Product Description
SALSA® MLPA® Probemix P072-D1 MSH6-MUTYH

To be used with the MLPA General Protocol.

Version D1
For complete product history see page 13.

Catalogue numbers:
- **P072-025R:** SALSA MLPA Probemix P072 MSH6-MUTYH, 25 reactions.
- **P072-050R:** SALSA MLPA Probemix P072 MSH6-MUTYH, 50 reactions.
- **P072-100R:** SALSA MLPA Probemix P072 MSH6-MUTYH, 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 P072 MSH6-MUTYH 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 *MSH6* gene and the *EPCAM/MSH2* region in order to confirm a potential cause of and clinical diagnosis for Lynch Syndrome (LS), as well as deletions and duplications in the *MUTYH* gene in order to confirm a potential cause of and clinical diagnosis for *MUTYH*-Associated Polyposis (MAP). In addition, the presence of the two most common point mutations in the *MUTYH* gene among people from European descent, c.536A>G (p.Tyr179Cys) and c.1187G>A (p.Gly396Asp), can be detected with this probemix. P072 MSH6-MUTYH is also intended for molecular genetic testing of at-risk family members. This assay is for use with genomic DNA isolated from human peripheral whole blood specimens.

Copy number variations (CNVs) detected with P072 MSH6-MUTYH should be confirmed with a different technique. In particular, CNVs detected by only a single probe as well as the two *MUTYH* point mutations always require confirmation by another method. Most defects in the *MSH6* gene, *EPCAM/MSH2* region and the *MUTYH* gene are point mutations, which will not be detected by MLPA, with exception of the two aforementioned *MUTYH* point mutations. It is therefore recommended to use this assay in combination with sequence analysis. Of note, not all exons of *EPCAM*, *MSH2* and *MUTYH* are covered in this probemix.

Assay results are intended to be used in conjunction 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, population screening, pre-implantation or prenatal testing. Only in a research setting can this device be used for the detection of, or screening for, acquired or somatic genetic aberrations, e.g. from DNA extracted from formalin-fixed paraffin embedded (FFPE) or fresh tumour materials.

[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, Coffalyser.Net analysis software, and SALSA Binning DNA SD022.

Clinical background
Lynch syndrome (LS; formerly known as hereditary non-polyposis colorectal cancer (HNPCC)), is an adult-onset hereditary cancer susceptibility syndrome predisposing to several cancer types, the most prevalent being colorectal cancer (CRC), endometrial cancer, gastric cancer and ovarian cancer. It is an autosomal dominantly inherited syndrome that is caused by heterozygous germline mutations in one of the four major DNA mismatch repair genes, *i.e. MLH1*, *MSH2*, *MSH6* or *PMS2*. The estimated contribution of the different genes to LS is 15-40% for *MLH1*, 20-40% for *MSH2*, 12-35% for *MSH6*, and 5-25% for *PMS2*. Germline deletions of the 3'-end of *EPCAM* result in inactivation of *MSH2* by hypermethylation of the *MSH2* promoter, and are the cause of LS in <10% of cases (Ligtenberg *et al.* 2009; Lynch *et al.* 2015; Tiwari *et al.* 2016). Although it is expected that deletion of exon 9 of *EPCAM* (including the transcription stop site) would be sufficient to lead to hypermethylation of the *MSH2* promoter, studies have shown that the identified *EPCAM* deletions included at least exon 8 and 9 (Kuiper *et al.* 2011, Rumilla *et al.* 2011). More information on Lynch syndrome is available on https://www.ncbi.nlm.nih.gov/books/NBK1211/.

