Product Description
SALSA® MLPA® Probemix P008-C1 PMS2

To be used with the MLPA General Protocol.

Version C1
For complete product history see page 13.

Catalogue numbers:
- **P008-025R:** SALSA MLPA Probemix P008 PMS2, 25 reactions.
- **P008-050R:** SALSA MLPA Probemix P008 PMS2, 50 reactions.
- **P008-100R:** SALSA MLPA Probemix P008 PMS2, 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.

This product requires the identification of suitable reference samples for proper data analysis. For more information, see appendix (page 15).

Intended purpose
The SALSA MLPA Probemix P008 PMS2 is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in exons 1-11 of the *PMS2* gene and in exons 12-15 of the *PMS2* or *PMS2CL* genes in genomic DNA isolated from human peripheral whole blood specimens. P008 PMS2 is intended to confirm a potential cause for and clinical diagnosis of Lynch syndrome or constitutional mismatch repair deficiency syndrome and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P008 PMS2 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 *PMS2* 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 of the *PMS2* gene. Suitable reference samples should be identified for proper data analysis. To determine whether a CNV of exons 12-15 is present in *PMS2* **or** *PMS2CL*, MLPA should always be combined with other methods, for example gene-specific long-range PCR and sequencing of the amplification products.

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 SALSA Reference Selection DNA SD082.

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, i.e. *MLH1*, *MSH2*, *MSH6* or *PMS2*. Another cause of Lynch syndrome is 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/.

Constitutional mismatch repair deficiency (CMMRD) syndrome is a rare inherited childhood cancer syndrome characterized by early-onset colorectal cancers, haematological malignancies, and brain tumours. These malignancies are often associated with features of neurofibromatosis type 1 (NF1), such as café-au-lait macules. CMMRD is caused by bi-allelic, i.e. homozygous or compound heterozygous, germline mutations in *MLH1*, *MSH2*, *MSH6* or *PMS2* (Wimmer and Etzler 2008). Mutations in *PMS2* are the most common cause of this recessive condition and are responsible for ~50-60% of the CMMRD cases reported thus far (Herkert et al. 2011; Wimmer et al. 2014).

Among the various defects in the *PMS2* gene that have been found in Lynch syndrome and CMMRD patients are deletions and duplications of complete exons, which 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 *PMS2* gene.

Gene structure
The *PMS2* gene spans 36 kilobases (kb) on chromosome 7p22.1 and contains 15 exons. The *PMS2* LRG_161 is available at www.lrg-sequence.org and is identical to GenBank NG_008466.1.

Mutation analysis of the *PMS2* gene is complicated due to the presence of multiple pseudogenes. One of these pseudogenes, *PMS2CL*, has an almost 100% sequence identity with *PMS2* exons 12-15 and is located at only 760 kb distance from *PMS2* (De Vos et al. 2004). For *PMS2* exons 1-5, a large number of different pseudogenes exist, each having a very high sequence homology to the real gene.

Transcript variants
For *PMS2,* multiple transcript variants have been described: http://www.ncbi.nlm.nih.gov/gene/5395. Transcript variant 1 is the predominant transcript and encodes isoform a (NM_000535.7; 5093 nt; coding sequence 31-2619). The ATG translation start site is located in exon 1 and the stop codon is located in exon 15.

Exon numbering
The *PMS2* exon numbering used in this P008-C1 PMS2 product description is the exon numbering from the LRG_161 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 P008-C1 PMS2 contains 47 MLPA probes with amplification products between 128 and 483 nucleotides (nt). This includes 34 probes for the *PMS2*/*PMS2CL* genes. In addition, 13 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).

Detection of abnormal copy numbers of *PMS2* exons is complicated due to the existence of many pseudogenes. In exons 12-15 no reliable sequence differences exist between *PMS2* and the *PMS2CL* pseudogene (van der Klift et al. 2010). As a consequence, it is not possible to design MLPA probes that are 100% specific for these *PMS2* exons. However, the combined copy number of *PMS2* and *PMS2CL* is very consistent among healthy individuals. For this reason, five probes are included that detect sequences present in exons 12-15 of both *PMS2* and *PMS2CL*. As these probes detect both genes, a normal individual has four copies per cell and a deletion or duplication of one copy results in a probe ratio of 0.75 or 1.25, respectively. Together with the exon 1-11 probes, the analysis of these probes should exclude *PMS2* copy number changes in the great majority of samples tested.

In case one or more of these five probes do indicate a copy number change in the exon 12-15 region, it should be determined whether the copy number change is in *PMS2* or in *PMS2CL*. To facilitate this, ten probes are included that detect the copy number of both allelic forms of five SNPs in exons 11-15 of *PMS2*/*PMS2CL*. The distribution of these SNP alleles among *PMS2* and *PMS2CL* varies. More information can be found in the "Interpretation of results" section, and in the appendix. Analysis of these SNP-specific probes, complemented with gene-specific long range PCR and (next generation) sequencing analysis, can give further indications on whether a copy number change of exons 12-15 is in the *PMS2* gene or in *PMS2CL* (Li et al. 2015, Vaughn et al. 2011). As reported by Vaughn et al. (2011 and 2013), the great majority of deletions detected in exons 12-15 are in fact located in the *PMS2* gene.

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 (with the exception of the ten SNP-specific 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.

Reference samples
A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. Reference samples should be derived from different unrelated individuals who are from families without a history of hereditary predisposition to cancer. Selecting suitable reference samples for the P008 PMS2 probemix is complicated due to the presence of ten SNP-specific probes in this probemix. Suitable

reference samples have two copies for each of the ten SNP probe targets. In our experience, only 25% of normal samples tested has this genotype and can thus be used as reference samples.

