Product Description
SALSA® MLPA® Probemix P062-D2 LDLR

To be used with the MLPA General Protocol.

Version D2
For complete product history see page 9.

Catalogue numbers:
- **P062-025R:** SALSA MLPA Probemix P062 LDLR, 25 reactions.
- **P062-050R:** SALSA MLPA Probemix P062 LDLR, 50 reactions.
- **P062-100R:** SALSA MLPA Probemix P062 LDLR, 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.

Intended purpose
The SALSA MLPA Probemix P062 LDLR is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in the *LDLR* gene in genomic DNA isolated from human peripheral whole blood specimens. P062 LDLR is intended to confirm a potential cause for and clinical diagnosis of familial hypercholesterolaemia and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P062 LDLR should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *LDLR* gene are point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

Assay results are intended to be used in combination with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1]Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).
[2]To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

Clinical background
The low-density lipoprotein receptor (LDLR) is a cell surface receptor that plays an important role in cholesterol homeostasis. The receptor binds and internalises LDL cholesterol particles by endocytosis. This process takes place in all nucleated cells, but mainly in the liver, which is responsible for ~70% of the total clearance of LDL cholesterol from the circulation.

Familial hypercholesterolaemia (FH) is a disease characterised by significantly elevated LDL cholesterol levels that cause atherosclerotic plaque deposition in arteries, which may lead to coronary artery disease (CAD) or other cardiovascular disease manifestations at an early age. Heterozygous FH is an autosomal dominant disease with a worldwide prevalence of ~1:250 that is caused by pathogenic mutations in the *LDLR*, *APOB* or *PCSK9* genes. Mutations in *LDLR* are the most common, with >50% of FH cases caused by pathogenic variants in the *LDLR* gene. Mutations in *APOB* and *PCSK9* are found in 5-10% and <1% of FH cases, respectively. In the remaining ~40% of FH cases, the underlying genetic defect is unknown. Homozygous FH is much rarer (1:160,000-400,000) and results from biallelic mutations (homozygous or compound heterozygous) in one of the aforementioned genes or one mutation in each of two different genes. Autosomal recessive FH has a prevalence lower than 1:1,000,000 and is caused by biallelic mutations in the LDL-receptor adaptor protein 1 (*LDLRAP1*) gene. Less than 1% of FH cases are due to mutations in *LDLRAP1*.

The severity of symptoms is dependent on the type of mutation; complete loss of function variants usually lead to more severe disease. Heterozygous FH patients are at an approximately 20-fold increased risk for CAD, whereas most homozygous FH patients experience severe CAD in their mid-20s and undergo coronary bypass surgery in their teenage years (https://www.ncbi.nlm.nih.gov/books/NBK174884/; Nordestgaard et al. 2013; Raal et al. 2011; Kassner et al. 2014).

Gene structure
The *LDLR* gene spans ~44.4 kilobases (kb) on chromosome 19p13.2 and contains 18 exons. The *LDLR* LRG_274 is available at www.lrg-sequence.org and is identical to GenBank NG_009060.1.

Transcript variants
For *LDLR,* multiple variants have been described (https://www.ncbi.nlm.nih.gov/gene/3949). Transcript variant 1 is the longest transcript and encodes the longest isoform (NM_000527.5; 5173 nt; coding sequence 87-2669). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site is located in exon 1 and the stop codon is located in exon 18.

Exon numbering
The *LDLR* exon numbering used in this P062-D2 LDLR product description is the exon numbering from the LRG_274 sequence. 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 P062-D2 LDLR contains 33 MLPA probes with amplification products between 136 and 490 nucleotides (nt). This includes 20 probes for the *LDLR* gene and one flanking probe for the *SMARCA4* gene located upstream of the *LDLR* gene. In addition, 12 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 from peripheral blood, 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 familial hypercholesterolaemia. 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.

Performance characteristics
Most of the pathogenic mutations in the *LDLR* gene are point mutations that can be identified by sequencing analysis. It is estimated that ~10% of pathogenic mutations in the *LDLR* gene are attributed to large rearrangements that can be detected with this MLPA probemix (Alonso et al. 2009; Futema et al. 2013; Iacocca et al. 2017; Marduel et al. 2010). The exact percentage of large rearrangements varies, however, between populations. The combined use of sequencing and MLPA analysis of the *LDLR* gene leads to a definitive diagnosis in >50% of FH patients. The analytical sensitivity and specificity for the detection of deletions and duplications in the *LDLR* gene is very high and can be considered >99% (based on a 2005-2022 literature review).

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

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 expected results for *LDLR*-specific probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion) or 3 (heterozygous duplication). In rare cases, copy numbers of 0 (homozygous deletion) or 4 (heterozygous triplication/homozygous duplication) may be obtained.

