Product Description
SALSA® MLPA® Probemix P074-A4 AR

To be used with the MLPA General Protocol.

Version A4
For complete product history see page 7.

Catalogue numbers:
- **P074-025R:** SALSA MLPA Probemix P074 AR, 25 reactions.
- **P074-050R:** SALSA MLPA Probemix P074 AR, 50 reactions.
- **P074-100R:** SALSA MLPA Probemix P074 AR, 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 P074 AR is a **research use only (RUO)** assay for the detection of deletions or duplications in the *AR* gene, which is associated with androgen insensitivity syndrome (AIS) and spinal and bulbar muscular atrophy (SBMA).

AIS, formerly known as the testicular feminization syndrome, is a recessive disorder in which affected males have female external genitalia, female breast development, blind vagina and female adnexa, and abdominal or inguinal testes, despite a normal male (XY) karyotype. Partial androgen insensitivity results in hypospadias and micropenis with gynecomastia (Reifenstein syndrome). AIS is caused by mutations in the *AR* gene.

X-linked spinal and bulbar muscular atrophy (SBMA, SMAX1), also known as Kennedy disease, is a recessive form of spinal muscular atrophy, which only occurs in men. Age at onset is usually in the third to fifth decade of life, but earlier involvement has been reported. The disorder is characterized by slowly progressive limb and bulbar muscle weakness with fasciculations, muscle atrophy, and gynecomastia. The disorder is clinically similar to, but genetically distinct from, classic forms of autosomal spinal muscular atrophy. It is caused by a trinucleotide CAG repeat expansion in exon 1 of the *AR* gene. SBMA patients usually harbour 38 or more CAG repeats, whereas healthy individuals have 10 to 36 CAG repeats. Note that the length of the trinucleotide repeat cannot be measured by MLPA.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1429/ and https://www.ncbi.nlm.nih.gov/books/NBK1333/ .

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 *AR* exon numbering used in this P074-A4 AR product description is the exon numbering from the LRG_1406 sequence. Moreover, exon 1 of NM_001011645.3 (transcript variant 2) is annotated as exon 1b in this product description (Table 1 and 2). 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 P074-A4 AR contains 25 MLPA probes with amplification products between 178 and 418 nucleotides (nt). This includes 16 probe(s) for the *AR* gene. Two probes for all the eight exons with the exception of exon 6 and 7, for these exons one probe each is included, and two probes have been included for the first exon of transcript variant 2 (NM_001011645.3), which is located in intron 1 of the reference standard transcript variant 1. In addition, nine reference probes are included that detect locations on the X-chromosome. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains ten quality control fragments generating amplification products between 64 and 121 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and two chromosome Y-specific fragments (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)
105-121	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 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 AIS and SBMA. 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:

Copy Number status: Male samples	Final ratio
Normal	0.80 < FR < 1.20
Deletion	FR = 0
Duplication	1.65 < FR < 2.25
Ambiguous copy number	All other values

Copy Number status: Female samples	Final ratio
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 *AR* gene are small (point) mutations, none of which will not be detected by using SALSA MLPA Probemix P074 AR.
- 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.

***AR* mutation database**

http://androgendb.mcgill.ca and https://databases.lovd.nl/shared/genes/AR. We strongly encourage users to deposit positive results in the Androgen Receptor Gene Mutations Database and 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 *AR* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P074-A4 AR

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*AR*
64-121	Control fragments – see table in probemix content section for more information		
178	Reference probe 07093-L06673	Xp22	
184	**AR probe** 12594-L13678		**Exon 2**
190	**AR probe** 12595-L13679		**Exon 6**
202	**AR probe** 12596-L13680		**Exon 3**
208	**AR probe** 12597-L13681		**Exon 2**
220	Reference probe 18221-L25392	Xp21	
226 «	**AR probe** 12598-L13682		**Exon 1**
238	Reference probe 06910-L06490	Xq22	
247	**AR probe** 12599-L13683		**Exon 5**
258	**AR probe** 12600-L13684		**Exon 3**
274	**AR probe** 12601-L13685		**Exon 5**
283	Reference probe 10761-L12953	Xq28	
292 «	**AR probe** 12602-L13686		**Exon 1**
301 +	**AR probe** 12603-L13687		**Exon 1b**
310	Reference probe 08257-L07954	Xq26	
319	**AR probe** 12604-L13688		**Exon 8**
328	**AR probe** 12605-L13689		**Exon 4**
337	Reference probe 07656-L07362	Xp11	
355	**AR probe** 12606-L13690		**Exon 7**
364	Reference probe 02641-L02108	Xp11	
373 +	**AR probe** 12607-L13691		**Exon 1b**
382	**AR probe** 12608-L13692		**Exon 8**
399	**AR probe** 12609-L13693		**Exon 4**
409	Reference probe 08634-L08650	Xq28	
418	Reference probe 06473-L05999	Xp22	