SALSA Reference Selection DNA SD082 (provided with this probemix; see SD082 section below) is an aid that can be used to select suitable reference samples from your own sample collection. Include three reactions with Reference Selection DNA SD082 as well as reactions on a number of DNA samples from healthy unrelated individuals in an initial experiment. Suitable reference samples provide results equivalent to Reference Selection DNA SD082 for each probe in the P008 probemix, including the ten SNP-specific probes. Note that SD082 should only be used to identify reference samples and not as reference sample for data normalisation purposes in routine use. More information on the selection of suitable reference samples, both with and without the use of Reference Selection DNA SD082, can be found in the appendix of this product description (see page 15).

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. 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.

SALSA Reference Selection DNA SD082
The selection of suitable reference DNA samples that can be used with P008 PMS2 is complicated. To facilitate the selection of suitable reference DNA samples from your own sample collection, a reference selection DNA sample (catalogue number SD082) is provided with this probemix from MRC Holland. Reference Selection DNA SD082 should only be used for initial experiments on DNA samples from healthy individuals with the intention to identify suitable reference samples. **SD082 should not be used as a reference sample in subsequent experiments.** For further details, consult the Reference Selection DNA SD082 product description, available online: www.mrcholland.com.

Performance characteristics
Large deletions account for 20-55% of the variants in *PMS2* that cause Lynch syndrome (Dong et al. 2020; Rosty et al. 2016; Senter et al. 2008; van der Klift et al. 2010; van der Klift et al. 2016; Vaughn et al. 2013; http://www.ncbi.nlm.nih.gov/books/NBK1211/). The overall frequency of *PMS2* deletions in CMMRD patients is ~12% (Bodo et al. 2015; Herkert et al. 2011). The analytical sensitivity and specificity for the detection of deletions or duplications in exons 1-11 of *PMS2* and the detection of deletions or duplications in exons 12-15 of *PMS2* or *PMS2CL* is very high and can be considered >99% (based on a 2005-2021 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

For the *PMS2* probes targeting exons 1-10, as well as for the *PMS2* exon 11 probes at 133 nt and 364 nt, the expected results are allele copy numbers of 0 (very rare homozygous deletions), 1 (heterozygous deletion), 2 (normal), 3 (heterozygous duplication) and occasionally 4 (heterozygous triplication or homozygous duplication).

Five probes targeting exons 12-15 (labelled with + in Table 1 and 2) detect a sequence that is present in both *PMS2* and its *PMS2CL* pseudogene. In case of normal copy numbers these probes detect 4 copies / cell. The expected results for these five probes are allele copy number of 0, 1, 2, 3, 4 (normal), 5, 6, 7 or 8, corresponding to probe ratios of 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2, respectively. For example, a heterozygous deletion or duplication of either *PMS2* **OR** *PMS2CL* results in probe ratios of 0.75 (3 copies) or 1.25 (5 copies), respectively. A heterozygous deletion or duplication of *PMS2* **AND** *PMS2CL*, or a homozygous deletion or duplication of *PMS2* **OR** *PMS2CL* (extremely rare), will result in probe ratios of 0.5 (2 copies) or 1.5 (6 copies). The probe ratios of these five probes should be interpreted together with the results of the surrounding probes (when arranged according to chromosomal location) to determine the copy number.

Ten probes (labelled with ~ in Table 1 and 2) target each allele of five different polymorphic sequences in exons 11-15. Since these SNPs are present in both *PMS2* as well as *PMS2CL*, the copy numbers detected by these probes in **normal** individuals can be 0, 1, 2, 3 or 4. The combined copy number of the two probes of one pair of SNP probes should be 4 in normal individuals. These SNP-specific probes are used in combination with other techniques such as long range PCR, in order to determine whether copy number changes are present in *PMS2* or in *PMS2CL*.

The six SNP-specific probes in exons 13-15 provide extremely variable results, as only 25% of normal samples have a copy number of 2 for each allele of these three SNPs. In contrast, the four SNP-specific probes in exon 11 and intron 12 show an aberrant copy number in less than 2% of the samples tested. For these probes, the 165 nt and 238 nt probes indicate the *PMS2* copy number in the majority of samples, while the 171 nt and 244 nt probes are indicative of the *PMS2CL* copy number. In some samples, however, the *PMS2* sequence is also present in *PMS2CL* or vice versa, resulting in a probe ratio of 0.5 for one probe and a probe ratio of 1.5 for the other probe of that SNP probe pair.

Examples of probe ratios in heathy individuals and copy number changes in *PMS2*/*PMS2CL* can be found in Table 3 and Table 4, respectively.

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 those probes with a normal copy number of 2:

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 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.

P008 specific note

- Several PMS2 probes depend for their specificity on a single nucleotide difference between the *PMS2* gene and the pseudogenes (see Table 1 and 2). As a result, an apparent duplication of those probes can be the result of a clinically non-significant single nucleotide sequence change in one of the pseudogenes.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *PMS2* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P008 PMS2.
- 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 cannot indicate whether a deletion or duplication of exons 12-15 is present in *PMS2* or in *PMS2CL*. To determine whether a copy number change in the exons 12-15 region is present in *PMS2* or *PMS2CL*, the results of the P008 PMS2 probemix in general and the SNP-specific probes in particular should be complemented with gene-specific long range PCR and sequencing of the amplification products (see confirmation of results section; Li et al. 2015; Vaughn et al. 2011). Allocation of the copy number change to either *PMS2* or *PMS2CL* will not be possible when all alleles of *PMS2* and *PMS2CL* share the same variant targeted by the SNP-specific probes (Vaughn et al. 2011). In situations like these, family studies will be required to determine whether the copy number change is present in *PMS2* or *PMS2CL*.