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 *LDLR* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P062 LDLR.
- 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.

***LDLR* mutation database**

https://databases.lovd.nl/shared/genes/LDLR. We strongly encourage users to deposit positive results in the UCL/LOVD *LDLR* gene variant 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 *LDLR* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P062-D2 LDLR

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*LDLR*
64-105	Control fragments – see table in probemix content section for more information		
136	Reference probe 09824-L10234	10q	
142 ¬	SMARCA4 probe 02488-L13996		Upstream
148	**LDLR probe** 02309-L01800		**Exon 1**
154	Reference probe 14813-L16521	1p	
160	**LDLR probe** 02310-L01801		**Exon 2**
166	**LDLR probe** 19517-L26011		**Exon 18**
184 Ж ± +	**LDLR probe** 19518-SP0828-L26012		**Exon 3**
193 +	**LDLR probe** 02313-L01804		**Exon 12**
202	Reference probe 01116-L00620	8q	
212	**LDLR probe** 02314-L01805		**Exon 4**
218 +	**LDLR probe** 19333-L12635		**Exon 14**
229	Reference probe 04360-L03780	7p	
238 +	**LDLR probe** 19519-L26013		**Exon 5**
247 +	**LDLR probe** 19520-L26014		**Exon 15**
256	Reference probe 06007-L05432	2q	
265	**LDLR probe** 19521-L26015		**Exon 7**
283	Reference probe 14477-L16197	4q	
290 Δ	**LDLR probe** 19523-L26017		**Exon 6**
310	Reference probe 15380-L17211	3p	
319 Ж Δ	**LDLR probe** 19522-SP0893-L28967		**Exon 17**
328 +	**LDLR probe** 19526-L26020		**Exon 11**
335 Ж ±	**LDLR probe** 19524-SP0864-L26573		**Exon 16**
352 Ж	**LDLR probe** 19525-SP0892-L26886		**Exon 8**
364	**LDLR probe** 02324-L26888		**Exon 9**
372	Reference probe 15433-L17263	5p	
390	**LDLR probe** 19640-L26021		**Exon 10**
400 Ж +	**LDLR probe** 19528-SP0829-L26022		**Exon 13**
415	Reference probe 18255-L22981	12q	
427 +	**LDLR probe** 10781-L11396		**Exon 15**
445	Reference probe 16634-L19164	17q	
460	Reference probe 16287-L25505	20q	
472 Ж +	**LDLR probe** 06281-SP0876-L26683		**Exon 1**
490	Reference probe 12461-L21828	22q	

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

± SNP rs559091708 influences the 184 nt probe signal. SNPs rs543852919 and rs750518671 influence the 335 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by these probes.

Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.

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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

\+ Ligation site is located outside the exonic sequence.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

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

Length (nt)	SALSA MLPA probe	*LDLR* exon[a]	Ligation site NM_000527.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
142 ¬	02488-L13996	*SMARCA4* gene	-	CGTCTTGCAGTC-GGTCTTCACCAG	29.5 kb
		start codon	*87-89 (Exon 1)*		
472 Ж +	06281-SP0876-L26683	Exon 1	188 nt before exon 1; 155 nt before exon 1	CAAGTCGCCTGC-33 nt spanning oligo-TCAGAGCTTCAC	0.2 kb
148	02309-L01800	Exon 1	49-50	CAGCAGGTCGTG-ATCCGGGTCGGG	10.7 kb
160	02310-L01801	Exon 2	199-200	CCAAGACGGGAA-ATGCATCTCCTA	2.4 kb
184 Ж ± +	19518-SP0828-L26012	Exon 3	41 nt before exon 3; 8 nt before exon 3	GGCCTCAGTGGG-33 nt spanning oligo-TTCTGTAGTGTC	2.7 kb
212	02314-L01805	Exon 4	477-478	TCTGTGACTCAG-ACCGGGACTGCT	1.2 kb
238 +	19519-L26013	Exon 5	18 nt before exon 5	AAAATCAACACA-CTCTGTCCTGTT	0.9 kb
290 Δ	19523-L26017	Exon 6	951-952	ACAGCGGCGAAT-GCATCACCCTGG	3.3 kb
265	19521-L26015	Exon 7	1093-1094	TAAGATCGGCTA-CGAGTGCCTGTG	0.9 kb
352 Ж	19525-SP0892-L26886	Exon 8	1199-1200; 1229-1230	TGCGTGAACCTG-30 nt spanning oligo-GGCTTCCAGCTG	1.7 kb
364	02324-L26888	Exon 9	1329-1330	AGATGACGCTGG-ACCGGAGCGAGT	0.3 kb
390	19640-L26021	Exon 10	1560-1561	TCTACTGGACCG-ACTCTGTCCTGG	2.4 kb
328 +	19526-L26020	Exon 11	7 nt before exon 11	TTCTCTGTCCTC-CCACCAGCTTCA	0.8 kb
193 +	02313-L01804	Exon 12	18 nt before exon 12	TGACCTCTCCTT-ATCCACTTGTGT	3.4 kb
400 Ж +	19528-SP0829-L26022	Exon 13	9 nt after exon 13; 39 nt after exon 13	GAGGTAAGGGTG-30 nt spanning oligo-TCCTTGTGGAAA	0.4 kb
218 +	19333-L12635	Exon 14	100 nt after exon 14, reverse	CTCATGAGTCCT-TACAACGACCTT	2.5 kb
247 +	19520-L26014	Exon 15	20 nt before exon 15	GGCACTCAGAAG-ACGTTTATTTAT	0.5 kb
427 +	10781-L11396	Exon 15	260 nt after exon 15	CTGGTTCCCAAA-GTCAGCCACGCA	4.4 kb
335 Ж ±	19524-SP0864-L26573	Exon 16	2449-2450; 2475-2476	CAGTAGCGTGAG-26 nt spanning oligo-GTAAGCGCGGGC	1.6 kb
319 Ж Δ	19522-SP0893-L28967	Exon 17	2600-2601; 6 nt after exon 17	GAGGTCCACATT-39 nt spanning oligo-GACCCTCTTTAG	2.1 kb
166	19517-L26011	Exon 18	3107-3108	ACTCAGGAGTCA-ACGTGTTTACCT	
		stop codon	*2667-2669 (Exon 18)*		

