Product Description
SALSA® MLPA® Probemix P464-A1 INSR

To be used with the MLPA General Protocol.

Version A1
For complete product history see page 7.

Catalogue numbers:
- **P464-025R:** SALSA MLPA Probemix P464 INSR, 25 reactions.
- **P464-050R:** SALSA MLPA Probemix P464 INSR, 50 reactions.
- **P464-100R:** SALSA MLPA Probemix P464 INSR, 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 P464 INSR is a **research use only (RUO)** assay for the detection of deletions or duplications in the *INSR* gene, which is associated with *INSR*-related severe syndromic insulin resistance.

INSR-related severe syndromic insulin resistance comprises a spectrum of milder to severe phenotypes, ranging from Donohue syndrome (most severe) to Rabson-Mendenhall syndrome (milder), based on the original clinical descriptions. Donohue syndrome is characterized by severe insulin resistance, severe growth failure, hypotonia, developmental delay, characteristic facial features and enlarged organs. Death usually occurs before the age of one. Rabson-Mendenhall syndrome is characterized by severe insulin resistance, with growth delay and intellectual disability varying from case to case. Facial features can be milder compared to Donohue syndrome. Life expectancy varies but death usually occurs in the second decade, most commonly because of complications of hyperglycemia. These disorders are extremely rare, with a prevalence estimated at under 1:1,000,000. *INSR* encodes the insulin receptor protein. This protein plays an important role in the insulin signalling pathway, which regulates glucose uptake and release, as well as the synthesis and storage of carbohydrates, lipids and protein.

The *INSR* gene (22 exons) spans ~182 kb of genomic DNA and is located on chromosome 19p13.2, ~7 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK476444/.

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
Matched Annotation from NCBI and EMBL-EBI (MANE): http://www.ncbi.nlm.nih.gov/refseq/MANE/
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering
The *INSR* exon numbering used in this P464-A1 INSR product description is the exon numbering from the MANE project (release version 1.0) based on MANE Select transcript NM_000208.4 as indicated in Table 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 MANE sequence. 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 P464-A1 INSR contains 39 MLPA probes with amplification products between 136 and 412 nucleotides (nt). This includes 30 probes for the *INSR* gene. 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 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 *INSR*-related severe syndromic insulin resistance. 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 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 *INSR* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P464 INSR.
- 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.

***INSR* mutation database**

https://databases.lovd.nl/shared/genes/INSR. 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 SNVs and unusual results (e.g., a duplication of *INSR* exons 4 and 6 but not exon 5) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P464-A1 INSR

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*INSR*
64-105	Control fragments – see table in probemix content section for more information		
136	Reference probe 20515-L28105	1q	
142	**INSR probe** 20675-L28092		**Exon 22**
149	**INSR probe** 23310-L28090		**Exon 20**
154	**INSR probe** 20488-L28069		**Exon 4**
160	**INSR probe** 20506-L28087		**Exon 17**
166	Reference probe 09267-L20529	10q	
172	**INSR probe** 20485-L28066		**Exon 2**
178	**INSR probe** 20495-L28076		**Exon 10**
184	**INSR probe** 20505-L28086		**Exon 16**
191	**INSR probe** 20486-L28067		**Exon 3**
196	Reference probe 12538-L23846	11p	
202	**INSR probe** 20504-L28085		**Exon 15**
209	**INSR probe** 20490-L28071		**Exon 6**
216	**INSR probe** 20512-L28093		**Exon 22**
222	**INSR probe** 20507-L28088		**Exon 18**
229	**INSR probe** 20492-L28073		**Exon 8**
236	Reference probe 10329-L23575	7q	
244	**INSR probe** 20500-L28081		**Exon 13**
250	**INSR probe** 20508-L28089		**Exon 19**
258	**INSR probe** 20496-L28077		**Exon 11**
265	**INSR probe** 20491-L28072		**Exon 7**
271	**INSR probe** 22165-L31483		**Exon 1**
278	Reference probe 18594-L28268	2q	
286	**INSR probe** 20497-L28078		**Exon 11**
292	**INSR probe** 20487-L28068		**Exon 3**
300	**INSR probe** 20499-L28080		**Exon 12**
307	**INSR probe** 23352-L33007		**Exon 9**
317	Reference probe 11898-L24065	6p	
325	**INSR probe** 20503-L31406		**Exon 14**
337	**INSR probe** 23312-L28091		**Exon 21**
348	Reference probe 19887-L26752	10q	
357	**INSR probe** 20484-L28065		**Exon 2**
364	**INSR probe** 20489-L28070		**Exon 5**
373	**INSR probe** 20502-L28083		**Exon 14**
380	Reference probe 16932-L19875	4q	
389	**INSR probe** 20494-L28075		**Exon 10**
399	**INSR probe** 23311-L28079		**Exon 12**
404	**INSR probe** 20501-L28082		**Exon 13**
412	Reference probe 09793-L12593	15q	

