Product Description
SALSA® MLPA® Probemix P003-D1 MLH1/MSH2

To be used with the MLPA General Protocol.

Version D1
For complete product history see page 14.

Catalogue numbers:
- **P003-025R:** SALSA MLPA Probemix P003 MLH1/MSH2, 25 reactions.
- **P003-050R:** SALSA MLPA Probemix P003 MLH1/MSH2, 50 reactions.
- **P003-100R:** SALSA MLPA Probemix P003 MLH1/MSH2, 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 P003 MLH1/MSH2 is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in specific regions of the *MLH1*, *MSH2* and *EPCAM* genes, as well as a recurrent 10 Mb inversion on chromosome arm 2p which disrupts the *MSH2* gene, in genomic DNA isolated from human peripheral whole blood specimens. P003 MLH1/MSH2 is intended to confirm a potential cause for and clinical diagnosis of Lynch syndrome and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P003 MLH1/MSH2 should be confirmed with the SALSA MLPA Probemix P248 MLH1-MSH2 Confirmation assay or a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *MLH1* and *MSH2* genes are point mutations, none of which will be detected by MLPA. P248 MLH1-MSH2 Confirmation cannot be used to verify deletions or duplications in the *EPCAM* gene. It is therefore recommended to use this assay in combination with sequence analysis.

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, pre-implantation or prenatal testing, population screening, or 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 SD052 Binning DNA.

Clinical background

Lynch syndrome, 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, endometrial cancer, ovarian cancer, gastric cancer and small bowel 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: *MLH1*, *MSH2*, *MSH6* or *PMS2*. Another cause of Lynch syndrome is a deletion of the 3' part of *EPCAM*, leading to constitutional epigenetic silencing of the downstream *MSH2* gene (Lynch et al. 2015). The estimated contribution of the different genes to Lynch syndrome is 15-40% for *MLH1*, 20-40% for *MSH2*, 12-35% for *MSH6*, 5-25% for *PMS2* and <10% for *EPCAM*. More information about Lynch syndrome is available on http://www.ncbi.nlm.nih.gov/books/NBK1211/.

Among the various defects in the *MLH1* and *MSH2* genes that have been found in patients, deletions and duplications of complete exons are usually missed by standard sequence analysis. The MLPA technique can detect most of these deletions and duplications and therefore complements sequence analysis of the *MLH1* and *MSH2* genes.

Gene structure

The *MLH1* gene spans ~57 kb on chromosome 3p22.2 containing 19 exons, *MSH2* spans ~80 kb on chromosome 2p21 containing 16 exons and *EPCAM* spans ~18 kb on chromosome 2p21 containing 9 exons. The *MLH1* LRG_216 is identical to GenBank NG_007109.2, the *MSH2* LRG_218 is identical to GenBank NG_007110.2, and the *EPCAM* LRG_215 is identical to GenBank NG_012352.2. All LRGs are available at www.lrg-sequence.org/.

Transcript variants

For *MLH1*, multiple variants have been described. Transcript variant 1 represents the most abundant transcript, encoding the full length protein (NM_000249.4; 2494 nt; coding sequence 31-2301; http://www.ncbi.nlm.nih.gov/gene/4292). 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 19.

For *MSH2*, two transcript variants have been described. Transcript variant 1 represents the longer transcript, encoding the longer isoform (NM_000251.3; 3115 nt; coding sequence 37-2841; http://www.ncbi.nlm.nih.gov/gene/4436). 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 16.

For *EPCAM*, one transcript variant has been described encoding the full length protein (NM_002354.3; 1547 nt; coding sequence 196-1140; http://www.ncbi.nlm.nih.gov/gene/4072). 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 9.