[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.

± SNP rs559091708 influences the 184 nt probe signal. SNPs rs543852919 and rs750518671 influence the 335 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by these probes.

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P062 product history	
Version	*Modification*
D2	Length of one target probe has been changed (no change in sequence detected).
D1	Twelve target probes for *LDLR* and ten reference probes have been replaced.
C2	Four reference probes have been replaced and four reference probes have been removed. In addition, the 88 and 96 nt control fragments have been replaced (QDX2).
C1	The exon 14 LDLR probe has been replaced and one extra probe for exon 15 has been added. In addition, one reference probe has been removed, one reference probe has been replaced, and four extra control fragments at 88-96-100-105 nt have been included.
B	One new LDLR probe and one new reference probe have been added.
A	Two new LDLR probes (exon 10 & 13) and two extra reference probes have been added.
'O'	First release.

Implemented changes in the product description
Version D2-06 – 09 February 2023 (04P) - Information about the prevalence of FH and the percentage of FH cases explained by mutations in *LDLR*, *APOB*, *PCSK9* and *LDLRAP1* updated in the Clinical background section. - Percentage of FH patients with mutations in *LDLR* updated in the Performance characteristics section. - Remark added to Table 1 and Table 2 about the influence of SNPs on the signal of the 335 nt probe 19524-SP0864-L26573.
Version D2-05 – 14 September 2021 (04P) - Product description rewritten and adapted to a new template. - UK has been added to the list of countries in Europe that accept the CE mark.
Version D2-04 — 13 August 2020 (02P) - Product description rewritten and adapted to a new template. - Intended use updated; the intended use now includes testing of at-risk family members. - Prevalence of heterozygous and homozygous FH updated in clinical background section. - Link updated to the *LDLR* mutation database. - Remark added to 184 nt probe 19518-SP0828-L26012 about a SNP influencing the probe signal. - Remark added to 290 nt probe 19523-L26017 and 319 nt probe 19522-SP0893-L28967 about probe sensitivity to experimental variation. - The target sequence of the 472 nt probe 06281-SP0876-L26683 is now indicated as *LDLR* exon 1 rather than promoter to make the indication of probe target sequences more consistent. - Symbol used for probe remark about ligation site located outside the exonic sequence changed in Table 1 and Table2. - Ligation site of SMARCA4 flanking probe removed from Table 2. - Ligation sites of the probes targeting the *LDLR* gene updated according to new version of the NM_ reference sequence. - Minor adjustment to partial sequence of 319 nt probe 19522-SP0893-L28967 in Table 2 to meet current product description guidelines of MRC Holland. - List with selected publications using SALSA MLPA Probemix P062 LDLR updated. - P062 product history corrected for version C1.
Version D2-03 – 29 January 2019 (03) - Product is now registered for IVD use in Israel.

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IVD	EUROPE* CE ISRAEL
RUO	ALL OTHER COUNTRIES

*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.