[a] See section Exon numbering on page 2 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. *INSR* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*INSR* exon[a]	Ligation site NM_000208.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*524-526 (Exon 1)*		
271	22165-L31483	Exon 1	325-326	CTCAGTATTTGT-AGCTGGCGAAGC	26.2 kb
357	20484-L28065	Exon 2	633-634	AGTGTGTCCCGG-CATGGATATCCG	0.4 kb
172	20485-L28066	Exon 2	1073-1072 *reverse*	CGGACAGATGTC-TCCACACTCCTC	83.0 kb
191	20486-L28067	Exon 3	1359-1360	CCAGGACTGGCG-CTGTGTGAACTT	0.1 kb
292	20487-L28068	Exon 3	4 nt after exon 3	TCCAGCAAGTGA-GTTCTGGATGTG	9.6 kb
154	20488-L28069	Exon 4	1543-1544	AAGGTGTGCCAC-CTCCTAGAAGGC	2.3 kb
364	20489-L28070	Exon 5	1677-1678	AGAAGCCAACCT-CGGCCTCATTGA	1.8 kb
209	20490-L28071	Exon 6	1915-1914 *reverse*	TTGTGGATTTCT-GACAAGCAGAGT	2.6 kb
265	20491-L28072	Exon 7	2085-2086	GCCGTACTGGCC-CCCCGACTTCCG	1.8 kb
229	20492-L28073	Exon 8	2304-2305	CATCTTTGTGAA-GACCCTGGTCAC	3.1 kb
307	23352-L33007	Exon 9	2497-2498	TCTAACTCATCA-TCCCAGATTATT	10.3 kb
389	20494-L28075	Exon 10	2649-2650	GGCCGGCGAATG-CTGCTCCTGTCC	0.2 kb
178	20495-L28076	Exon 10	59 nt after exon 10 *reverse*	CTAAGTAATGAC-CTTCCACCAACA	2.2 kb
258	20496-L28077	Exon 11	2774-2775	CTTCAGGCACTG-GTGCCGAGGACC	0.2 kb
286	20497-L28078	Exon 11	146 nt after exon 11	TTCACCAATTCA-GAAATCCAATGC	7.3 kb
399	23311-L28079	Exon 12	2792-2793	GTCGTTCCAGGC-CATCTCGGAAAC	0.2 kb
300	20499-L28080	Exon 12	2980-2981	ACGGGCTATCGC-ATCGAGCTGCAG	1.2 kb
244	20500-L28081	Exon 13	3136-3137	CACTTGATGTGG-CAGGAGCCGAAG	0.1 kb
404	20501-L28082	Exon 13	57 nt after exon 13	CCCCTTCAGAGT-TGCACTTGGTAC	9.2 kb
325	20503-L31406	Exon 14	65 nt before exon 14	GAGCTCCCTGCG-AGGGGTGGACAC	0.2 kb
373	20502-L28083	Exon 14	3295-3296	TACAGCGTGCGA-ATCCGGGCCACC	3.3 kb
202	20504-L28085	Exon 15	3422-3421 *reverse*	AACACTGAAGAG-AAAGACAAAGAT	2.3 kb
184	20505-L28086	Exon 16	3532-3531 *reverse*	TACTCACCATCA-CTGGCACTGAGA	1.3 kb
160	20506-L28087	Exon 17	3751-3752	AATGAGGCCTCG-GTCATGAAGGGC	2.3 kb
222	20507-L28088	Exon 18	3796-3797	CGCCTCCTGGGA-GTGGTGTCCAAG	0.2 kb
250	20508-L28089	Exon 19	3924-3925	CCCTACCCTTCA-AGAGATGATTCA	2.1 kb
149	23310-L28090	Exon 20	4111-4110 *reverse*	ACAGGGAGCAGA-CCCTTGCCCCCT	1.2 kb
337	23312-L28091	Exon 21	3 nt after exon 21	AGAGAGAGTGTA-AGTGTAGAAAGG	2.2 kb
142	20675-L28092	Exon 22	4503-4502 *reverse*	GGTCCAGGGGCA-CATTCTCCATGT	0.2 kb
216	20512-L28093	Exon 22	4727-4728	CTTTCCTCTGGT-TTGAAAGCCTCT	
		stop codon	*4670-4672 (Exon 22)*		

[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.

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

- P117: contains probes for the *ABCC8* gene, involved in familial hyperinsulinemic hypoglycemia 1.
- P217: contains probes for the *IGF1R* gene and other genes related to prenatal and postnatal growth failure.

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.

P464 product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-01 – 08 December 2023 (04P) - Not applicable, new document.

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