Product Description
SALSA® digitalMLPA Probemix D001-C1 Hereditary Cancer Panel 1

To be used with the digitalMLPA General Protocol.

Version C1
Compared to version B1, 14 target probes were adjusted (not in sequence detected), two target probes were replaced, one target probe was removed and 19 target probes were added. The reference probe selection was adjusted and an updated set of control probes was included. Details about the added and changed probes can be found in the D001-C1 probemix specific Probe Information File. For complete product history see page 14.

Catalogue numbers
- **D001-025R:** SALSA digitalMLPA Probemix D001 Hereditary Cancer Panel 1, 25 reactions.
- **D001-050R:** SALSA digitalMLPA Probemix D001 Hereditary Cancer Panel 1, 50 reactions.
- **D001-100R:** SALSA digitalMLPA Probemix D001 Hereditary Cancer Panel 1, 100 reactions.

To be used in combination with:
1. SALSA® digitalMLPA reagent kit DRK01-IL (100 reactions) or DRK05-IL (500 reactions).
2. SALSA® Barcode Solutions Plates for Illumina instruments (768 reactions/plate)
   BP01-IL from lot number 03-009-xxxxxx and higher
   BP02-IL from lot number 03-008-xxxxxx and higher
   BP03-IL from lot number 03-010-xxxxxx and higher
   BP04-IL from lot number 03-011-xxxxxx and higher
   N.B. The three-digit number between dashes (e.g. -009-) will increase with every new barcode plate lot.
3. Coffalyser digitalMLPA software.

Certificate of Analysis
Information regarding storage conditions and quality tests 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 digitalMLPA General Protocol before use: www.mrcholland.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

General information
This SALSA digitalMLPA probemix D001-C1 Hereditary Cancer Panel 1 is a **research use only (RUO) assay** for the detection of exon deletions or duplications in the genes mentioned in Table 2, which are associated with hereditary predisposition for formation of breast, ovarian, colorectal, gastric, prostate, pancreatic or endometrial tumours, or for melanoma.

This assay is intended for use with human genomic DNA isolated from peripheral whole blood and is not intended to be used with genomic DNA extracted from formalin-fixed paraffin embedded or fresh tumour materials. Copy number variations (CNVs) detected with the D001 Hereditary Cancer Panel 1 probemix should be confirmed with a different technique or with conventional MLPA probemixes when possible. In particular, CNVs detected by only a single probe always require confirmation by another method. For many genes included in this D001 Hereditary Cancer Panel 1, the most frequent germline defects are point mutations, the majority of which will not be detected by this probemix. It is therefore strongly recommended to use this D001 digitalMLPA probemix in combination with sequence analysis.

This SALSA digitalMLPA Probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Probemix content
A total number of approximately 725 probes is included in this D001 Probemix, this consists of:
- 576 probes detecting copy number alterations involved in hereditary cancer (Table 2).
- Five mutation-specific probes, which will only generate probe reads when that particular mutation is present (Table 2). For more information see the D001-C1 probemix specific Probe Information File.
- Three wild type specific probes, which detect the wild type sequence of a particular mutation (Table 2). For more information see the D001-C1 probemix specific Probe Information File.
- More than 120 control probes and fragments: these include probes for sample identification and probes for detection of errors or deviations when performing digitalMLPA assays, impurities in and fragmentation of the DNA samples, ligase and polymerase activity and extent of hybridisation.

Reference probes
As the target probes are spread over a large number of different autosomal chromosomal regions, no separate reference probes have been included in this D001-C1 Hereditary Cancer Panel 1 probemix. Instead, a selection of 290 target probes is used as reference probes for data normalisation.

digitalMLPA technique
The digitalMLPA technique is a variant on the widely used conventional MLPA technique (Schouten et al. 2002, Benard-Slagter et al. 2017). In digitalMLPA, Illumina sequencers, instead of capillary electrophoresis instruments, are used for relative quantification of amplicons. The principles of the digitalMLPA technique are described in the digitalMLPA General Protocol (www.mrcholland.com).

digitalMLPA technique validation
Internal validation of the digitalMLPA technique using 16 DNA samples from healthy individuals is required, in particular when using digitalMLPA for the first time, or when pre-analytical steps, DNA extraction method or the instruments used are changed. This validation experiment should result in a standard deviation ≤0.10 for all probes with the exception of SNP- and mutation-specific probes.