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

+ Probes noted as detecting exon 1b detect the first exon of transcript variant 2 (NM_001011645.3).
« 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. *AR* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*AR* exon[a]	Ligation site NM_000044.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*1127-1129 (Exon 1)*		
292 «	12602-L13686	Exon 1	932-933	CGTGTGTCTTCT-TCTGCACGAGAC	1.1 kb
226 «	12598-L13682	Exon 1	2041-2042	AGCACTGAAGAT-ACTGCTGAGTAT	22.8 kb
373 +	12607-L13691	Exon 1b	7 nt before exon 2 (NM_001011645.3)	AAGTTGCTCTCT-TCTCCAGTAGGT	0.2 kb
301 +	12603-L13687	Exon 1b	2301-2302 (NM_001011645.3)	TGCCTGTCACTT-TTTCCCATGATA	74.3 kb
208	12597-L13681	Exon 2	2766-2767	CAGGGACCATGT-TTTGCCCATTGA	0.1 kb
184	12594-L13678	Exon 2	2835-2836	AGCTTCTGGGTG-TCACTATGGAGC	42.7 kb
258	12600-L13684	Exon 3	2920-2921	CTGTGCGCCAGC-AGAAATGATTGC	0.1 kb
202	12596-L13680	Exon 3	3003-3004	AGCAGGGATGAC-TCTGGGAGGTAA	25.5 kb
328	12605-L13689	Exon 4	3183-3184	GCCAGGTGTAGT-GTGTGCTGGACA	0.1 kb
399	12609-L13693	Exon 4	3267-3268	GAGACAGCTTGT-ACACGTGGTCAA	5.9 kb
247	12599-L13683	Exon 5	3340-3341	ATGGCTGTCATT-CAGTACTCCTGG	0.1 kb
274	12601-L13685	Exon 5	3410-3411	TCAACTCCAGGA-TGCTCTACTTCG	4.3 kb
190	12595-L13679	Exon 6	3505-3506	CACCTCTCTCAA-GAGTTTGGATGG	1.1 kb
355	12606-L13690	Exon 7	4 nt after exon 7	TGCAGCCTGTAA-GCAAACGATGGA	0.7 kb
319	12604-L13688	Exon 8	3760-3761	CATCAGTTCACT-TTTGACCTGCTA	0.1 kb
382	12608-L13692	Exon 8	3894-3895	CCAGTGAAGCAT-TGGAAACCCTAT	
		stop codon	*3887-3889 (Exon 8)*		

[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.

+ Probes noted as detecting exon 1b detect the first exon of transcript variant 2 (NM_001011645.3).
« 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.

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 P074 AR

- Chan et al. (2015). Aetiological bases of 46,XY disorders of sex development in the Hong Kong Chinese population. *Hong Kong Med J* 21:499-510.
- Li Y et al. (2012). AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression. *Oncogene* 31:4759-67.
- Teklu S et al. (2016). Three siblings with adrogen insensitivity syndrome. *Ethiop Med J* 54:229-36.

P074 product history	
Version	*Modification*
A4	Three reference probes have been replaced.
A3	Three reference probes have been replaced.
A2	QDX2 fragments have been added.
A1	First release.

Implemented changes in the product description

Version A4-02 – 7 March 2022 (04P)
- Product description rewritten and adapted to a new template.

Version A4-01 – 25 March 2020 (02P)
- Product description 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 *AR* gene updated according to new version of the NM_ reference sequence.

Version A3-01 – 19 April 2018 (01P)
- Product description restructured and adapted to a new template.
- Ligation sites of the probes targeting the *AR* gene updated according to new version of the NM_reference sequence.

Version 07 (53)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new pictures included).
- Adjustment of the exon numbering to the NCBI exon numbering that is present in the NM_ sequences for this gene.
- Various minor textual changes.
- Updated link for “Database of Genomic Variants”.

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