Product Description SALSA® MLPA® Probemix P328-A3 EYS

To be used with the MLPA General Protocol.

Version A3. As compared to version A2, two reference probes have been replaced and one reference probe added, in addition several probe lengths have been adjusted.

Catalogue numbers:
- **P328-025R:** SALSA® MLPA® probemix P328 EYS, 25 reactions.
- **P328-050R:** SALSA® MLPA® probemix P328 EYS, 50 reactions.
- **P328-100R:** SALSA® MLPA® probemix P328 EYS, 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.mlpa.com).

Certificate of Analysis: Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mlpa.com.

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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 P328-A3 EYS is a **research use only (RUO)** assay for the detection of deletions or duplications in the *EYS* gene.

The EYS gene (43 exons) spans ~2 Mb of genomic DNA, and is located on chromosome 6q12, ~64 Mb from the p-telomere.

Mutations in the *EYS* (eyes shut homolog) gene, originally designated as the RP25 locus on chromosome 6q12, are a major cause of autosomal recessive retinitis pigmentosa 25 (RP25). RP25 is characterised by progressive peripheral vision loss and night vision difficulties (nyctalopia) that can lead to central vision loss.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1417

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 exon numbering used in this P328-A3 product description is the exon numbering from the RefSeq transcript NM_001142800.2. The exon numbering and NM sequence used have been retrieved in 12/2019. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P328-A3 EYS contains 55 MLPA probes with amplification products between 128 and 500 nt. This P328-A3 contains one probe for each exon of the gene or in an intron close to an exon except for exon 9 and 27. Additionally, this probemix includes a probe for intron 11 and 27. Exon 12, 17 and 28 are covered by two probes, a normal copy number probe as well as a wildtype probe for three pathogenic mutations described in Abd El-Aziz M.M. et al. (2008. *Nat Genet.* 40(11):1285-1287). In addition, nine reference probes are included, that detect several different autosomal chromosomal locations.

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, 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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment 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.mlpa.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 unrelated individuals who are from families without a history of Retinitis Pigmentosa. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample 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.mlpa.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 dosage quotient (DQ) 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 DQ of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

- 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. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the *EYS* 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.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. 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: lower injection voltage / injection time settings, 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 *EYS* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P328.
- 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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.

***EYS* mutation database:** https://databases.lovd.nl/shared/genes/EYS. 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 SNPs and unusual results (e.g., a duplication of *EYS* exons 4 and 6 but not exon 5) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P328-A3 EYS probemix