Exon numbering

The *MLH1, MSH2* and *EPCAM* exon numbering used in this P003-D1 MLH1/MSH2 product description is the exon numbering from the LRG_216, LRG_218 and LRG_215 sequences, respectively. 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 P003-D1 MLH1/MSH2 contains 50 MLPA probes with amplification products between 130 and 499 nucleotides (nt). This includes 19 probes for the *MLH1* gene, 18 probes for the *MSH2* gene and two probes for the *EPCAM* gene (formerly known as *TACSTD1*). *EPCAM* is a gene located just upstream of *MSH2*. Deletions of the last exon of *EPCAM* can result in silencing of the *MSH2* gene. Furthermore, this probemix contains two probes specific for the recurrent 10 Mb inversion that disrupts the *MSH2* gene (Wagner et al. 2002, Chen 2008, Rhees et al. 2014), which will only generate a signal when the inversion is present. In addition, nine reference probes are included that detect autosomal chromosomal locations.

Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal 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 human 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.

The P003-D1 probemix is not suitable for use on DNA extracted from formalin-fixed paraffin embedded (FFPE) or fresh tumour materials. The probemix does not contain enough reference probes to ensure proper data analysis when using these specimens.

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. The NIBSC Institute in the U.K. provides a kit with five DNA samples (NIBSC code: 11/218-XXX) harbouring heterozygous *MLH1* or *MSH2* exon deletions or amplifications: http://www.nibsc.org/documents/ifu/11-218-xxx.pdf. These have been tested with this P003-D1 probemix at MRC Holland and can be used as positive control samples for deletions of *MSH2* exons 1-6, *MSH2* exon 7, *MSH2* exons 1-2, *MSH2* exon 1 and amplification of *MLH1* exon 13. The quality of cell lines can change; therefore samples should be validated before use.

SALSA Binning DNA SD052

The SD052 Binning DNA provided with this probemix can be used for binning of all probes including the two inversion-specific probes (265 nt probe 20091-SP0917-L28216 and 317 nt probe 20090-SP0916-L28222 for a 10 Mb inversion with one breakpoint in intron 7 of *MSH2*). SD052 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 SD052 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation 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 SD052 Binning DNA product description, available online: www.mrcholland.com.

Performance characteristics

The diagnostic sensitivity of P003 is ~12%, as this is the total portion of Lynch syndrome which is caused by large rearrangements in *MLH1*, *MSH2* and *EPCAM*. Large deletions account for ~20-40% of variants in *MSH2,* ~10-20% of variants in *MLH1* and for all *EPCAM* variants that cause Lynch syndrome (http://www.ncbi.nlm.nih.gov/books/NBK1211/). Furthermore, the recurrent 10 Mb inversion of chromosome 2, which has one breakpoint in intron 7 of the *MSH2* gene, can be detected by this probemix (Wagner et al. 2002, Chen 2008, Rhees et al. 2014). *MLH1* or *MSH2* duplications have been rarely reported, but can also be detected with this probemix. Note that the P003 MLH1/MSH2 probemix does not contain probes for the detection of *MSH6* and *PMS2* genes, which are also implicated in Lynch syndrome. The analytical sensitivity and specificity for the detection of deletions in the *MSH2*, *MLH1* and *EPCAM* genes using point mutation negative samples with Lynch syndrome is very high and can be considered >99%.

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 *MLH1/MSH2/EPCAM* region specific MLPA probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication) and occasionally 4 (heterozygous triplication). Homozygous deletions may occur, but are extremely rare. 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

Copy number status	Final ratio (FR)
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 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.

P003 specific notes:

- Lynch syndrome due to *MLH1/MSH2/EPCAM* gene defects is an autosomal dominant disorder. A heterozygous deletion of one or more *MLH1* or *MSH2* exons that are present in the major transcript variants, NM_000249.4 (*MLH1*) and NM_000251.3 (*MSH2*), is expected to result in Lynch syndrome.
- Simultaneous deletion of the 472 and 481 nt *EPCAM* probes is a strong indication that the last *EPCAM* exon (exon 9) is disrupted, which can lead to methylation of the adjacent *MSH2* promoter and inactivation of *MSH2* (Ligtenberg et al. 2009, Kovacs et al. 2009).
- The presence of a peak at 265 nt and/or 317 nt is an indication that the 10 Mb chromosome 2 inversion is present in the sample DNA (Wagner et al. 2002, Chen 2008, Rhees et al. 2014). These probes do not generate a signal on normal samples. Please note that the peak height of these two probes can be strongly affected by DNA fragmentation. The 20091-SP0917-L28216 probe present at 265 nt and the 20090-SP0916-L28222 probe present at 317 nt require an intact DNA fragment to bind to of at least 220 nt or 520 nt, respectively, in inversion positive DNA samples, which is much longer than the length requirements for other MLPA probes (60-80 nt). DNA samples in which both probes show a clear peak, as well as samples in which a clear peak for only one of the two probes is observed, should be further investigated even when