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.

For copy number changes detected in exons 12-15, the MLPA results will not indicate whether a deletion or duplication is present in *PMS2* or in *PMS2CL*. To determine whether the copy number change is in *PMS2* or *PMS2CL*, MLPA can be complemented with gene-specific long range PCR, followed by (next generation) sequencing of the amplification products (Li et al. 2015; Vaughn et al. 2011). MLPA will determine the copy number for each of the allelic variants detected by the P008-C1 probemix. Sequence analysis will subsequently show which allelic variants are present in *PMS2* and *PMS2CL*. By combining this information, it will be possible to establish whether a copy number change is present in *PMS2* or *PMS2CL*. MRC Holland can only provided limited support on the methods used to allocate copy number changes in the exon 12-15 region to either *PMS2* or *PMS2CL*. More information and suitable PCR primer pairs for long range PCR can be found in the publications by Vaughn et al. (2011) and Li et al. (2015).

***PMS2* mutation database**

https://databases.lovd.nl/shared/genes/PMS2; http://insight-database.org/. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD) or in the International Society for Gastrointestinal Hereditary Tumours (InSiGHT). 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 *PMS2* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P008-C1 PMS2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*PMS2*	*PMS2CL*
64-105	Control fragments – see table in probemix content section for more information			
128	Reference probe 00797-L00093	5q		
133 #	**PMS2 probe** 14452-L00900		Exon 11	
140 #	**PMS2 probe** 14448-L16160		Exon 9	
146 #	**PMS2 probe** 07935-L16148		Exon 1	
154	Reference probe 02417-L04306	6p		
160	Reference probe 08583-L08584	17q		
165 ~	**PMS2/PMS2CL probe** 14453-L16164		Exon 11 (SNP)	Exon 11 (SNP)
171 ~	**PMS2/PMS2CL probe** 14453-L16165		Exon 11 (SNP)	Exon 11 (SNP)
177	Reference probe 04359-L03779	7p		
184 #	**PMS2 probe** 01176-L16620		Exon 2	
190 +	**PMS2/PMS2CL probe** 15768-L18167		**Exon 14**	**Exon 14**
196	Reference probe 07510-L07172	14q		
202 ~	**PMS2/PMS2CL probe** 14458-L16176		Exon 14 (SNP)	Exon 14 (SNP)
208 ~	**PMS2/PMS2CL probe** 14458-L16177		Exon 14 (SNP)	Exon 14 (SNP)
214 ~	**PMS2/PMS2CL probe** 14456-L16511		Exon 13 (SNP)	Exon 13 (SNP)
220 ~	**PMS2/PMS2CL probe** 14456-L16512		Exon 13 (SNP)	Exon 13 (SNP)
226	Reference probe 07083-L06712	11p		
232 ‡ #	**PMS2 probe** 14445-L16154		Exon 5	
238 ~	**PMS2/PMS2CL probe** 14455-L16168		Intron 12 (SNP)	Intron 12 (SNP)
244 ~	**PMS2/PMS2CL probe** 14455-L16169		Intron 12 (SNP)	Intron 12 (SNP)
250	**PMS2 probe** 01180-L16157		Exon 6	
261 +	**PMS2/PMS2CL probe** 15767-L17448		**Exon 13**	**Exon 13**
268	Reference probe 19040-L09299	3q		
276	**PMS2 probe** 01181-L16158		Exon 7	
283 +	**PMS2/PMS2CL probe** 15769-L17786		**Exon 12**	**Exon 12**
292	Reference probe 11087-L11770	2p		
299	**PMS2 probe** 01182-L16159		Exon 8	
310 ° #	**PMS2 probe** 19910-L26895		Exon 3	
319	**PMS2 probe** 01184-L00745		Exon 10	
328	Reference probe 08543-L08544	3q		
338 #	**PMS2 probe** 07934-L16147		Exon 1	
349 ~	**PMS2/PMS2CL probe** 14460-L04046		Exon 15 (SNP)	Exon 15 (SNP)
356 ~	**PMS2/PMS2CL probe** 14460-L16180		Exon 15 (SNP)	Exon 15 (SNP)
364 #	**PMS2 probe** 14451-L16163		Exon 11	
373	Reference probe 02528-L01959	17q		
382 +	**PMS2/PMS2CL probe** 15293-L17051		**Exon 14**	**Exon 14**
390 ° #	**PMS2 probe** 19915-L26898		Exon 3	
400 #	**PMS2 probe** 14441-L16150		Exon 2	
409 +	**PMS2/PMS2CL probe** 01189-L00750		**Exon 15**	**Exon 15**
418 #	**PMS2 probe** 19906-L26893		Exon 4	
427	Reference probe 06029-L05485	11p		
436	**PMS2 probe** 14447-L16623		Exon 6	
445 #	**PMS2 probe** 14449-L16622		Exon 9	
454 ‡ #	**PMS2 probe** 14446-L16621		Exon 5	
463	**PMS2 probe** 14450-L16162		Exon 10	
472	Reference probe 15978-L18133	8q		
483	Reference probe 08480-L08491	10p		

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

For explanation of symbols, see below Table 2.

