Product Description SALSA® MLPA® Probemix P436-A2 ANO5

To be used with the MLPA General Protocol.

Version A2. For complete product history see page 7.

Catalogue numbers:
- **P436-025R:** SALSA MLPA Probemix P436 ANO5, 25 reactions.
- **P436-050R:** SALSA MLPA Probemix P436 ANO5, 50 reactions.
- **P436-100R:** SALSA MLPA Probemix P436 ANO5, 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 P436 ANO5 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ANO5* gene, which is associated with limb girdle muscular dystrophy type 2L (LGMD2L). This probemix can also be used to detect the presence of the *ANO5* c.191dupA point mutation.

LGMD2L or anoctaminopathy is a condition mainly characterised by adult onset proximal lower limb muscular weakness and raised creatine kinase (CK) values, due to recessive anoctamin 5 (*ANO5*) gene mutations. This gene encodes a member of the anoctamin family of transmembrane proteins, and the encoded protein is likely a calcium activated chloride channel. An exon 5 founder mutation (c.191dupA) represents 61% of mutated alleles and appears to be more prevalent in Northern European populations (Sarkozy et al. 2013). The c.191dupA mutation leads to a frameshift and to premature truncation, which strongly suggests that c.191dupA is associated with a loss of *ANO5* function (Bolduc et al. 2010).

The *ANO5* gene (22 exons) consists of ~90 kb of genomic DNA and is located at 11p14.3, 22 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK114459/.

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 *ANO5* exon numbering used in this P436-A2 ANO5 product description is the exon numbering from RefSeq transcript NM_213599.2, which is identical to the exon numbering from the LRG_868 sequence. The exon numbering and NM_ sequence used have been retrieved on 09/2020. 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 P436-A2 ANO5 contains 37 MLPA probes with amplification products between 136 and 391 nucleotides (nt). This includes 27 probes for *ANO5* copy number detection.

This probemix contains one probe for every exon, two probes for exon 1, 7 and 19 and three probes for exon 22. Furthermore, this probemix contains one probe specific for the *ANO5* c.191dupA mutation which will only generate a signal when the mutation is present (see P436 specific notes). The exon 5 probe detects the wild-type sequence of the c.191dupA mutation, which means that its signal will decrease when the mutation is present. In addition, nine 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 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.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 unrelated individuals who are from families without a history of LGMD2L. 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.

SALSA Binning DNA SD101: The **SD101** Binning DNA provided with this probemix can be used for binning of the *ANO5* c.191dupA mutation-specific probe (198 nt probe, 18658-SP0690-L24012). **SD101** Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probe. Inclusion of one reaction with 5 µl **SD101** Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation signals, as for this purpose true mutation positive patient samples or cell lines should be used. It is strongly advised that all samples tested are extracted with the

same method and derived from the same source of tissue. For further details, please consult the **SD101** Binning DNA product description, available online: www.mrcholland.com.

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 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. 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.

P436 specific notes:

- The presence of a clear signal for the 198 nt *ANO5* c.191dupA probe (at least 10% of the mean peak height of all reference probes in the sample), indicates the presence of the c.191dupA mutation, either heterozygous or homozygous. Presence of the mutation should always be confirmed by sequencing analysis. In a heterozygous c.191dupA sample, the ratio of the 204 nt wildtype specific probe is ~0.5, whereas there will be only a very small signal for this wildtype probe in a homozygous c.191dupA

sample. Please note that other mutations near the ligation site of the 204 nt probe also result in a lower signal for this probe.
- WARNING: The 198 nt *ANO5* c.191dupA probe and the 204 nt *ANO5* exon 5 probe are designed to target the same location on the genomic DNA. Therefore, the wild type exon 5 amplification probe gives a small signal at 204 nt when a homozygous c.191dupA mutation is present! Also, the mutation specific probe gives a small signal around 198 nt when the mutation is not present.

Limitations of the procedure:
- In most populations, the major cause of genetic defects in the *ANO5* gene are small (point) mutations. Except for the common c.191dupA mutation, point mutations will not be detected by using SALSA MLPA Probemix P436 ANO5.
- 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.