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a] Reference	*EYS*
64-105	Control fragments – see table in probemix content section for more information		
128	Reference probe 00797-L00093	5q31	
131	**EYS probe** 13373-L20974		**Exon 16**
136	**EYS probe** 13374-L14831		**Exon 14**
142	**EYS probe** 13375-L14832		**Exon 11**
149	**EYS probe** 13376-L14833		**Exon 34**
154	**EYS probe** 13377-L14834		**Exon 13**
160	**EYS probe** 13378-L14835		**Exon 4**
166	**EYS probe** 13379-L14836		**Intron 7**
172 *	Reference probe 08222-L23547	10q26	
179	**EYS probe** 13381-L16559		**Intron 27**
184 §	**EYS probe** 13462-L14922		**WT for c.1971delT**
190	**EYS probe** 13382-L14839		**Exon 19**
196	**EYS probe** 13383-L14840		**Exon 41**
202	**EYS probe** 13384-L14841		**Exon 31**
208 ¥	**EYS probe** 13385-L32010		**Exon 22**
214	**EYS probe** 13386-L14843		**Exon 15**
220	**EYS probe** 13463-L14923		**Exon 17**
226	**EYS probe** 13387-L14844		**Exon 20**
232	Reference probe 11997-L21978	8p11	
238	**EYS probe** 14794-L14845		**Exon 35**
244	**EYS probe** 13389-L14846		**Exon 43**
250 ¥	**EYS probe** 13390-L32011		**Exon 29**
256	**EYS probe** 13391-L14848		**Exon 38**
263	**EYS probe** 17026-L22534		**Exon 5**
268	**EYS probe** 13392-L22535		**Exon 26**
274	**EYS probe** 13393-L14850		**Exon 40**
280 *	Reference probe 16907-L21371	12q24	
285 ¥	**EYS probe** 13394-L32012		**Exon 36**
292 ¥ §	**EYS probe** 14688-L32014		**WT for c.2710_2726del17**
298 ¥	Reference probe 03796-L32015	21q22	
301	**EYS probe** 13395-L20978		**Exon 25**
310	**EYS probe** 13396-L14853		**Exon 42**
319	**EYS probe** 20640-L29703		**Exon 37**
328	**EYS probe** 13398-L14855		**Exon 30**
333 Ж	**EYS probe** 17029-SP0435-L20979		**Exon 18**
341	**EYS probe** 13399-L20980		**Exon 21**
346	**EYS probe** 15831-L20981		**Exon 3**
352	**EYS probe** 13400-L20982		**Exon 8**
361	Reference probe 10086-L20983	8q22	
371	**EYS probe** 13403-L14860		**Intron 27**
379	**EYS probe** 17027-L21925		**Exon 6**
386	**EYS probe** 13404-L17499		**Exon 39**
392 §	**EYS probe** 13466-L14926		**WT for c.5857G>T**
400	**EYS probe** 13405-L14862		**Exon 23**
409	**EYS probe** 13467-L14927		**Exon 12**
419	**EYS probe** 13407-L14864		**Exon 33**
427	**EYS probe** 13408-L14865		**Intron 1**
436 Ж	**EYS probe** 17028-SP0434-L20084		**Exon 10**
445	Reference probe 11256-L11939	11q21	
454	**EYS probe** 15832-L17898		**Exon 1**
466 ¥	**EYS probe** 15640-L32016		**Exon 32**
472	**EYS probe** 13413-L14870		**Exon 24**
481	**EYS probe** 17030-L20086		**Intron 11**
494 *	Reference probe 21924-L31471	9p22	
500	Reference probe 10218-L14675	7q22	

a) See above section on exon numbering for more information.

* New in version A3 (from lot A3-1019 onwards).
¥ Changed in version A3 (from lot A3-1019 onwards). Small change in length, no change in sequence detected.
§ Wild type sequence detected. The presence of the c.1971delT mutation **or** a deletion of exon 12 will result in a decreased probe signal (184 nt). The presence of the c.2710_2726del17 mutation **or** a deletion of exon 17 will result in a decreased probe signal (292 nt). The presence of the c.5857G>T mutation close to the ligation site may **or** a deletion of exon 28 will result in a decreased probe signal (392 nt).
Ж This probe consists of three parts and has two ligation sites.