Table 2. *PMS2* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*PMS2* exon[a]	Normal copy number	Ligation site NM_000535.7	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon		*31-33 (Exon 1)*		
338 #	07934-L16147	Exon 1	2	276 nt before exon 1	CGCGTGCCAAAG-GCCAACGCTCAG	0.2 kb
146 #	07935-L16148	Exon 1	2	32 nt before exon 1	TCAGGAGGCGGA-GCGCCTGTGGGA	3.1 kb
400 #	14441-L16150	Exon 2	2	3 nt before exon 2	AACTGATTTCTC-TAGTACAGAACC	0.1 kb
184 #	01176-L16620	Exon 2	2	147-148	AGCACTGCGGTA-AAGGAGTTAGTA	1.7 kb
390 º #	19915-L26898	Exon 3	2	147 nt before exon 3	GGACTTTATAAC-AATGAAATATAC	0.3 kb
310 º #	19910-L26895	Exon 3	2	80 nt after exon 3, reverse	ACTGTTTTTGCA-TTTCCCAAGACA	0.3 kb
418 #	19906-L26893	Exon 4	2	77 nt after exon 4, reverse	ATAGAAAACTGA-AAATAATAATGA	1.0 kb
232 ‡ #	14445-L16154	Exon 5	2	436-437	GAACTCGACTGA-TGTTTGATCACA	0.1 kb
454 ‡ #	14446-L16621	Exon 5	2	508-509	CAGTCAGCGTGC-AGCAGTTATTTT	3.3 kb
436	14447-L16623	Exon 6	2	610-609, reverse	GCCTGCTGAAAT-GATACAGTATGC	0.1 kb
250	01180-L16157	Exon 6	2	658-659	TTGGACAAGGAA-AACGACAGCCTG	1.8 kb
276	01181-L16158	Exon 7	2	784-785	GTGACTCCGTGT-GTGAAGAGTACG	1.8 kb
299	01182-L16159	Exon 8	2	879-880	GTTGGAAGGAGT-TCAACAGACAGA	3.5 kb
445 #	14449-L16622	Exon 9	2	30 nt before exon 9, reverse	GTAGAAAAGAAT-AAATGACAAATG	0.1 kb
140 #	14448-L16160	Exon 9	2	954-955	CTCGTGAATGAG-GTCTACCACATG	2.1 kb
463	14450-L16162	Exon 10	2	1044-1045	AATGTTACTCCA-GATAAAAGGCAA	0.1 kb
319	01184-L00745	Exon 10	2	1142-1141, reverse	GCTGACTGACAT-TTAGCTTGTTGA	2.4 kb
133 #	14452-L00900	Exon 11	2	1390-1391	AAAGGGGTATGC-TGTCTTCTAGCA	0.1 kb
165 ~	14453-L16164	Exon 11	0-4	1467-1468 rs63750685, C-allele	AGTTCCAGTCA**C**-GGACCCAGTGAC	0.4 kb
171 ~	*14453-L16165*	*Exon 11*	*0-4*	*1467-1468 rs63750685, G-allele*	*AGTTCCAGTCA**G**-GGACCCAGTGAC*	
364 #	14451-L16163	Exon 11	2	1893-1894	CTGGACTTTTCT-ATGAGTTCTTTA	4.0 kb
283 +	15769-L17786	Exon 12	4	2119-2118, reverse	CTGGTCCACTAT-GAAGATATCCTC	1.2 kb
238 ~	14455-L16168	Intron 12	0-4	1096 nt after exon 12, reverse rs35748755, GTA-allele	AGC**G**AAAAA**TAC**-CTGATACTTAGA	3.0 kb
244 ~	*14455-L16169*	*Intron 12*	*0-4*	*1096 nt after exon 12, reverse rs35748755, ACC-allele*	*AGC**A**AAAAA**GGT**-CTGATACTTAGA*	
261 +	15767-L17448	Exon 13	4	18 nt before exon 13, reverse	AACACAAAAATG-ATTCAAACCATA	0.1 kb
214 ~	14456-L16511	Exon 13	0-4	2283-2282, reverse rs1805325, T-allele	ATAACAAAATC**A**-AAGCCATTCTTT	0.9 kb
220 ~	14456-L16512	Exon 13	0-4	2283-2282, reverse rs1805325, C-allele	ATAACAAAATC**G**-AAGCCATTCTTT	
202 ~	14458-L16176	Exon 14	0-4	2354-2355 rs17420802, A-allele	AACTAGTAAAA**A**-CTGGACCTTCGG	0.1 kb
208 ~	14458-L16177	Exon 14	0-4	2354-2355 rs17420802, G-allele	AACTAGTAAAA**G**-CTGGACCTTCGG	
382 +	15293-L17051	Exon 14	4	2461-2462	TTGCCTCCAGAG-CCTGCCGGAAGT	0.1 kb
190 +	15768-L18167	Exon 14	4	62 nt after exon 14	AGGAAGGTCTCA-GCCCAGCTCCTG	4.0 kb
409 +	01189-L00750	Exon 15	4	2542-2543	TGGGGGAGATGG-ACCACCCCTGGA	0.2 kb
349 ~	14460-L04046	Exon 15	0-4	2711-2712 rs267608145	TTTTAAAATGAA-CCTGCTACTTAA	
356 ~	14460-L16180	Exon 15	0-4	2711-2712 rs267608145, +A-allele	TTTAAAATGAA**A**-CCTGCTACTTAA	
		stop codon		*2617-2619 (Exon 15)*		

[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.

+ Detects *PMS2* **and** its pseudogene *PMS2CL*.