Mutations in the *MUTYH* gene also result in a hereditary predisposition to colon and gastric cancer, which is referred to as MAP. In contrast to LS, MAP is an autosomal recessive disorder. In MAP patients, ten to several hundred colonic adenomatous polyps develop and these become evident at a mean age of 50 years. However, colon cancer can also develop in the absence of polyposis. A single defective copy of the *MUTYH* gene may result in no, or only a small increase in risk for CRC. Phenotypes of Lynch (like) syndrome and MAP can partly overlap. There are two common *MUTYH* mutations, c.536A>G (p.Tyr179Cys) and c.1187G>A (p.Gly396Asp), that are carried by ~1%-2% of the general population and account for ≥90% of all *MUTYH* pathogenic variants in northern European populations. Up to 70% of MAP patients harbor at least one of these variants (Aretz *et al.* 2013). Since the *MUTYH* gene is small (11 kilobases (kb)), the eleven copy number detection MLPA probes present in this P072 MSH6-MUTYH probemix are expected to detect most of the *MUTYH* copy number changes as 11/16 exons are covered. For instance, the most frequent CNV in *MUTYH* – a deletion of exon 4-16 that is reported in multiple patients (Castillejo *et al.* 2014) – can be detected with nine probes in this probemix. More information on MAP is available at http://www.ncbi.nlm.nih.gov/books/NBK107219/. For complete exon coverage of *MUTYH* the SALSA MLPA probemix P378 MUTYH is available.

Gene structure
The *MSH6* gene spans ~24 kb on chromosome 2p16.3 and contains 10 exons. The *MSH6* LRG_219 is available at www.lrg-sequence.org and is identical to GenBank NG_007111.1.

The *MUTYH* gene spans ~11 kb on chromosome 1p34.1 and contains 16 exons. The *MUTYH* LRG_220 is available at www.lrg-sequence.org and is identical to GenBank NG_008189.1.

The *EPCAM* gene spans ~18 kb and has 9 exons, and the *MSH2* gene spans ~80 kb and has 16 exons. Both genes are located on chromosome 2p21; *EPCAM* is located ~16 kb upstream of *MSH2*. The *EPCAM* LRG_215 (identical to GenBank NG_012352.2) and the *MSH2* LRG_218 (identical to GenBank NG_007110.2) are available at www.lrg-sequence.org

Transcript variants
For *MSH6,* multiple variants have been described. Transcript variant 1 is the longest transcript and encodes the longest protein isoform (NM_000179.3; 4265 nucleotides (nt); coding sequence (CDS) 90-4172). In Table 2a, the ligation sites of the *MSH6* MLPA probes are indicated according to this sequence. More information can be found on the NCBI gene page: https://www.ncbi.nlm.nih.gov/gene/2956.

For *MUTYH,* multiple variants have been described. Transcript variant alpha5 (NM_001128425.2; 1900 nt; CDS 187-1836) represents the longest transcript and encodes the long isoform 5. In Table 2b, the ligation sites of

the *MUTYH* MLPA probes are indicated according to this sequence. More information can be found on the NCBI gene page: https://www.ncbi.nlm.nih.gov/gene/4595.

For *EPCAM,* one transcript variant has been described encoding the full-length protein (NM_002354.3; 1547 nt; CDS 196-1140). In Table 2a, the ligation sites of the *EPCAM* MLPA probes are indicated according to this sequence. More information can be found on the NCBI gene page: https://www.ncbi.nlm.nih.gov/gene/4072.

For *MSH2,* two variants have been described. Transcript variant 1 is the longest transcript and encodes the longest protein isoform (NM_000251.3; 3115 nt; CDS 37-2841). In Table 2a, the ligation sites of the *MSH2* MLPA probes are indicated according to this sequence. More information can be found on the NCBI gene page: https://www.ncbi.nlm.nih.gov/gene/4436.

Exon numbering

The *MSH6* exon numbering used in this P072-D1 MSH6-MUTYH product description is the exon numbering from the LRG_219 sequence. For *MUTYH* the exon numbering from the LRG_220 was used, for *EPCAM* the exon numbering from the LRG_215 was used and for *MSH2* the exon from the LRG_218 was used. The exon numbering of the NM_ sequences that were used for determining the probe ligation sites 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 P072-D1 MSH6-MUTYH contains 48 MLPA probes with amplification products between 130 and 500 nt. This includes 15 probes for the *MSH6* gene, eleven probes for the *MUTYH* gene, five probes for the *EPCAM* gene and three probes that target sequences in, down- and upstream of the *MSH2* gene. Furthermore, this probemix also contains two probes specific for the c.536A>G (p.Tyr179Cys) and c.1187G>A (p.Gly396Asp) *MUTYH* mutations, which will only generate a signal when the mutation is present. In addition, twelve reference probes are included that target relatively copy number stable regions in various cancer types associated with LS and MAP. Complete probe sequences and the identity of the genes detected by the reference probes are available in Table 3 and 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 (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). More information on the use of MLPA in tumour applications can be found in Hömig-Hölzel and Savola (2012).

