Product Description
SALSA® MLPA® Probemix P456-A1 EVC EVC2

To be used with the MLPA General Protocol.

Version A1
For complete product history see page 7.

Catalogue numbers:
- **P456-025R:** SALSA MLPA Probemix P456 EVC EVC2, 25 reactions.
- **P456-050R:** SALSA MLPA Probemix P456 EVC EVC2, 50 reactions.
- **P456-100R:** SALSA MLPA Probemix P456 EVC EVC2, 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 P456-A1 EVC EVC2 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *EVC* and *EVC2* genes, which are associated with Ellis-van Creveld syndrome.

The Ellis-van Creveld syndrome is a skeletal dysplasia characterized by short limbs and ribs, postaxial polydactyly and dysplastic nails and teeth. Additionally, congenital cardiac defects occur in 60% of affected individuals. The *EVC* and *EVC2* genes, located on chromosome 4p16.2 in a head-to-head configuration, encode transmembrane proteins that are involved in the Hedgehog signalling pathway, involved in cellular organization and morphology during embryogenesis. Mutations in the *EVC* or *EVC2* gene might also cause Weyers Acrodental Dysostosis, an autosomal dominant condition, having characteristics very similar to those of Ellis-van Creveld syndrome.

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 *EVC* and *EVC2* exon numbering used in this P456-A1 EVC EVC2 product description is the exon numbering from the NG_008843.1 and NG_015821.1 sequences. 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/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 P456-A1 EVC EVC2 contains 52 MLPA probes with amplification products between 122 and 500 nucleotides (nt). This includes 19 probes for the *EVC* gene, which is one probe for every

exon with the exception of exons 6 and 12. Furthermore, 23 probes for the *EVC2* gene, one probe for every exon and one probe upstream of exon 1 are include in P456-A1 EVC EVC2. 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-fragments (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 Ellis-van Creveld 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 *EVC* and *EVC2* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P456-A1 EVC EVC2.
- 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.

***EVC* and *EVC2* mutation databases**

https://databases.lovd.nl/shared/genes/ EVC2. 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 *EVC2* exons 11 and 13 but not exon 12) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P456-A1 EVC EVC2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*EVC*	*EVC2*
64-105	Control fragments – see table in probemix content section for more information			
122	Reference probe 02844-L02274	18q		
130	**EVC probe** 19963-L27025		**Exon 5**	
136	**EVC2 probe** 19976-L27038			**Exon 22**
142	**EVC probe** 19962-L27024		**Exon 20**	
148	**EVC2 probe** 19979-L27041			**Exon 9**
154	**EVC probe** 19965-L27774		**Exon 13**	
160	**EVC2 probe** 19970-L27032			**Exon 11**
166	**EVC2 probe** 19972-L28611			**Exon 21**
172	Reference probe 04948-L04334	1p		
178	**EVC probe** 19964-L27026		**Exon 14**	
184	**EVC probe** 19961-L27023		**Exon 9**	
189	**EVC2 probe** 19988-L27050			**Exon 7**
196	**EVC probe** 19957-L27019		**Exon 7**	
202	**EVC2 probe** 19992-L27054			**Exon 8**
208	**EVC probe** 19960-L27022		**Exon 18**	
215	Reference probe 06675-L06253	11p		
221	**EVC2 probe** 19987-L27049			**Exon 13**
226	**EVC2 probe** 19981-L27316			**Exon 2**
232	**EVC2 probe** 19986-L27317			**Exon 18**
238 «	**EVC probe** 19959-L27775		**Exon 1**	
244	Reference probe 13389-L14846	6q		
250	**EVC2 probe** 19985-L27047			**Exon 15**
256	**EVC2 probe** 19984-L27776			**Exon 5**
263	**EVC probe** 19966-L27028		**Exon 15**	
270	**EVC2 probe** 19993-L27320			**Exon 20**
276	**EVC2 probe** 19989-L27051			**Exon 6**
284	**EVC2 probe** 19971-L27033			**Exon 10**
292	**EVC probe** 19968-L27777		**Exon 8**	
299 «	**EVC probe** 19967-L27029		**Exon 2**	
307	Reference probe 19016-L24829	21q		
316	**EVC probe** 19969-L27031		**Exon 11**	
328	**EVC2 probe** 19975-L27037			**Exon 4**
341	**EVC probe** 19954-L27379		**Exon 4**	
351	**EVC2 probe** 19991-L27053			**Exon 16**
359 «	**EVC2 probe** 19977-L27039			**Upstream**
369	**EVC probe** 19956-L27018		**Exon 16**	
375	Reference probe 16748-L27324	22q		
382 «	**EVC2 probe** 20177-L27450			**Exon 1**
391	Reference probe 06672-L27891	10p		
398	**EVC2 probe** 19983-L27045			**Exon 14**
405	**EVC probe** 19955-L27017		**Exon 10**	
415	**EVC probe** 19953-L27015		**Exon 17**	
426	Reference probe 07719-L07429	7p		
436	**EVC2 probe** 19990-L27052			**Exon 19**
442	**EVC probe** 19952-L27014		**Exon 21**	
451	**EVC2 probe** 19973-L27035			**Exon 3**
459	Reference probe 18002-L22334	14q		
463	**EVC2 probe** 19978-L27040			**Exon 17**
476	**EVC2 probe** 19980-L27325			**Exon 12**
481	**EVC probe** 19949-L27011		**Exon 19**	
492	**EVC probe** 19950-L27012		**Exon 3**	
500	Reference probe 09682-L22509	3p		