these peaks are much lower than obtained on the SD052 Binning DNA sample. A non-specific peak close to the 265 nt peak can appear if the ligation reaction is performed at a temperature below 54°C.
- A duplication of an internal part of a gene usually results in a defective copy of that gene, as the duplicated sequence is typically located directly adjacent to the original sequence, resulting in a defective transcript. However, duplication of the complete *MLH1* or *MSH2* gene is not expected to result in disease. Please note the remark above on duplications that include the first or last exon of a gene.

Limitations of the procedure
- In most populations, the major cause of genetic defects in the *MLH1* and *MSH2* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P003 MLH1/MSH2.
- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations, except the 10 Mb inversion with one breakpoint in intron 7 of *MSH2*. 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 SALSA MLPA Probemix P248 MLH1-MSH2 Confirmation or 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 SALSA MLPA Probemix P248 MLH1-MSH2 Confirmation or 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. All probes included in P248 MLH1-MSH2 Confirmation have different ligation sites from those in P003, except for the 148 nt exon 1 MSH2 probe. The SALSA MLPA Probemix P072 MSH6 contains additional probes for *EPCAM* and the region between *MSH2* and *EPCAM*. In addition, the P072 probemix can be used to detect copy number variations in the *MSH6* gene.

***MLH1/MSH2* mutation database**

We strongly encourage users to deposit positive results in the International Society for Gastrointestinal Hereditary Tumours (INSiGHT) and/or LOVD database (http://www.insight-group.org; http://www.lovd.nl). 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 *MLH1* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P003-D1 MLH1/MSH2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*MLH1*	*MSH2/EPCAM*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 00797-L21056	5q		
136	Reference probe 00981-L00566	10p		
142	**MLH1 probe** 14701-L19726		**Exon 1**	
148 »	**MSH2 probe** 12036-L02162			**Exon 1**
154	**MLH1 probe** 01008-L00577		**Exon 2**	
160	**MSH2 probe** 00906-L00494			**Exon 2**
166	**MLH1 probe** 00888-L00476		**Exon 3**	
172	**MSH2 probe** 01029-L00601			**Exon 3**
178	**MLH1 probe** 00889-L23928		**Exon 4**	
184	**MSH2 probe** 00908-L00496			**Exon 4**
190	**MSH2 probe** 11287-L12006			**Exon 1**
196	Reference probe 17056-L20134	7q		
203	**MLH1 probe** 20360-L28308		**Exon 5**	
211 ±	**MSH2 probe** 00909-L00497			**Exon 5**
217	**MSH2 probe** 13145-L14624			**Exon 1**
224	**MLH1 probe** 14702-L28215		**Exon 6**	
231	**MSH2 probe** 15298-L17064			**Exon 6**
236	Reference probe 15941-L18067	2q		
242	**MLH1 probe** 00892-L16355		**Exon 7**	
249	**MSH2 probe** 11634-L16356			**Exon 7**
256	**MLH1 probe** 00893-L00481		**Exon 8**	
265 §	**MSH2 probe** 20091-SP0917-L28216			**Intron 7**
269	**MSH2 probe** 00912-L28217			**Exon 8**
278	**MLH1 probe** 00894-L28218		**Exon 9**	
287	Reference probe 18920-L25191	1p		
292	**MSH2 probe** 00913-L28221			**Exon 9**
301	**MLH1 probe** 00895-L00483		**Exon 10**	
310	**MSH2 probe** 11288-L12007			**Exon 10**
317 §	**MSH2 probe** 20090-SP0916-L28222			**Intron 7**
326	**MLH1 probe** 00896-L18364		**Exon 11**	
332	**MSH2 probe** 00915-L28223			**Exon 11**
340 ∫	**MLH1 probe** 14703-L28224		**Exon 12**	
346	**MSH2 probe** 18133-L23925			**Exon 12**
355	**MLH1 probe** 00898-L23926		**Exon 13**	
364	**MSH2 probe** 01013-L00575			**Exon 13**
375	Reference probe 00681-L11147	4q		
382	**MLH1 probe** 00899-L00586		**Exon 14**	
391	**MSH2 probe** 00918-L00506			**Exon 14**
401	**MLH1 probe** 00900-L00488		**Exon 15**	
409	**MSH2 probe** 00919-L00585			**Exon 15**
418	**MLH1 probe** 01009-L00576		**Exon 16**	
427	**MSH2 probe** 01053-L14623			**Exon 16**
436 ±	**MLH1 probe** 01030-L00602		**Exon 17**	
445	**MLH1 probe** 01031-L00603		**Exon 18**	
454	**MLH1 probe** 12094-L12994		**Exon 19**	
463	Reference probe 00979-L00568	10p		
472	**EPCAM probe** 13147-L14404			***EPCAM* exon 9**
481	**EPCAM probe** 18132-L24050			***EPCAM* exon 9**
490	Reference probe 04274-L24051	13q		
499 ±	Reference probe 14882-L21050	14q		