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. Note that the peaks of the mutation-specific probes are expected to be absent in the majority of samples from healthy individuals.

Required specimens

Extracted DNA from human peripheral blood or, in a research setting, DNA derived from fresh or FFPE tumour tissue, 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. More information on the use of FFPE tissue samples for MLPA can be found in Atanesyan *et al.* (2017).

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 hereditary predisposition to cancer. 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. Sample ID numbers from the Coriell Institute described in the table below have been tested with this P072-D1 Probemix at MRC Holland and can be used as positive control samples. Note that the two *MUTYH* mutation-specific probes are only intended to determine the presence of the mutation and should not be used to determine zygosity. The quality of cell lines can change; therefore, samples should be validated before use.

Coriell sample ID	Affected target genes	Expected result
NA13451	*EPCAM/MSH2/MSH6* region	Heterozygous deletion of all probes present in Table 2a
NA10401	*EPCAM/MSH2/MSH6* region*	Heterozygous duplication of all probes present in Table 2a
HG01918	*MUTYH*	Positive for the *MUTYH* c.536A>G (p.Tyr179Cys) mutation
HG00097; HG01095; HG01519	*MUTYH*	Positive for the *MUTYH* c.1187G>A (p.Gly396Asp) mutation

* Reference probes 155 nt (14199-L29775) and 190 nt (14330-L31002), the targets of which are located on chromosome 2 as well, are also affected in this sample.

SALSA Binning DNA SD022

The SALSA Binning DNA SD022 provided with this probemix can be used for binning of all probes, including the two *MUTYH* mutation-specific probes: the 184 nt probe 18416-SP0654-L23441, detecting the c.536A>G (p.Tyr179Cys) mutation; and the 258 nt probe 18417-SP0655-L23442, detecting the c.1187G>A (p.Gly396Asp) mutation. This Binning DNA is a mixture of genomic DNA from healthy individuals and synthetic DNA that contains the target sequence detected by the above mentioned probes. Inclusion of one reaction with 5 µl SD022 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 signal(s). 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 SALSA Binning DNA SD022 product description, available online: www.mrcholland.com.

Performance characteristics

12-35% of LS cases are caused by pathogenic mutations in *MSH6* and 0-10% of *MSH6* pathogenic variants are large deletions and duplications detectable with MLPA. Therefore 0-3.5% of LS cases are expected to be positive for a *MSH6* copy number variation when tested with P072 MSH6-MUTYH. For *EPCAM*, all reported pathogenic variants are large deletions, all of which can be detected with P072 MSH6-MUTYH and these explain 0-10% of LS patients (https://www.ncbi.nlm.nih.gov/books/NBK1211/).

Almost all MAP patients have biallelic point mutations in the *MUTYH* gene. Deletions in this gene have rarely been described: the percentage of cases explained by large deletions/duplications is estimated to be <1%. The two common *MUTYH* point mutations c.536A>G (p.Tyr179Cys) and c.1187G>A (p.Gly396Asp) account for ≥90% of all *MUTYH* pathogenic variants in northern European populations. Up to 70% of MAP patients harbor at least one of these variants. The presence of the c.536A>G (p.Tyr179Cys) and c.1187G>A (p.Gly396Asp) mutations, can be detected by thisprobemix. Based on the populations tested in scientific literature the diagnostic sensitivity of the P072-D1 MSH6-MUTYH Probemix for MAP is estimated at ~70%.

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 all copy number 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) can be obtained. The standard deviation of each individual probe (with exception of the mutation-specific probes) 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 copy number 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.

The above mentioned FR values do not apply to the mutations-specific probes. The peaks of the mutation-specific probes are expected to be absent in the majority of samples tested and therefore their standard deviation cannot be determined. Clear signal (at least 10% of the median peak height of all reference probes in that sample) for one of these probes indicates that the mutation is present.

Please note that these above mentioned final ratios are only valid for germline testing. Final ratios are affected both by percentage of tumour cells and by possible subclonality.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic or subclonal 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.