~ Shaded boxes. These ten probes each detect a SNP which is present in two allelic forms. Due to frequent gene conversion events, these probes cannot confidently be assigned to *PMS2* or its pseudogene *PMS2CL* (in particular the probes for exons 13, 14 and 15). When the combined copy number of the two alleles is four, a deletion or duplication is unlikely. The probe pairs for exon 11 (165 nt and 171 nt) and intron 12 (238 nt and 244 nt) are more stable. The 165 nt and 238 nt probes seem to indicate the copy number of *PMS2* in most cases, while the two probes marked in *italics* at 171 nt and 244 nt seem to show the copy numbers of *PMS2CL*. Please note that gene conversions may also occur for these probe pairs, and that the obtained results should always be confirmed.
‡ The two probes for exon 5 generate a (limited) signal on one of the many *PMS2* pseudogenes. When a sample with a homozygous deletion of *PMS2* was tested, the 232 nt exon 5 probe generated a signal of ~10% of the normal signal, while the 454 nt exon 5 probe generated a signal of ~20% of the normal signal. On samples with a heterozygous deletion of *PMS2* exon 5, we expect a signal of ~60-70% of the normal signal for these probes. When the ligation reaction is performed at room temperature, the 454 nt exon 5 probe is more prone to bind to homologous sequences. A deletion or duplication of exon 5 should be further investigated only if both exon 5 probes show the same result.
# 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.
º A deletion or duplication of exon 3 should be further investigated only if both exon 3 probes show the same result.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 3. Selected examples of probe ratios in healthy individuals

SALSA MLPA probe	Length (nt)	*PMS2* exon	A	B	C	D	E	F	G	H	I
07934-L16147	338	Exon 1	1	1	1	1	1	1	1	1	1
07935-L16148	146	Exon 1	1	1	1	1	1	1	1	1	1
14441-L16150	400	Exon 2	1	1	1	1	1	1	1	1	1
01176-L16620	184	Exon 2	1	1	1	1	1	1	1	1	1
19915-L26898	390	Exon 3	1	1	1	1	1	1	1	1	1
19910-L26895	310	Exon 3	1	1	1	1	1	1	1	1	1
19906-L26893	418	Exon 4	1	1	1	1	1	1	1	1	1
14445-L16154	232	Exon 5	1	1	1	1	1	1	1	1	1
14446-L16621	454	Exon 5	1	1	1	1	1	1	1	1	1
14447-L16623	436	Exon 6	1	1	1	1	1	1	1	1	1
01180-L16157	250	Exon 6	1	1	1	1	1	1	1	1	1
01181-L16158	276	Exon 7	1	1	1	1	1	1	1	1	1
01182-L16159	299	Exon 8	1	1	1	1	1	1	1	1	1
14449-L16622	445	Exon 9	1	1	1	1	1	1	1	1	1
14448-L16160	140	Exon 9	1	1	1	1	1	1	1	1	1
14450-L16162	463	Exon 10	1	1	1	1	1	1	1	1	1
01184-L00745	319	Exon 10	1	1	1	1	1	1	1	1	1
14452-L00900	133	Exon 11	1	1	1	1	1	1	1	1	1
14453-L16164 ~	165	Exon 11	**0.5**	**1.5**	1	1	1	1	1	1	1
14453-L16165 ~	171	Exon 11	**1.5**	**0.5**	1	1	1	1	1	1	1
14451-L16163	364	Exon 11	1	1	1	1	1	1	1	1	1
15769-L17786 +	283	Exon 12	1	1	1	1	1	1	1	1	1
14455-L16168 ~	238	Intron 12	1	1	**0.5**	1	1	1	1	1	1
14455-L16169 ~	244	Intron 12	1	1	**1.5**	1	1	1	1	1	1
15767-L17448 +	261	Exon 13	1	1	1	1	1	1	1	1	1
14456-L16511 ~	214	Exon 13	1	1	1	**0.5**	1	1	**1.5**	**2**	**1.5**
14456-L16512 ~	220	Exon 13	1	1	1	**1.5**	1	1	**0.5**	**0**	**0.5**
14458-L16176 ~	202	Exon 14	1	1	1	1	**0.5**	1	**1.5**	**2**	**1.5**
14458-L16177 ~	208	Exon 14	1	1	1	1	**1.5**	1	**0.5**	**0**	**0.5**
15293-L17051 +	382	Exon 14	1	1	1	1	1	1	1	1	1
15768-L18167 +	190	Exon 14	1	1	1	1	1	1	1	1	1
01189-L00750 +	409	Exon 15	1	1	1	1	1	1	1	1	1
14460-L04046 ~	349	Exon 15	1	1	1	1	1	**0.5**	**1.5**	**2**	**0**
14460-L16180 ~	356	Exon 15	1	1	1	1	1	**1.5**	**0.5**	**0**	**2**

