19770	Exon 8	1084-1083, reverse	AGAGGAATTTGT-GAGTGAATTATT	0.5 kb
234	20829-L28844	Exon 9	1192-1191, reverse	GCTGGTCATATA-ATGTGCTGGATA	27.4 kb
178	05666-L28847	Exon 10	1409-1410	CCGAAGAAACAA-TAATCCTTCACC	2.0 kb
196	04985-L23260	Exon 11	1478-1479	TGAGACAATCAT-TGTTTTCCACTC	25.1 kb
307	20726-L29019	Exon 12	1550-1551	TTCAGTTTCCCT-TGGACTGCGAAT	27.7 kb
136	16901-L19771	Exon 13	1672-1673	ACGGACAGGACC-TAAGGTGAGCAC	0.2 kb

Length (nt)	SALSA MLPA probe	*EYA1* exon[a]	Ligation site NM_000503.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
247	20830-L29018	Exon 14	1706-1707	AGATGGCTTTCC-TGCTGCAGCAAC	1.1 kb
279	04988-L20303	Exon 15	1862-1863	AGCTAAGAGGGA-AGCCTGGCTGCA	0.2 kb
220	16902-L19772	Exon 16	2002-2001, reverse	GACTTTCGCCAA-TGCTGGGATGAG	4.3 kb
256 #	20831-L28721	Exon 17	2136-2137	GTGTATGTTGTT-ATAGGAGATGGT	11.9 kb
322	04990-L28720	Exon 18	2298-2299	GCTCTGTGACCA-GGGACAGATCCA	
		stop codon	*2254-2256 (Exon 18)*		

[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.

This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

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

P163 GJB-WFS1	Contains probes for the *GJB2*, *GJB6*, *GJB3*, *WFS1* and *POU3F4* genes involved in prelingual deafness.
P280 SLC26A4	Contains probes for *SLC26A4* gene involved in DFNB4 and Pendred syndrome.
P461 STRC-CATSPER2-OTOA	Contains probes for the *STRC*, *CATSPER2* and *OTOA* genes associated with deafness-infertility syndrome, autosomal recessive deafness 16, and autosomal recessive deafness 22.

References

- Chang EH et al. (2004). Branchio-oto-renal syndrome: The mutation spectrum in EYA1 and its phenotypic consequences. *Hum mutat*, *23*(6), 582-589.
- 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.
- Vervoort VS et al. (2002). Genomic rearrangements of EYA1 account for a large fraction of families with BOR syndrome. *Eur J Hum Genet*., 10(11), 757.

Selected publications using SALSA MLPA Probemix P153 EYA1

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- Krug P et al (2011) Mutation screening of the EYA1, SIX1, and SIX5 genes in a large cohort of patients harboring branchio-oto-renal syndrome calls into question the pathogenic role of SIX5 mutations. *Hum Mutat*, 32(2):183-90.
- Masuda M et al. (2022). Phenotype–genotype correlation in patients with typical and atypical branchio-oto-renal syndrome. *Sci Rep*, 12(1), 1-10.
- Morisada N et al. (2010). Branchio-oto-renal syndrome caused by partial EYA1 deletion due to LINE-1 insertion. *Pediatr Nephrol*, 25(7), 1343-1348.
- Nozu K et al. (2018). Detection of copy number variations by pair analysis using next-generation sequencing data in inherited kidney diseases. *Clin Exp Nephrol*, 22(4), 881-888.

- Schmidt T et al. (2014) Branchio-Otic Syndrome Caused by a Genomic Rearrangement: Clinical Findings and Molecular Cytogenetic Studies in a Patient with a Pericentric Inversion of Chromosome 8. *Cytogenet Genome Res*, 142:1-6.
- Song M et al. (2013) Mutational Analysis of EYA1, SIX1 and SIX5 Genes and Strategies for Management of Hearing Loss in Patients with BOR/BO Syndrome. *PLoS ONE* 8(6):e67236.
- Stanton SG et al. (2014). X-linked hearing loss: two gene mutation examples provide generalizable implications for clinical care. *Am J Audiol*, 23(2), 190-200.
- Unzaki A et al. (2018). Clinically diverse phenotypes and genotypes of patients with branchio-oto-renal syndrome. *J Hum Genet*, 63(5), 647.

P153 product history	
Version	*Modification*
B2	One reference probe has been replaced.
B1	Probes for exon 6, 9 and 14 have been replaced. New probes for exon 1, 8, 13 and 16 have been added, and the second probes for exon 6, 9 and 10 removed. Four reference probes have been removed, and ten replaced.
A2	The 88, 96, 100 and 105 nt control fragments have been included.
A1	First release.

Implemented changes in the product description

Version B2-02 – 7 March 2022 (04P)
- Product description rewritten and adapted to a new template.

Version B2-01 — 18 September 2019 (02P)
- 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).
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.
- Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

Version 13 – 22 January 2016 (55)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).
- Various minor textual changes on page 1.
- Various minor layout changes.
- Ligation sites of the probes targeting the *EYA1* gene updated according to new version of the NM_reference sequence.

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