P072 specific notes

- Simultaneous deletion of the 470 and 429 nt exon 9 *EPCAM* probes is a strong indication that the 3' end of *EPCAM* is disrupted, which can lead to hypermethylation and inactivation of *MSH2* (Ligtenberg *et al.* 2009). We recommend using SALSA MLPA Probemix ME011 Mismatch Repair Genes to test the methylation status of the *MSH2* promoter and of other mismatch repair genes. The probes with genomic targets located between the *EPCAM* and *MSH2* gene can help determine the extent of a deletion/duplication.
- The two *MUTYH* mutation-specific probes are only intended to determine the presence (or absence) of the mutation.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *MSH6* and *MUTYH* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P072 MSH6-MUTYH, with the exception of the presence of the two most common point mutations in the *MUTYH* gene among Europeans (c.536A>G (p.Tyr179Cys) and c.1187G>A (p.Gly396Asp)).
- 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.

- MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. In addition, subclonality of the aberration affects the final ratio of the corresponding probe. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample, especially in solid tumours with more chaotic karyotypes.

Confirmation of results

Copy number changes detected by only a single probe as well as the two common *MUTYH* point mutations always require confirmation by another method. Because the two *MUTYH* mutation-specific probes are only intended to determine the presence of the mutation, positive results obtained for either of these probes need to be confirmed by sequence analysis to determine the zygosity of the mutation.

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.

Mutation databases

We strongly encourage users to deposit positive results in the *MSH6, MUTYH, MSH2* and *EPCAM* LOVD mutation databases at https://databases.lovd.nl/shared/genes. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report false positive results due to SNVs and unusual results (e.g., a duplication of *MSH6* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P072-D1 MSH6-MUTYH

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*MSH6*	*MUTYH*	*EPCAM/MSH2*
64-105	Control fragments – see table in probemix content section for more information				
130	Reference probe 18835-L24359	3p14			
135	**MUTYH probe** 20901-L28955			**Exon 1**	
142	**EPCAM probe** 04249-L29773				**Exon 3**
150	**MSH6 probe** 04243-L29774		**Exon 7**		
155	Reference probe 14199-L29775	2q13			
160	**MSH6 probe** 04244-L03599		**Exon 8**		
166	**MUTYH probe** 15780-L17837			**Exon 6**	
172	**MUTYH probe** 15781-L17838			**Exon 4**	
178	**MSH6 probe** 04245-L29776		**Exon 9**		
184 §	**MUTYH probe** 18416-SP0654-L23441			**c.536A>G mutation-specific**	
190	Reference probe 14330-L31002	2q32			
196	**MSH6 probe** 04246-L03601		**Exon 10**		
202	**MUTYH probe** 20902-L29777			**Exon 11**	
208 ◎	**MSH2 probe** 12006-L29778				**Upstream**
213	**MSH6 probe** 06230-L29780		**Exon 1**		
220	Reference probe 12424-L29630	22q12			
226	**MSH6 probe** 21977-L31340		**Exon 1**		
232	**MUTYH probe** 15788-L17845			**Exon 5**	
243	**MUTYH probe** 15787-L29781			**Exon 15**	
250	**MSH6 probe** 12011-L29631		**Exon 4**		
258 §	**MUTYH probe** 18417-SP0655-L23442			**c.1187G>A mutation-specific**	
267	Reference probe 17834-L22900	6q21			
274	**MSH6 probe** 12012-L31341		**Exon 2**		
283	**MSH6 probe** 12005-L29785		**Exon 5**		
288	**MUTYH probe** 15792-L29786			**Exon 2**	
295	Reference probe 14829-L17169	10q26			
303	**MSH6 probe** 20894-L24863		**Exon 3**		
310	**MSH6 probe** 21978-L31342		**Exon 5**		
318	**EPCAM probe** 20891-L29632				**Exon 8**
328	**MUTYH probe** 18355-L23309			**Exon 13**	
340 ¬	**KCNK12 probe** 08663-L26400				**Downstream of *MSH2***
349	**MSH6 probe** 18113-L29787		**Exon 2**		
358	**MSH2 probe** 02735-L29788				**Exon 1**
367	**MSH6 probe** 01252-L24416		**Exon 4**		
377	Reference probe 10693-L19115	6p12			
388	**MSH6 probe** 01253-L30953		**Exon 6**		
397	Reference probe 08792-L30954	10q21			
404	**MUTYH probe** 18420-L30955			**Exon 9**	
411 ◎	**EPCAM probe** 13146-L14626				**Downstream**
420	**MUTYH probe** 20900-L28954			**Exon 16**	
429	**EPCAM probe** 13215-L29506				**Exon 9**
436	Reference probe 06948-L29805	3q29			
445	**MSH6 probe** 18760-L24128		**Exon 4**		
454	Reference probe 16571-L29791	11q13			
470	**EPCAM probe** 18132-L03603				**Exon 9**
478	Reference probe 13539-L29861	19p13			
487 ±	**MUTYH probe** 20898-L29793			**Exon 8**	
500	Reference probe 19675-L27455	4p13			