Table 4. Selected examples of copy number changes in *PMS2/PMS2CL*

SALSA MLPA probe	Length (nt)	*PMS2* exon	1	2	3	4	5	6	7	8	9
07934-L16147	338	Exon 1	1	1	1	1	1	**0.5**	1	1	1
07935-L16148	146	Exon 1	1	1	1	1	1	**0.5**	1	1	1
14441-L16150	400	Exon 2	1	1	1	1	1	**0.5**	1	1	1
01176-L16620	184	Exon 2	1	1	1	1	1	**0.5**	1	1	1
19915-L26898	390	Exon 3	1	1	1	1	1	**0.5**	1	1	1
19910-L26895	310	Exon 3	1	1	1	1	1	**0.5**	1	1	1
19906-L26893	418	Exon 4	1	1	1	1	1	**0.5**	1	1	1
14445-L16154	232	Exon 5	1	1	1	1	1	**0.5**	1	**0.5**	1
14446-L16621	454	Exon 5	1	1	1	1	1	**0.5**	1	**0.5**	1
14447-L16623	436	Exon 6	1	1	1	1	1	**0.5**	1	**0.5**	1
01180-L16157	250	Exon 6	1	1	1	1	1	**0.5**	1	**0.5**	1
01181-L16158	276	Exon 7	**0.5**	1	1	**1.5**	1	**0.5**	1	**0.5**	1
01182-L16159	299	Exon 8	**0.5**	1	1	**1.5**	1	**0.5**	1	**0.5**	1
14449-L16622	445	Exon 9	1	1	1	**1.5**	**0.5**	**0.5**	1	**0.5**	1
14448-L16160	140	Exon 9	1	1	1	**1.5**	**0.5**	**0.5**	1	**0.5**	1
14450-L16162	463	Exon 10	1	1	**0.5**	**1.5**	**0.5**	**0.5**	1	**0.5**	1
01184-L00745	319	Exon 10	1	1	**0.5**	**1.5**	**0.5**	**0.5**	1	**0.5**	1
14452-L00900	133	Exon 11	1	1	1	**1.5**	**0.5**	**0.5**	**0.5**	**0.5**	1
14453-L16164 ~	165	Exon 11	1	1	1	**1.5**	**0.5**	**0.5**	**0.5**	**0.5**	1
14453-L16165 ~	171	Exon 11	1	1	1	**1**	**1**	**1**	**1**	**1**	1
14451-L16163	364	Exon 11	1	1	1	**1.5**	**0.5**	**0.5**	**0.5**	**0.5**	1
15769-L17786 +	283	Exon 12	1	1	1	**1.25**	**0.75**	**0.75**	**0.75**	**0.75**	1
14455-L16168 ~	238	Intron 12	1	1	1	**1.5**	**0.5**	**0.5**	**0.5**	**0.5**	1
14455-L16169 ~	244	Intron 12	1	1	1	**1**	**1**	**1**	**1**	**1**	1
15767-L17448 +	261	Exon 13	1	1	1	**1.25**	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**
14456-L16511 ~	214	Exon 13	1	1.5	1.5	**1**	**1.5**	**0.5**	**0.5**	**0.5**	**0**
14456-L16512 ~	220	Exon 13	1	0.5	0.5	**1.5**	**0**	**1**	**1**	**1**	**1.5**
14458-L16176 ~	202	Exon 14	1	**1**	1.5	**1**	**1.5**	**0.5**	**0.5**	**0.5**	**0**
14458-L16177 ~	208	Exon 14	1	**0.5**	0.5	**1.5**	**0**	**1**	**1**	**1**	**1.5**
15293-L17051 +	382	Exon 14	1	**0.75**	1	**1.25**	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**
15768-L18167 +	190	Exon 14	1	**0.75**	1	**1.25**	**0.75**	**0.75**	**0.75**	**0.75**	**0.75**
01189-L00750 +	409	Exon 15	1	1	1	1	**0.75**	1	**0.75**	**0.75**	1
14460-L04046 ~	349	Exon 15	0.5	1.5	1.5	0.5	**1.5**	0.5	**0.5**	**0.5**	0
14460-L16180 ~	356	Exon 15	1.5	0.5	0.5	1.5	**0**	1.5	**1**	**1**	2

For explanation of symbols, see below Table 2.

1. Heterozygous deletion of *PMS2* exon 7-8.
2. Heterozygous deletion of exon 14. The P008 probemix cannot determine if the deletion is present in *PMS2* or in *PMS2CL*. This needs to be investigated further by means of follow-up studies.
3. Heterozygous deletion of *PMS2* exon 10.
4. Heterozygous duplication of *PMS2* exon 7-14.
5. Heterozygous deletion of *PMS2* exon 9-15.
6. Heterozygous deletion of *PMS2* exon 1-14
7. Heterozygous deletion of *PMS2* exon 11-15.
8. Heterozygous deletion of *PMS2* exon 5-15.
9. Heterozygous deletion of exon 13-14. The P008 probemix cannot determine if the deletion is present in *PMS2* or in *PMS2CL*. This needs to be investigated further by means of follow-up studies.

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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P008 product history	
Version	*Modification*
C1	The exon 3 and 4 probes and one reference probe have been replaced by two new exon 3 probes and one new exon 4 probe. The new probes generate almost no signal on the many *PMS2* pseudogenes. One of the exon 2 probes was removed.
B2	One reference probe has a 1 nt shorter length.
B1	Product was completely redesigned and targets only *PMS2*.
A2	Two extra control fragments at 100-105 nt included.
A1	First release. Probemix contains probes for *PMS2*, *MSH6*, *MUTYH*, *MLH3* and *MSH3*.

Implemented changes in the product description
Version C1-05 – 04 November 2021 (04P) - Intended purpose updated; SALSA Reference Selection DNA SD082 added to footnote 2. - Information in Clinical background and Performance characteristics sections updated. - Reference Selection DNA SD072 replaced by Reference Selection DNA SD082 throughout the document. - Probemix names added to Table with Related SALSA MLPA Probemixes. - Various minor textual and layout changes. Version C1-04 – 22 March 2021 (04P) - Product description rewritten and adapted to a new template. - *PMS2* mutation database website links updated.

- Ligation sites of the probes targeting the *PMS2* gene updated according to new version of the NM_ reference sequence.
- Warning added to Table 1 and 2 for probe specificity of the 338 nt PMS2 exon 1 probe (07934-L16147) relying on a single nucleotide difference between target gene and related gene or pseudogene.
- Warning added to Table 1 and 2 for effect of room temperature ligation on the specificity of the 454 nt PMS2 exon 5 probe (14446-L16621)
- UK has been added to the list of countries in Europe that accept the CE mark.

Version C1-03 – 11 May 2020 (04)
- Colombia and Israel added as countries with IVD status.

Version C1-02 – 30 July 2018 (04)
- Product description adapted to a new template.
- Extra warning placed in Interpretation of Results section for probe specificity relying on a single nucleotide difference between target gene and pseudogene.
- Various minor textual changes.