***ANO5* mutation database:** https://databases.lovd.nl/shared/genes/ANO5. 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 SNPs and unusual results (e.g., a duplication of *ANO5* exons 2 and 4 but not exon 3) to MRC-Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P436-A2 ANO5

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a] Reference	*ANO5*
64-105	Control fragments – see table in probemix content section for more information		
136	Reference probe 00797-L24120	5q31	
142	**ANO5 probe** 18651-L24005		**Exon 7**
148	**ANO5 probe** 18652-L24006		**Exon 1**
155	**ANO5 probe** 18653-L24007		**Exon 16**
160	Reference probe 12741-L13835	21q22	
168	**ANO5 probe** 18654-L24797		**Exon 1**
172	**ANO5 probe** 18655-L24009		**Exon 10**
178	**ANO5 probe** 18656-L24010		**Exon 2**
184	**ANO5 probe** 18657-L24011		**Exon 17**
193 *	Reference probe 12422-L28134	14q24	
198 § Ж +	**ANO5 probe** 18658-SP0690-L24012		**c.191dupA**
204 ∞ Ж +	**ANO5 probe** 18658-SP0690-L25311		**Exon 5**
210	**ANO5 probe** 18659-L25310		**Exon 3**
219	**ANO5 probe** 18660-L25309		**Exon 8**
228	**ANO5 probe** 18661-L24015		**Exon 12**
232	**ANO5 probe** 18662-L24796		**Exon 18**
238	**ANO5 probe** 18663-L24017		**Exon 13**
244	**ANO5 probe** 18664-L24018		**Exon 22**
250	**ANO5 probe** 18665-L24019		**Exon 7**
256 *	Reference probe 02469-L01913	15q21	
262	**ANO5 probe** 18666-L24799		**Exon 9**
267	Reference probe 14110-L15943	8p21	
274	**ANO5 probe** 18667-L24021		**Exon 22**
281	**ANO5 probe** 18668-L24022		**Exon 15**
285	**ANO5 probe** 18669-L24023		**Exon 4**
292	**ANO5 probe** 18670-L24024		**Exon 19**
301	**ANO5 probe** 18671-L24025		**Exon 14**
313	Reference probe 06580-L24038	2q24	
319	**ANO5 probe** 18672-L24026		**Exon 21**
328	**ANO5 probe** 18673-L24027		**Exon 6**
339	**ANO5 probe** 18674-L24028		**Exon 20**
346	Reference probe 06015-L07508	19q13	
355	**ANO5 probe** 18675-L24029		**Exon 22**
364	**ANO5 probe** 18676-L24030		**Exon 11**
373	Reference probe 00655-L00183	4q27	
384	**ANO5 probe** 18677-L24031		**Exon 19**
391	Reference probe 07808-L22560	3p22	

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

* New in version A2.
§ Mutation-specific probe. This probe will only generate a signal when the c.191dupA mutation is present. It has been tested on artificial DNA **but not on positive human samples!**
∞ Wild type sequence detected. The presence of the c.191dupA mutation will result in a decreased probe signal.
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.
+ The *ANO5* c.191dupA and *ANO5* exon 5 probes target the same location in the genomic DNA. Therefore, they may give small signals (approximately 5%) on each other's target DNA. See the P436 specific notes in the section Interpretation of results.