[a] See section Exon numbering on page 3 for more information.

§ Mutation-specific probe. This probe will only generate a signal when the indicated mutation is present. Both mutation-specific probes consist of three parts and have 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. Masking of the mutation-specific signal due to another mutation or SNP in the probe target can only occur when both are present on the same allele.
± SNP rs200872702 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
¬ 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.
℗ Copy number variations detected by only this probe are of unknown clinical significance.

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. P072-D1 probes arranged according to chromosomal location

Table 2a. *EPCAM/MSH2/MSH6* region

Length (nt)	SALSA MLPA probe	Gene / exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		EPCAM	**NM_002354.3**		
		start codon	*196-198*		
142	04249-L29773	Exon 3	571-572	CTGGGGTCAGAA-GAACAGACAAGG	11.2 kb
318	20891-L29632	Exon 8	1065-1066	GTTATTTCCAGA-AAGAAGAGAATG	1.5 kb
		stop codon	*1138-1140*		
470 #	18132-L03603	Exon 9	1189-1190	AAATGGACACAA-ATTACAAATGTG	0.1 kb
429	13215-L29506	Exon 9	1320-1319 reverse	GGTCAAATTTCA-AGATTGGTAAAG	3.3 kb
411 ℗	13146-L14626	Downstream	3.1 kb after exon 9	TCATTCCCAGAA-TAACTAAAATCT	10.3 kb
		MSH2	**NM_000251.3**		
208 ℗	12006-L29778	Upstream	2.8 kb upstream of *MSH2*	AGAGATTTCTAA-ATTGAGCTGATT	2.6 kb
358	02735-L29788	Exon 1	156 nt before exon 1	GCGTGCGCGGGA-AGCTGGGCCGCG	118.3 kb
340 ¬	08663-L26400	Downstream (*KCNK12*)	Between *MSH2* and *MSH6*	CCTGCTCGGCGT-GTGCTGCATTTA	261.8 kb
		MSH6	**NM_000179.3**		
213	06230-L29780	Exon 1	37 nt before exon 1	TGATTGGCCACT-GGGGCCCGGGTT	0.1 kb
		start codon	*90-92*		
226	21977-L31340	Exon 1	101-102	ATGTCGCGACAG-AGCACCCTGTAC	7.7 kb
274	12012-L31341	Exon 2	391-392	GGCCAAGATGGA-GGGTTACCCCTG	0.1 kb
349	18113-L29787	Exon 2	475-476	ATCAGTCCGTGT-TCATGTACAGTT	5.0 kb
303	20894-L24863	Exon 3	679-680	GGCAGTTTGTGA-TGAGCCCTCAGA	2.8 kb
367	01252-L24416	Exon 4	977-978	CCTGTCAAAGTT-GCTCGAAAGCGG	0.6 kb
445	18760-L24128	Exon 4	1628-1629	AGGAGGGAGATC-TGTAGGATCATT	0.9 kb
250	12011-L29631	Exon 4	2560-2561	GCTACTCAGTAA-AATTCATAATGT	3.0 kb
310	21978-L31342	Exon 5	3310-3309 reverse	CTGGGCGACACA-TAGGACCATCAC	0.2 kb
283	12005-L29785	Exon 5	3465-3466	AGGAAAATGGCA-AAGCCTATTGTG	1.3 kb
388	01253-L30953	Exon 6	3563-3564	CAGATGGGTTGT-TACGTCCCTGCT	0.7 kb
150	04243-L29774	Exon 7	3698-3699	ATACTCATGCAT-GCAACAGCACAT	0.7 kb
160	04244-L03599	Exon 8	3865-3864 reverse	GCACAGCAACAT-TTTGAGAATAAT	0.2 kb
178	04245-L29776	Exon 9	3987-3988	AAAGCTATGGCT-TTAATGCAGCAA	0.3 kb
		stop codon	*4170-4172*		
196	04246-L03601	Exon 10	4184-4183 reverse	TCAAAGCTTCCA-ATGTAGTCAGTC	-