Version C1-01 – 11 December 2017 (03)
- Product description adapted to a new template.
- Warning added to page 1 regarding the identification of suitable reference samples.
- For P008, SD072 should be used instead of SD019.
- SD027: information removed as product is obsolete.
- Additional symbols and remarks added to tables 1 and 2.
- Tables 3 and 4 are new.
- rs3214300 updated to rs267608145.
- Various minor textual or layout changes.

Version 34 – 05 December 2016 (55)
- COA adapted to a new lot (lot number and sample picture changed).
- Various textual changes.
- Product description restructured.
- New publications with SALSA MLPA probemix P008 added.
- Ratios corrected in Table 4 of the appendix.

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Appendix – How to select suitable reference samples

I. Five SNP sites, two biallelic forms, in two genes

The P008-C1 probemix contains ten probes that detect *both forms* of five polymorphic sequences. Each of the two forms of these SNPs can be present in either *PMS2* or *PMS2CL*. These five probe pairs are (1) 165-171 nt, (2) 202-208 nt, (3) 214-220 nt, (4) 238-244 nt, and (5) 349-356 nt. One probe of the pair detects one SNP variant (allele I), the other probe detects the other SNP variant (allele II). In a normal individual, the total copy number of each probe *pair* (allele I + II) is four: two copies in the *PMS2* gene and two in the pseudogene. However, it cannot be established which allelic form is present in which gene.

Although the combined copy number of each probe pair is highly constant, each individual SNP-specific probe (e.g. detecting allele I) may detect 0, 1, 2, 3 or 4 copies in normal individuals.

Examples (normal individuals):
If the 165 nt probe shows 3 copies, the corresponding 171 nt probe should show 1 copy (3+1=4).
If the 202 nt probe shows 2 copies, the corresponding 208 nt probe should also show 2 copies (2+2=4).
If the 214 nt probe shows 0 copies, the corresponding 220 nt probe should show 4 copies (0+4=4).

In particular the copy numbers detected by the probe pairs at 202-208 nt, 214-220 nt and 349-356 nt are very variable in normal individuals. For probe pairs at 165-171 nt and 238-244 nt, most individuals have two copies. In these probe pairs, the 165 nt and 238 nt probes appear to be specific for *PMS2*, while the 171 nt and 244 nt probes appear to be specific for *PMS2CL*. However, please note that gene conversions for these allelic variants do occur. Any copy number aberrations observed for these probes should, therefore, be further investigated to determine if they are in *PMS2* or in the *PMS2CL* pseudogene.

For P008-C1 data analysis, it is important to select proper reference samples. **Suitable reference samples have 2 copies for each allele detected by these ten SNP-specific probes.**

II. How to select suitable reference samples using Reference Selection DNA SD082

MRC Holland has selected a cell line with two copies for each of the allelic variants. This Reference Selection DNA SD082 is provided with every order of the probemix. **Reference Selection DNA SD082 should only be used for the selection of suitable reference samples from your own collection and not as a reference DNA sample.** In our experience, approximately one in four DNA samples tested is suitable as a reference sample. By testing 21 different DNA samples from healthy individuals with P008-C1, and including three reactions with SD082, the chance of finding at least three different suitable reference samples is high. Suitable reference samples provide results equivalent to the Reference Selection DNA SD082 for each probe in the P008-C1 probemix, including the ten SNP-specific probes.

III. How to select suitable reference samples without the use of Reference Selection DNA SD082

In order to select suitable reference samples without the use of Reference Selection DNA SD082, first run an MLPA experiment on DNA samples from at least 16 healthy individuals. During the first analysis, use all 16 samples as reference samples (in Coffalyser.Net all samples must be set to 'sample'). The results of the first analysis can be erratic at first sight.

For the extremely variable SNP-specific probes at 202-208 nt, 214-220 nt and 349-356 nt, the ratio showing the *lowest value* (zero values excluded) will usually indicate 1 copy. Twice this value indicates 2 copies, three times this value corresponds to 3 copies, etc.

Table 5 (below) contains an example of this procedure. For example, the lowest value >0 for the 356 nt probe is 0.35. This likely corresponds to 1 copy. Dividing all other ratios for this probe by 0.35 leads to the approximate copy numbers shown in Table 6. This way, the initially calculated ratios of 0, 0.35, 0.75, 1.17 and 1.52 are found to correspond to 0, 1, 2, 3 and 4 copies. The procedure should be repeated for all variable SNP-specific probes separately.

When the copy numbers have been established this way, the samples *containing two copies for each of the ten allelic variants* can be identified.

Subsequently, data analysis can be repeated using just these selected samples as reference samples. If the correct reference samples have been chosen, all ten SNP-specific probes, including the six highly variable SNP-specific probes, should give probe ratios close to 0, 0.5, 1.0, 1.5 or 2 upon reanalysis. A wrong choice of reference samples may for instance result in probe ratios of 0, 0.33, 0.67, 1 and 1.33 for one allele while the probe for the other allele may show probe ratios of 0, 1, 2, 3 and 4.

The reference samples identified can be used in all following P008-C1 MLPA experiments. To ensure that your reference samples do not deteriorate due to a large number of freeze-thaw cycles, divide them over smaller aliquots.

In Tables 5-7, column J shows the results obtained on a sample with an exon 14 deletion. This sample shows: (1) a combined copy number of only 3 for the 202-208 nt SNP probe pair (i.e. a combined probe ratio of 1 + 0.5 = 1.5 instead of 2.0 for the SNP pair), and (2) a ratio of 0.75, corresponding to a total of 3 copies (0.75 x 4 copies), for the 190 nt and 382 nt probes that each detect a sequence in exon 14 of both *PMS2* and *PMS2CL*. Follow-up tests should be done to determine whether this exon 14 deletion is present in the *PMS2* gene or in the *PMS2CL* pseudogene.