Table 2. *ANO5* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ANO5* exon[a]	Ligation site NM_213599.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*409-411 (Exon 1)*		
168	18654-L24797	Exon 1	313-314	GGCGGCCCACAG-TCAGATTCAGCA	0.1 kb
148	18652-L24006	Exon 1	391-392	GCACCAGTGCCA-TTAACGAGCTGG	10.3 kb
178	18656-L24010	Exon 2	474-475	AAGCATATAGAC-TACTCTTTCCAA	7.5 kb
210	18659-L25310	Exon 3	499-500	ACTGTTAGCAGA-GCCTGAGCAGCA	7.0 kb
285	18669-L24023	Exon 4	586-587	GGCGGCGGCTTA-TGGTAAAACCAG	2.8 kb
198 § Ж +	18658-SP0690-L24012	c.191dupA	595-596; 629-630	GCAGTTTCAAAA-34 nt spanning oligo-AGATGGGATTAG	-
204 ∞ Ж +	18658-SP0690-L25311	Exon 5	595-596; 629-630	TGCAGTTTCAAA-34 nt spanning oligo-AGATGGGATTAG	4.9 kb
328	18673-L24027	Exon 6	745-744 *reverse*	TTCCAACTCAAG-ACCTGTTTTTCT	1.3 kb
250	18665-L24019	Exon 7	788-789	GGAAGATGGAAG-AACTTATTTTGT	0.1 kb
142	18651-L24005	Exon 7	859-860	TGGGAATCAAAA-TGCCTATTAAGG	8.9 kb
219	18660-L25309	Exon 8	1150-1151	ACACTTACTCAT-CTGCCTATCCAC	3.3 kb
262	18666-L24799	Exon 9	1181-1182	GGGCCAATATTG-GAAGCCATCAGA	10.7 kb
172	18655-L24009	Exon 10	1359-1360	CTATTCTTTGCA-GCTGTAGTTGGC	0.4 kb
364	18676-L24030	Exon 11	12 nt before exon 11	ATAACTTTGCTG-TTCCTCTTGCAG	0.3 kb
228	18661-L24015	Exon 12	6 nt after exon 12	TTTGGGGTGAGT-AAATAGTCCCAT	4.4 kb
238	18663-L24017	Exon 13	1659-1660	CTGGTGGACTTT-GAAGAGGAACAG	2.2 kb
301	18671-L24025	Exon 14	15 nt before exon 14	TTCTTTGTGATT-TCTTCAATATTA	1.9 kb
281	18668-L24022	Exon 15	1866-1867	TACCGCCTGTCA-GTCTTTGCTACA	2.7 kb
155	18653-L24007	Exon 16	2166-2167	GTAGGCTATCCT-GGAAAATACACA	0.7 kb
184	18657-L24011	Exon 17	2220-2221	TGTGATCCTGGA-GGCTGTCTTATA	7.4 kb
232	18662-L24796	Exon 18	2375-2374 *reverse*	CATGATCCTGCT-CCCATCGACTAT	2.5 kb
292	18670-L24024	Exon 19	2507-2508	TGCTCTCATAAA-TAATATTGTAGA	0.1 kb
384	18677-L24031	Exon 19	2599-2600	TAGGTGTTTGGC-AAGACATTCTTT	1.7 kb
339	18674-L24028	Exon 20	2735-2736	TATGACAGGATA-TGTGAATAATAG	1.5 kb
319	18672-L24026	Exon 21	2863-2864	ACGAGAATAAAT-ATTTTCATAATA	3.6 kb
355	18675-L24029	Exon 22	3117-3118	ATGATTGAGGAA-AACAAAGCACAG	0.4 kb
244	18664-L24018	Exon 22	3467-3468	AGAAACACTGGC-CTTGGGCTGTCC	1.5 kb
274	18667-L24021	Exon 22	4967-4968	TGGCTTGTCAAA-TCAGATTCTCCA	
		stop codon	*3148-3150 (Exon 22)*		

a) See above section on exon numbering 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.

§ Mutation-specific probe. This probe will only generate a signal when the c.191dupA mutation is present. It has been tested on artificial DNA **but not on positive human samples!**
∞ Wild type sequence detected. The presence of the c.191dupA mutation will result in a decreased probe signal.
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.
+ The *ANO5* c.191dupA and *ANO5* exon 5 probes target the same location in the genomic DNA. Therefore, they may give small signals (approximately 5%) on each other's target DNA. See the P436 specific notes in the section Interpretation of results.

Related SALSA MLPA probemixes

- P048 LMNA/MYOT/ZMPSTE24: Contains probes for the *MYOT* gene, involved in Limb Girdle Muscular Dystrophy 1A (LGMD1A).
- P116 SGC: Contains probes for the *SGCA*, *SGCB*, *SGCD*, *SGCG* and *FKRP* genes, involved in various types of Limb Girdle Muscular Dystrophy.
- P176 CAPN3: Contains probes for the *CAPN3* gene, involved in Limb Girdle Muscular Dystrophy 2A (LGMD2A).

- P268 DYSF: Contains probes for the *DYSF* gene, involved in Limb Girdle Muscular Dystrophy 2B (LGMD2B).

References

- Bolduc V et al. (2010). Recessive mutations in the putative calcium-activated chloride channel Anoctamin 5 cause proximal LGMD2L and distal MMD3 muscular dystrophies. *Am J Hum Genet*. 86:213-221.
- Sarkozy A et al. (2013). ANO5 gene analysis in a large cohort of patients with anoctaminopathy: confirmation of male prevalence and high occurrence of the common exon 5 gene mutation. *Hum mutat*. 34:1111-1118.
- 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 P436 ANO5

- Ten Dam L et al. (2019). Autosomal recessive limb-girdle and Miyoshi muscular dystrophies in the Netherlands: The clinical and molecular spectrum of 244 patients. *Clin genet*. 96:126-133.

P436 Product history	
Version	*Modification*
A2	Two reference probes have been replaced and one reference probe has been removed.
A1	First release.

Implemented changes in the product description
Version A2-02 — 23 April 2026 (02P) - Replaced SD033 with SD101.

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