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

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

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. P456-A1 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
EVC2 gene (NM_147127.5)					
		Stop Codon	*3971-3973 (Exon 22)*		
136	19976-L27038	Exon 22	3778-3779	TGGCCACACCTG-TCACTGGAGCCC	2.3 kb
166	19972-L28611	Exon 21	3649-3648 reverse	CTTATCAGATCT-CCTCGCAGTTTG	3.2 kb
270	19993-L27320	Exon 20	3488-3489	GTGTGGTACTGC-CCACAGCCTCAC	6.1 kb
436	19990-L27052	Exon 19	3389-3388 reverse	GCACAGGGTTGC-AAAGGTGTCTGC	1.6 kb
232	19986-L27317	Exon 18	3224-3225	GGATTCTGAACG-AACCTGGGGAGG	8.4 kb
463	19978-L27040	Exon 17	2996-2997	AGACTCTGTCGG-CCTACACCGCCC	30.7 kb
351	19991-L27053	Exon 16	2821-2822	AAGAAGTGCATC-GAAGACAAAATT	3.2 kb
250	19985-L27047	Exon 15	2572-2573	TCAGTCTTCTCC-CTGTCTGAAGAG	4.3 kb
398	19983-L27045	Exon 14	2166-2167	CGGCCAGTACCT-GCACCAGAAGAG	3.0 kb
221	19987-L27049	Exon 13	1961-1960 reverse	CAATAGCATTTC-CATTTGGTCTTC	2.8 kb
476	19980-L27325	Exon 12	1855-1856	CAGAACCTCCAG-TCATCAGAGACC	3.2 kb
160	19970-L27032	Exon 11	1724-1725	CAGAGGCAGCTA-AAATGCTGCTGC	8.8 kb
284	19971-L27033	Exon 10	1368-1367 reverse	CTTGTATTTCAT-TTTCCAGCAATA	22.6 kb
148	19979-L27041	Exon 9	1063-1064	GTTTGGCAGTAT-GAGAGCAAGCTG	2.3 kb
202	19992-L27054	Exon 8	1014-1013 reverse	TCAGACACTGAT-AGCGAACCATGA	15.6 kb
189	19988-L27050	Exon 7	75 nt after exon 7 reverse	GGCCGCTCCCCA-TGGCTCCAAGGA	4.2 kb
276	19989-L27051	Exon 6	825-824 reverse	GGGCAGGAAGCT-TGAGGCTCTCCC	3.8 kb
256	19984-L27776	Exon 5	672-673	GCTGGACAGCAT-TGCTGGTCTCAC	1.9 kb
328	19975-L27037	Exon 4	72 nt after exon 4 reverse	TCTTAAATACAT-GACTCTAATAAA	3.2 kb
451	19973-L27035	Exon 3	423-424	ATGGGCTCATTC-CTTATTTGCTTT	3.2 kb
226	19981-L27316	Exon 2	327-326 reverse	AAAACTCACCTG-CAGTCTTAAAGT	10.7 kb
382 «	20177-L27450	Exon 1	3 nt after exon 1	AGCACGCAGGTA-AGGAGGGCCCGA	1.0 kb
359 « ‡	19977-L27039	Upstream	229-230 (in NM_001166136.2)	GCAGGGATGAGG-AAGCACACAAAA	2.2 kb
		Start Codon	*47-49 (Exon 1)*		
EVC gene (NM_153717.3)					
		Start Codon	*181-183 (Exon 1)*		
238 «	19959-L27775	Exon 1	5 nt after exon 1 reverse	CTGCTCGGCCGA-CCCACCTGGTGT	7.6 kb