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

Table 2. *EYS* gene

Length (nt)	SALSA MLPA probe	*EYS* exon[a]	Ligation site NM_001142800.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		Start Codon	*540-542 (exon 4)*		
454	15832-L17898	Exon 1	72 nt after exon 1	TAGCTGAACACA-ACTACAAAGATC	66.7 kb
427	13408-L14865	Intron 1	415 nt before exon 2	GGTTTCCTGCCA-AGTTGAAGCCCT	144.5 kb
346	15831-L20981	Exon 3	40 nt after exon 3	AACCAGAACTTT-TCAGAATCAGGT	0.4 kb
160	13378-L14835	Exon 4	554-555	ACTGACAAATCA-ATCGTCATTCTG	4.8 kb
263	17026-L22534	Exon 5	24 nt after exon 5	TATGTATACAAC-CATATACACAAG	85.3 kb
379	17027-L21925	Exon 6	1495-1496	TTATACTTATGA-ATGCCCAAAAGG	3.2 kb
166	13379-L14836	Intron 7	419 nt after exon 7	AGCAGATAGATA-TTAGTTAGGGTC	17.6 kb
352	13400-L20982	Exon 8	1807-1808	TCTGAATGAAGA-ATGGTGTTTCAA	40.3 kb
	No probe	Exon 9			
436 Ж	17028-SP0434-L20084	Exon 10	2066-2065, 2036-2035 reverse	TAGGTTGCATCT-30nt spanning oligo-GCATCAATAACC	9.0 kb
142	13375-L14832	Exon 11	2202-2203	GGTATCTATGTT-TTCTCAGATGGG	5.6 kb
481 ^	17030-L20086	Intron 11	5.5 kb after exon 11 (NM_001142801.2; 5215-5216)	GCCAGTCTTGAA-ACCACTAGATCA	33.6 kb
184 §	13462-L14922	Exon 12	2510-2511	AATGGAACAACT-AGTACACATTTA	0.2 kb
409	13467-L14927	Exon 12	177 nt after exon 12	GGATTGCTTCTT-TGGGCAATTTAA	238.1 kb
154	13377-L14834	Exon 13	2674-2675	GCCTCCATTTAA-AGGTAATGAACA	60.0 kb
136	13374-L14831	Exon 14	2744-2743 reverse	AGGATGCAGTCA-TCAATGTCCTGT	51.8 kb
214	13386-L14843	Exon 15	2890-2891	CAAGAACAATTC-CACCTGTACTGA	33.2 kb
131	13373-L20974	Exon 16	3008-3009	AATGGAGGTCTT-TGTCATGAATCT	9.9 kb
220	13463-L14923	Exon 17	233 nt before exon 17	TCTCAGGGGATT-TAGTGGAGTATT	0.3 kb
292 ¥ §	14688-L32014	Exon 17	3263-3264	TGTGAAGATATG-GTCAACAATTTC	0.3 kb
333 Ж	17029-SP0435-L20979	Exon 18	3308-3309, 3344-3345	TTTTCTGGATCT-36nt spanning oligo-CCTTGCAAAAAT	15.4 kb
190	13382-L14839	Exon 19	3504-3505	CTGATGGATACA-ACTGCCTCTGTG	64.0 kb
226	13387-L14844	Exon 20	3633-3634	GTGACTGCAAGA-GTGGGTTTTTTG	1.0 kb
341	13399-L20980	Exon 21	3744-3745	GTTCATGTGATG-CAGATGGGACTA	8.1 kb
208 ¥	13385-L3210	Exon 22	3811-3812	TTGTATGAATGA-AGGCTTCTGTCA	187.6 kb
400	13405-L14862	Exon 23	132 nt after exon 23	GACTACTAATCA-AGTTTTAAGCAA	8.5 kb
472	13413-L14870	Exon 24	4147-4148	GCTTGAGTGCAT-TCCCAACTCATG	24.4 kb
301	13395-L20978	Exon 25	4416-4417	CAGTTGATCAAG-GTAAGTAGAAGT	2.0 kb
268	13392-L22535	Exon 26	5329-5330	AGCCAGCTGGTA-TGCACTAATGGG	153.5 kb
	No probe	Exon 27			
179	13381-L16559	Intron 27	1.3 kb before exon 28	ATTTTAGCACAA-AGCACATGTGAA	0.9 kb
371	13403-L14860	Intron 27	367 nt before exon 28	CTAGATTGTATT-AACCATTCTTTG	0.4 kb
392 §	13466-L14926	Exon 28	6399-6400	GTCCTGGTGAAG-CAAAATTTAAAA	47.5 kb
250 ¥	13390-L32011	Exon 29	6541-6542	ATCTATCAATCA-TGTACTCGGAAA	80.5 kb
328	13398-L14855	Exon 30	6711-6712	CAGTGAAAAACT-ATCACATTAACA	76.2 kb
202	13384-L14841	Exon 31	6799-6800	ACAAGGGGTTGA-TACCATGTGGAC	148.8 kb
466 ¥	15640-L32016	Exon 32	7004-7005	GGGAATTCCTAT-TTAGAACTGCCC	15.6 kb
419	13407-L14864	Exon 33	28 nt after exon 33	ACGGTAGTTCTT-TCGTGCTGAAAT	67.2 kb
149	13376-L14833	Exon 34	1 nt after exon 34	TTTGCATACCTA-CCTGAGAGGCAT	14.5 kb