Table 2b. *MUTYH*

Length (nt)	SALSA MLPA probe	*MUTYH* exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
			NM_001128425.2		
135	20901-L28955	Exon 1	133-132 reverse	TTCCGACGGTGA-GCGGCTTCCCAG	5.9 kb
		start codon	*187-189 (exon 1)*		
288	15792-L29786	Exon 2	322-323	ACAACAGTCAGG-CCAAGCCTTCTG	1.1 kb
	No probe	Exon 3			
172	15781-L17838	Exon 4	9 nt after exon 4	CTGGTCAGTACA-TCTCCTGAGAGC	0.1 kb
232	15788-L17845	Exon 5	605-606	GCTGCAGCAGAC-CCAGGTTGCCAC	0.2 kb
166	15780-L17837	Exon 6	3 nt before exon 6	TGCCTGTGGCTA-TAGAAGTGGCCT	0.2 kb
184 §	18416-SP0654-L23441	c.536A>G (p.Tyr179Cys) in exon 7	722-721 & 694-693 reverse	CACGAGAATAG**C**-28 nt spanning oligo-CTCCTGTGGGTA	0.2 kb
487 ±	20898-L29793	Exon 8	853-854	CAGCTGGGGCCA-TTGCCTCTATCG	0.2 kb
404	18420-L30955	Exon 9	941-942	CATTGGTGCTGA-TCCCAGCAGCAC	0.3 kb
	No probe	Exon 10			
202	20902-L29777	Exon 11	1129-1128 reverse	TAAGAGCTGTTC-CTGCTCCACCTG	0.6 kb
	No probe	Exon 12			
258 §	18417-SP0655-L23442	c.1187G>A (p.Gly396Asp) in exon 13	1373-1374 & 1408-1409	CTCCCTCTCAG**A**-35 nt spanning oligo-CCTGGGAGCCCT	0.1 kb
328	18355-L23309	Exon 13	1505-1504 reverse	CACTTACCTCCC-CAAGGTGCCGGA	0.9 kb
	No probe	Exon 14			
243	15787-L29781	Exon 15	intron 14-1663	CTTCTTGTCTAG-GTTTTCCGTGTG	1.2 kb
420	20900-L28954	Exon 16	1823-1822 reverse	ACTGGGCTGCAC-TGTTGAGGCTGT	-
		stop codon	*1834-1836 (exon 16)*		

[a] See section Exon numbering on page 3 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 indicated mutation is present. Both mutation-specific probes consist of three parts and have 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. Masking of the mutation-specific signal due to another mutation or SNP in the probe target can only occur when both are present on the same allele.
± SNP rs200872702 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
¬ 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.
ⵙ Copy number variations detected by only this probe are of unknown clinical significance.
This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

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 3. Reference probes arranged according to chromosomal location.

Length (nt)	SALSA MLPA probe	Gene	Chromosomal band (hg18)	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
155	14199-L29775	*EDAR*	2q13	GAGAGTTCTGTG-GGTGGAGAGAAG	02-108,893937
190	14330-L31002	*SLC40A1*	2q32	GATTGACCAGTT-AACCAACATCTT	02-190,138497
130	18835-L24359	*FLNB*	3p14	TCACAGCCAAGA-TCACAGGTAGGG	03-058,107720
436	06948-L29805	*OPA1*	3q29	ACAGCAATGGGA-TGCAGCTATTTA	03-194,859398
500	19675-L27455	*ATP8A1*	4p13	CAGATTCTTCTT-CGAGGAGCTCAG	04-042,278439