299 «	19967-L27029	Exon 2	88 nt before exon 2	ACTGGGGGAGTT-GACTGGCAAAAG	10.2 kb
492	19950-L27012	Exon 3	548-549	CATCTACCCCAT-CAATCAGAAGTT	2.1 kb
341	19954-L27379	Exon 4	586-587	ATGGCTCCTCCA-ACCCGTCTCTGC	2.1 kb
130	19963-L27025	Exon 5	65 nt after exon 5	CAAGGACTCTGT-GTGCAGTGAGTC	11.7 kb
	no probe	Exon 6			
196	19957-L27019	Exon 7	1016-1017	ACTTCAGGTCAA-ACTGTCAAACAC	2.9 kb
292	19968-L27777	Exon 8	2 nt before exon 8 reverse	AAGCTTCCATCT-GTGAAATGAGCA	4.8 kb
184	19961-L27023	Exon 9	1355-1356	AGTCCAGGAGGA-GACCAGGTGCCG	1.0 kb
405	19955-L27017	Exon 10	1587-1588	CAAGAGGAGGAA-CAGAGAAGCTTC	2.4 kb
316	19969-L27031	Exon 11	1644-1645	TTTTCCTCACAG-GCTTTTCATGAG	37.5 kb
	no probe	Exon 12			
154	19965-L27774	Exon 13	2066-2067	CCTCACTGAAGA-GTGAGTACAGCT	3.5 kb
178	19964-L27026	Exon 14	2253-2254	CTGGACGAGCAT-CAGTGGCAGCTG	1.5 kb
263	19966-L27028	Exon 15	2395-2394 reverse	GCACTCCATGTG-CTGCTGCAGAAG	3.4 kb
369	19956-L27018	Exon 16	2595-2596	GAGGACCACGAG-GAGAGAAAACTG	2.7 kb
415	19953-L27015	Exon 17	2629-2630	TAAATGGTCTAG-GTGAGAGGATGG	3.6 kb
208	19960-L27022	Exon 18	2858-2557 reverse	CCTGTAGCTGGG-TCTCCAGCTGGG	1.3 kb
481	19949-L27011	Exon 19	2937-2938	GAAAGCAAACTG-TTGCCTGCTAAG	0.8 kb
142	19962-L27024	Exon 20	2965-2966	CCTCTACAGAGA-AGCCCCTAAGGA	1.1 kb
442	19952-L27014	Exon 21	3563-3564	CATAATGACACC-TGCAGAAATGTA	
		Stop Codon	*3157-3159 (Exon 21)*		

[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.

« 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.

‡ This probe is located in exon 1 of transcript variant 2 (NM_001166136.2) and is located ~0.8 kb upstream of exon 1 of transcript variant 1 (NM_147127.5) of EVC2.

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

- 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.

P456 Product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-02 – 28 September 2022 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the EVC and EVC2 genes updated according to new versions of the of the NM_ reference sequence. Version A1-01 — 10 May 2019 (02P) - Product description rewritten and adapted to a new template.

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