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

§ Mutation-specific probe. This probe will only generate a signal when the 10 Mb inversion with one breakpoint in intron 7 of *MSH2* is present. This probe consists of three parts and has two ligation sites. See note about this probe on page 5, section P003 specific notes.
± SNP rs147586703 could influence the probe signal of the 499 nt reference probe. SNPs rs1800146 and rs55907433 could influence the probe signal of the 436 nt target probe. SNP rs146577635 could influence the probe signal of the 211 nt target probe. In case of apparent deletions, it is recommended to sequence the regions targeted by these probes.
» Detects the same sequence as the 355 nt probe in SALSA MLPA Probemix P248 MLH1-MSH2 Confirmation.
∫ See note on the 340 nt MLH1 exon 12 probe below Table 2a.

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. P003-D1 probes arranged according to chromosomal location

Table 2a. *MLH1* gene

Length (nt)	SALSA MLPA probe	*MLH1* exon[a]	Ligation site NM_000249.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*31-33 (Exon 1)*		
142	14701-L19726	Exon 1	37-38	CCAAAATGTCGT-TCGTGGCAGGGG	3.1 kb
154	01008-L00577	Exon 2	215-216	GATTCAGATCCA-AGACAATGGCAC	4.3 kb
166	00888-L00476	Exon 3	295-296	TGCAGTCCTTTG-AGGATTTAGCCA	3.4 kb
178	00889-L23928	Exon 4	359-360	CATAAGCCATGT-GGCTCATGTTAC	2.6 kb
203	20360-L28308	Exon 5	459-460	AAACCATGTGCT-GGCAATCAAGGG	1.8 kb
224	14702-L28215	Exon 6	496-497	TGGAGGACCTTT-TTTACAACATAG	3.0 kb
242	00892-L16355	Exon 7	596-597	ACACAATGCAGG-CATTAGTTTCTC	0.2 kb
256	00893-L00481	Exon 8	650-651	TGTTAGGACACT-ACCCAATGCCTC	2.4 kb
278	00894-L28218	Exon 9	752-753	CCTAGCCTTCAA-AATGAATGGTTA	3.1 kb
301	00895-L00483	Exon 10	851-852	TTCCTTGAGAAA-AGCCATAGAAAC	2.9 kb
326	00896-L18364	Exon 11	1022-1023	GCAGCACATCGA-GAGCAAGCTCCT	5.5 kb
340 ∫	14703-L28224	Exon 12	1314-1315	AGGCAGCAAGAT-GAGGAGATGCTT	3.0 kb
355	00898-L23926	Exon 13	1497-1498	TCCCGAAAGGAA-ATGACTGCAGCT	11.4 kb
382	00899-L00586	Exon 14	1631-1632	CGTGGGCTGTGT-GAATCCTCAGTG	2.1 kb
401	00900-L00488	Exon 15	1724-1725	CCAGATACTCAT-TTATGATTTTGC	5.3 kb
418	01009-L00576	Exon 16	1799-1800	CATGCTTGCCTT-AGATAGTCCAGA	1.0 kb
436 ±	01030-L00602	Exon 17	2004-2005	ATCTTCATTCTT-CGACTAGCCACT	0.4 kb
445	01031-L00603	Exon 18	2101-2100 reverse	CTCCTCAGATAT-GTACTGCTTCCG	1.8 kb
		stop codon	*2299-2301 (Exon 19)*		
454	12094-L12994	Exon 19	2427-2426 reverse	TATCAGAAGGCA-AGTATAAGTCTT	