Length (nt)	SALSA MLPA probe	Gene	Chromosomal band (hg18)	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
377	10693-L19115	*PKHD1*	6p12	TTGTACTCATCT-GTTGAATTCAGT	06-051,721327
267	17834-L22900	*FYN*	6q21	CCTGTCACAAAG-GGATGCCAAGGC	06-112,128077
397	08792-L30954	*PCDH15*	10q21	GTCGTAGTGGAG-TCCATTGGAGCT	10-055,270172
295	14829-L17169	*UROS*	10q26	AGTCCCTCTGGC-CTCACATACAGT	10-127,473456
454	16571-L29791	*SHANK2*	11q13	TCGAGGTACGAT-GCGAAGGCAGAA	11-070,014011
478	13539-L29861	*CACNA1A*	19p13	GCCATTACATCC-TGAACCTGCGCT	19-013,255144
220	12424-L29630	*LARGE1*	22q12	CAGTGAGGCTGA-TGTCAACAGTGA	22-032,063613

Complete probe sequences are available at www.mrcholland.com.

Related SALSA MLPA probemixes

Condition		Gene	SALSA MLPA Probemix
Lynch syndrome (HNPCC)		*MLH1*	P003 MLH1/MSH2 P248 MLH1-MSH2 Confirmation ME011 Mismatch Repair Genes ME042 CIMP
		MSH2	P003 MLH1/MSH2 P248 MLH1-MSH2 Confirmation ME011 Mismatch Repair Genes
		MSH6	P072 MSH6-MUTYH ME011 Mismatch Repair Genes
		PMS2	P008 PMS2 ME011 Mismatch Repair Genes
		EPCAM	P003 MLH1/MSH2 P072 MSH6-MUTYH ME011 Mismatch Repair Genes
Polyposis syndrome	MAP	*MUTYH*	P378 MUTYH P043 APC P072 MSH6-MUTYH
	FAP	*APC*	P043 APC
	AFAP	*APC*	P043 APC

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P072 product history	
Version	*Modification*
D1	Two mutation specific probes and eight probes for *MUTYH* have been added and three *MUTYH* probes have been replaced. One *MSH6* and all *MLH1* probes have been removed. All reference probes have been adjusted. Several probes have a small change in length but no change in sequence detected.
C1	One additional probe for *MSH6* (exon 4) and three extra reference probes have been included. Five reference probes have been replaced.
B2	The 88 and 96 nt control fragments have been replaced (QDX2).
B1	Extra *TACSTD1* (*EPCAM*) probes have been added.
A1	First release.

Implemented changes in the product description

Version D1-04 – 11 October 2022 (04P)
- Sections *Clinical background* and *Performance characteristics* were updated according to recent literature.
- *References* and *Selected Publications* were curated and new literature was included.
- Table with positive samples was adjusted.
- Minor textual and lay-out changes throughout document.

Version D1-03 – 22 July 2021 (04P)
- Product description rewritten and adapted to a new template.
- Intended use has become Intended purpose and was rewritten using new template.
- Sections *Clinical background* and *Transcript variants* are rewritten for clarification and to include recent knowledge.
- Performance characteristics updated.
- Throughout document several statement are included/rephrased emphasizing that only the presence of the common *MUYTH* mutations can be detected and not the zygosity.
- Ligation sites of the probes targeting the *MSH6*, *MUTYH*, *EPCAM* and *MSH2* genes are updated according to new versions of the NM_ reference sequences.
- Recommended positive samples have been updated and are now listed in a table.
- Remarks in Table 1 and 2 for salt sensitivity was removed because no probes are sensitive according to current criteria and recent quality testing.
- Table containing the related probemixes has been updated.
- *References* and *Selected publications* were curated and new literature was included.
- UK has been added to the list of countries in Europe that accept the CE mark.

Version D1-02 – 15 June 2020 (04)
- Product is now registered for IVD use in Israel.

Version D1-01 – 07 November 2018 (04)
- The name of the probemix is changed.
- Product description restructured and adapted to a new template.

- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - P072 is now CE-marked. - Various minor textual or layout changes. - References to SD027 artificial duplication DNA have been removed as this product is obsolete. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. - Warning added to Table 1 and 2 for flanking probes and probes of unknown clinical significance.

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