Table 5. Typical initial results when analysed using <u>all samples</u> as reference sample

Gene-exon	Length	A	B	C	D	E	F	G	H	I	J
PMS2-15 (allele I)	356	1.17	0	0.75	0.81	0	0.75	1.52	0.35	0.39	0.76
PMS2-15 (allele II)	349	0.54	2.18	1.14	1.07	2.20	1.13	0	1.68	1.75	1.08
PMS2-15 (4 copies)	**409**	1.01	0.99	1.02	1.07	1.04	1.02	1.01	1.04	1.01	0.99
PMS2-14 (4 copies)	**190**	0.97	1.07	1.00	1.02	1.02	0.98	0.97	1.02	0.98	0.76
PMS2-14 (4 copies)	**382**	0.99	1.01	1.01	1.02	1.01	0.99	1.00	0.99	1.00	0.76
PMS2-14 (allele I)	202	1.15	1.62	1.21	1.24	2.38	1.16	0.56	1.73	2.30	0.64
PMS2-14 (allele II)	208	0.75	0.35	0.74	0.77	0	0.77	1.07	0.38	0	0.82
PMS2-13 (allele I)	220	0.80	0.49	0.80	0.83	0	0.80	1.15	0.44	0	0.80
PMS2-13 (allele II)	214	1.12	1.80	1.09	1.06	2.13	1.15	0.54	1.70	2.27	1.13
PMS2-13 (4 copies)	**261**	1.02	1.09	1.04	1.05	1.02	1.05	1.00	1.02	1.04	1.03

The relative value of each probe in exon 13-15 is shown. All samples tested were used as reference sample. For each probe, the lowest probe value in samples A-I is indicated in grey. Sample J is a known positive sample, and was therefore excluded from the initial analysis.

Table 6. Same results when each probe value is divided by the lowest probe value >0

Gene-exon	Length	A	B	C	D	E	F	G	H	I	J
PMS2-15 (allele I)	356	3.3	0	2.1	2.3	0	2.1	4.3	1.0	1.1	2.2
PMS2-15 (allele II)	349	1	4.0	2.1	2.0	4.1	2.1	0	3.1	3.2	2.0
PMS2-15 (4 copies)	**409**	1	1	1	1.1	1.1	1	1	1.1	1	1
PMS2-14 (4 copies)	**190**	1	1.1	1	1.1	1.1	1	1	1.1	1	0.8
PMS2-14 (4 copies)	**382**	1	1	1	1	1	1	1	1	1	0.8
PMS2-14 (allele I)	202	2.1	2.9	2.2	2.2	4.3	2.1	1	3.1	4.1	1.1
PMS2-14 (allele II)	208	2.1	1	2.1	2.2	0	2.2	3.1	1.1	0	2.3
PMS2-13 (allele I)	220	1.8	1.1	1.8	1.9	0	1.8	2.6	1	0	1.8
PMS2-13 (allele II)	214	2.1	3.3	2	2	3.9	2.1	1	3.1	4.2	2.1
PMS2-13 (4 copies)	**261**	1	1.1	1	1.1	1	1.1	1	1	1	1

When sufficient samples are tested, it is likely that the lowest probe value >0 obtained for the SNP-specific probes represents the presence of 1 copy. Dividing each probe value by the lowest value obtained (grey in Table 5), provides the copy number of the sequence detected by that probe in each sample. Samples C, D and F appear to be suitable reference samples for further tests as they detect two copies for each allele detected by the SNP-specific probes (grey in Table 6).

Table 7. Same results analysed using selected samples C, D & F as reference samples

Gene-exon	Length	A	B	C	D	E	F	G	H	I	J
PMS2-15 (allele I)	356	1.45	0	0.93	1	0	0.93	1.88	0.44	0.49	0.94
PMS2-15 (allele II)	349	0.48	1.93	1.01	0.95	1.95	1	0	1.49	1.55	0.96
PMS2-15 (4 copies)	**409**	1	0.98	1.01	1.06	1.03	1.01	1	1.03	1	0.98
PMS2-14 (4 copies)	**190**	0.98	1.08	1.01	1.03	1.03	0.99	0.98	1.03	0.99	**0.77**
PMS2-14 (4 copies)	**382**	1	1.02	1.02	1.03	1.02	1	1.01	1	1.01	**0.77**
PMS2-14 (allele I)	202	0.97	1.37	1.02	1.05	2.01	0.98	0.47	1.46	1.95	**0.54**
PMS2-14 (allele II)	208	1	0.44	0.99	1.03	0	1.02	1.43	0.5	0	**1.09**
PMS2-13 (allele I)	220	1.02	0.63	1.03	1.06	0	1.02	1.47	0.56	0	1.02
PMS2-13 (allele II)	214	0.94	1.61	0.92	0.89	1.79	0.97	0.44	1.43	1.94	0.95
PMS2-13 (4 copies)	**261**	1	1.07	1.02	1.03	1	1.03	0.98	1	1.02	1.01

Probes detecting both *PMS2* and *PMSCL* are in bold. The reference samples C, D and F show a ratio around 1.0 for each probe. Please note that a probe ratio of 1 indicates 2 copies for the SNP-specific probes but indicates 4 copies for the probes that detect both *PMS2* and *PMS2CL*.

Samples A, B, E, G, H and I show a combined ratio of ~2.0 for the 356-349 nt, 202-208 nt and 220-214 nt probe pairs, as well as a ratio around 1.0 for the 409, 190, 382 and 261 nt probes that detect a sequence in *PMS2* and *PMS2CL*. **Note that sample J is a positive sample with an exon 14 deletion.**