± SNPs rs1800146 and rs55907433 could influence the probe signal of the 436 nt probe. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
∫ The 340 nt MLH1 exon 12 probe has been found to give false positive deletion/duplication results. This is probably due to an unusual low sensitivity of this probe to sample DNA depurination. Reduced signals of other probes caused by sample depurination lead to seemingly high signals of the 340 nt probe. Depurination of sample DNA can occur in samples with insufficient buffering capacity. The MLH1 exon 12 probe in the P248 probemix does not have this problem and can be used for confirmation. More information on sample DNA depurination is available on www.mrcholland.com.

Table 2b. *MSH2* gene

Length (nt)	SALSA MLPA probe	*MSH2* exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
			***EPCAM* gene**		
			NM_002354.3		
		stop codon	*1138-1140 (Exon 9)*		
481 #	18132-L24050	Exon 9	1189-1190	AAATGGACACAA-ATTACAAATGTG	0.1 kb
472	13147-L14404	Exon 9	1320-1319 reverse	GGTCAAATTTCA-AGATTGGTAAAG	16.0 kb
			***MSH2* gene**		
			NM_000251.3		
217	13145-L14624	Exon 1	346 nt before exon 1	CCGGGCACATTA-CGAGCTCAGTGC	0.2 kb
148 »	12036-L02162	Exon 1	156 nt before exon 1	GCGTGCGCGGGA-AGCTGGGCCGCG	0.7 kb
		start codon	*37-39 (Exon 1)*		
190	11287-L12006	Exon 1	269 nt after exon 1 reverse	GAACTAGAACAA-TGCATTAAAATG	4.8 kb
160	00906-L00494	Exon 2	339-340	TATAGAGTTGAA-GTTTATAAGAAT	1.7 kb
172	01029-L00601	Exon 3	487-488	TTGTGGGTGTTA-AAATGTCCGCAG	2.3 kb
184	00908-L00496	Exon 4	774-775	CGGTTGTTGAAA-GGCAAAAAGGGA	1.8 kb
211 ±	00909-L00497	Exon 5	854-855	ACTGTCTGCGGT-AATCAAGTTTTT	2.1 kb
231	15298-L17064	Exon 6	1029-1030	GCCTTGCTGAAT-AAGTGTAAAACC	13.5 kb
249	11634-L16356	Exon 7	1233-1234	AGACAAGCAGCA-AACTTACAAGAT	15.7 kb
269	00912-L28217	Exon 8	1360-1361	CTCCTCTTACTG-ATCTTCGTTCTG	17.5 kb
292	00913-L28221	Exon 9	1447-1448	AATTCCTTGTAA-AACCTTCATTTG	3.9 kb
310	11288-L12007	Exon 10	190 nt after exon 10	GACTGAAGTGGT-ACTTTGGGTCTA	4.0 kb
332	00915-L28223	Exon 11	1743-1742 reverse	GCTTCTTCATAT-TCTGTTTTATTT	4.1 kb
346	18133-L23925	Exon 12	1891-1892	CACCTGTTCCAT-ATGTACGACCAG	1.3 kb
364	01013-L00575	Exon 13	2107-2108	TCATGGCCCAAA-TTGGGTGTTTTG	1.9 kb
391	00918-L00506	Exon 14	2314-2315	CCTACGATGGAT-TTGGGTTAGCAT	2.5 kb
409	00919-L00585	Exon 15	2597-2598	ACTTGAGGAGTT-TCAGTATATTGG	2.0 kb
427	01053-L14623	Exon 16	2673-2674	GTGTTTCAGCAA-GGTGAAAAAATT	
		stop codon	*2839-2841 (Exon 16)*		
265 §	20091-SP0917-L28216	Intron 7	This probe will only generate a signal on mutation positive DNA samples (10 Mb inversion with one breakpoint in *MSH2* intron 7).		
317 §	20090-SP0916-L28222	Intron 7	This probe will only generate a signal on mutation positive DNA samples (10 Mb inversion with one breakpoint in *MSH2* intron 7).		