			reverse		
238	14794-L14845	Exon 35	7446-7445 reverse	CCTGAACCCATA-AACAGGACCTGC	120.3 kb
285 ¥	13394-L32012	Exon 36	7736-7737	TGCCTCTGCCCA-TATGGGAGGTCT	58.0 kb
319	20640-L29703	Exon 37	9 nt after exon 37	ATGGTAAGATTA-ACTGAACCCTCT	17.1 kb
256	13391-L14848	Exon 38	12 nt after exon 38	GTAAAGAGTCAT-TCCTTCGTCACT	0.9 kb
386	13404-L17499	Exon 39	8209-8210	TGGCTACAGTGA-ATACACTCCAGA	10.1 kb
274	13393-L14850	Exon 40	8426-8427	ATAGAGAGTGGA-ACTAGTGTTTAG	15.4 kb
196	13383-L14840	Exon 41	8489-8490	GAGACAGTTTCT-ACCTGTGATCCT	35.8 kb
310	13396-L14853	Exon 42	133 nt before exon 42	AAGGTTTGATGT-ACTCACCTACAA	5.2 kb
244	13389-L14846	Exon 43	8932-8933	AGGCTACCTGGA-TCTAGATGGGAT	
		Stop Codon	*9972-9974 (exon 43)*		

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

* New in version A3 (from lot A3-1019 onwards).
¥ Changed in version A3 (from lot A3-1019 onwards). Small change in length, no change in sequence detected.
§ Wild type sequence detected. The presence of the c.1971delT mutation **or** a deletion of exon 12 will result in a decreased probe signal (184 nt). The presence of the c.2710_2726del17 mutation **or** a deletion of exon 17 will result in a decreased probe signal (292 nt). The presence of the c.5857G>T mutation close to the ligation site may **or** a deletion of exon 28 will result in a decreased probe signal (392 nt).
Ж This probe consists of three parts and has two ligation sites.
^ This probe detects the coding sequence of NM_001142801.1, which represents transcript variant 2 of the EYS gene.

Related SALSA MLPA probemixes

- P235 Retinitis pigmentosa: contains probes for the autosomal genes *IMPDH1*, *PRPF31*, *RHO* and *RP1*.
- P366 CHM-RP2-RPGR: contains probes for the *CHM*, *RP2* and *RPGR* genes, which are involved in X-linked retinitis pigmentosa.

References

- 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 P328 EYS

- Jespersgaard C et al. 2019. Molecular genetic analysis using targeted NGS analysis of 677 individuals with retinal dystrophy. *Sci Rep.* 9(1):1219. doi: 10.1038/s41598-018-38007-2.
- Coppieters F et al. (2014). Identity-by-descent–guided mutation analysis and exome sequencing in consanguineous families reveals unusual clinical and molecular findings in retinal dystrophy. G*enet in Med.* 16:671-680.

P328 Product history	
Version	*Modification*
A3	Two reference probes have been replaced and one reference probe added, in addition several probe lengths have been adjusted.
A2	Two reference probes have been replaced and one probe length has been adjusted.
A1	First release.

Implemented changes in the product description

Version A3-01 — 9 January 2020 (02P)
- Product description restructured and adapted to a new template.
- Product description adapted to a new product version.
- Changes in Table 1 and Ligation sites and exon numbers of the probes targeting the *EYS* gene are updated according to NM_001142800.2 in Table 2.
- Three probes have been designated Intronic probes while in previous product descriptions these were exonic probes (13379-L14836, 166 nt; 13403-L14860, 371 nt; 13408-L14865, 427 nt)

Version 06 – 23 March 2017 (55)
- Product description adapted to a product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).
- New reference added on page 1.
- Various minor textual and layout changes.

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