[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.

§ Mutation-specific probe. This probe will only generate a signal when the 10 Mb inversion with one breakpoint in intron 7 of *MSH2* is present. This probe consists of three parts and has two ligation sites. See note about this probe on page 5, section P003 specific notes.
± SNP rs146577635 could influence the probe signal of the 211 nt probe. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
» Detects the same sequence as the 355 nt probe in SALSA MLPA Probemix P248 MLH1-MSH2 Confirmation.
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.

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

Related SALSA MLPA probemixes

Condition		Gene	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
	AFAP	*APC*	P043 APC
	FAP	*APC*	P043 APC

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P003 product history	
Version	*Modification*
D1	Two probes specific for the recurrent 10 Mb inversion on chr. 2p have been added; and one reference probe has been replaced.
C1	Two reference probes have been replaced and two extra reference probes have been added.
B2	The 88 and 96 nt DNA denaturation control fragments have been replaced (QDX2).
B1	One MLH1 probe (exon 19) and four MSH2 probes (exons 1, 1, 7 and 10) have been replaced. In addition, one extra MSH2 exon 1 probe, two extra EPCAM (formerly known as TACSTD1) probes and two extra control fragments at 100 and 105 nt have been included.
A2	One extra MSH2 exon 1 probe has been included.
A1	First release.

Implemented changes in the product description
Version D1-08 – 07 May 2024 (04P) - Warning relating to salt sensitivity («) removed for probes LATS2-D08-CC-490-M (Table 1) and MSH2-D07CC-GT-249-M (Table 1 and Table 2). - Footnote related to salt sensitivity of probes removed from Table 1 and Table 2. Version D1-07 – 24 February 2023 (04P) - Clinical background updated according to the most recent literature. - Morocco has been removed from the list of countries with IVD status. - Selected publications updated (five articles removed). - Minor textual or layout changes.

Version D1-06 – 29 October 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.
- Clinical background updated according to the most recent literature.
- Added information on Gene structure and Transcript variants for the *EPCAM* gene
- Ligation sites of the probes targeting the *MLH1*, *MSH2* and *EPCAM* genes updated according to new version of the NM_ reference sequence.
- Various minor textual or layout changes.
- SNP warning added to table 1 and 2 for the 436 nt and 211 nt probes.
- Note on the 278 nt MLH1 exon 9 probe removed from table 1 and 2, as it is not relevant anymore for the P003-D1 version.
- Selected publications updated (three recent articles added, ten articles removed).

Version D1-05 — 22 June 2020 (02P)
- Product description rewritten and adapted to a new template.
- Minor textual changes.
- Colombia added as country with IVD status.
- Warning added to Table 1 for the 499 nt reference probe.
- Removed phrase on FFPE derived samples from the footnote for the 340 nt probe below Table 2a.
- Added explanation on why P003 is not suitable for use on DNA extracted from FFPE or fresh tumour materials to 'required specimens' section.
- Note on interpretation of results of the 265 nt and 317 nt probes rephrased for clarity purposes.
- New references added.

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

IVD	EUROPE* CE ISRAEL COLOMBIA
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.