Required specimens
Extracted DNA, free from impurities known to affect digitalMLPA reactions. For more information see the digitalMLPA General Protocol, section DNA sample treatment.

Reference samples
When sufficient DNA samples from unrelated families are tested with the D001-C1 probemix, there is a low probability that the majority of the samples will have the same copy number change and no separate reference samples are required. In this case, for data analysis using Coffalyser digitalMLPA, the sample type should be set to "Test" (not "Reference") for all samples.

When the testing sample set is small or includes many samples from the same family, inclusion of at least three separate reference DNA samples in the experiment is required.

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 digitalMLPA General Protocol.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample in your digitalMLPA experiments. The NIBSC Institute in the U.K. provides a kit with 5 DNA samples which contain CNVs in the *MLH1* or *MSH2* genes: https://www.nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=11/218. The quality of

cell lines can change, therefore positive samples should be either acquired from quality assessed biological sample repositories or validated before use. Table 1 contains a list of positive control samples that have been tested with the D001-C1 probemix at MRC Holland.

Table 1. Positive samples from biobanks tested by MRC Holland

Coriell sample ID	Genomic aberration
NA13451	14 Mb 2p deletion including *MSH2*, *EPCAM*, and *MSH6* (heterozygous)
HG00259	*MITF* E318K mutation present
NA04127	51 Mb 3p duplication including *MLH1* (heterozygous)
NA11570	22 Mb 5q deletion including *APC* (heterozygous)
NA14234	31 Mb 5q deletion including *APC* (heterozygous)
NA02030	Trisomy 8 sample with *NBN* duplication (heterozygous)
GM03226	40 Mb 9p duplication including *CDKN2A* (heterozygous)
NA08618	23 Mb 11q duplication including *ATM* (heterozygous)
NA09596	32 Mb 11q deletion including *ATM* (heterozygous)
HG03694	*ATM* exons 62 and 63 duplication (heterozygous, benign)
NA07891	7 Mb 12q duplication including *POLE* (heterozygous) and 31 Mb 18q deletion of *SMAD4* (heterozygous)
NA01535	1 Mb 12q deletion including *POLE* (heterozygous)
HG00634	*PALB2* exon 13 duplication (heterozygous)
NA02718	28 Mb 13q deletion including *BRCA2* (heterozygous)
NA03184	82 Mb 15q duplication including *SCG5* and *GREM1* (heterozygous)
NA12074	8 Mb 16q deletion including *CDH1* (heterozygous)
NA20539	*PALB2* exons 5 and 6 deletion (heterozygous)
HG03857	*PALB2* exons 5-7 deletion (heterozygous)
NA18949	*BRCA1* exon 15 and 16 deletion (heterozygous)
NA14626	*BRCA1* exon 13 duplication (heterozygous)
NA01359	Trisomy 18 sample with *SMAD4* duplication (heterozygous)
NA07106	35 Mb 22q duplication including *CHEK2* (heterozygous)
HG00187	*CHEK2* 1100delC mutation present
NIBSC sample ID	**Genomic aberration**
Sample 3	*MSH2* exons 1-6 deletion (heterozygous)
Sample 4	*MSH2* exon 7 deletion (heterozygous)
Sample 5	*MSH2* exons 1 and 2 deletion (heterozygous)
Sample 6	*MSH2* exon 1 deletion (heterozygous)
Sample 7	*MLH1* exon 13 triplication (heterozygous) or homozygous duplication

Data analysis
Coffalyser digitalMLPA software must be used for data analysis in combination with the appropriate lot-specific Coffalyser digitalMLPA product sheet. Coffalyser digitalMLPA software is freely downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. The digitalMLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Interpretation of results
The expected results for (pseudo)autosomal probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 0 (homozygous deletion), 3 (heterozygous duplication) or ≥4 (heterozygous triplication/homozygous duplication).

The standard deviation of all probes in the reference samples should be ≤0.10. When this criterion is fulfilled, the following cut-off values for the inter ratio of the probes can be used to interpret digitalMLPA results for autosomal or pseudo-autosomal chromosomes:

Copy number status	Inter ratio

Normal	0.80 < ratio < 1.20
Homozygous deletion	ratio = 0
Heterozygous deletion	0.40 < ratio < 0.65
Heterozygous duplication	1.30 < ratio < 1.65
Heterozygous triplication/ Homozygous duplication	1.75 < ratio < 2.15
Ambiguous copy number	All other values

The following non-standard probes in this D001-C1 probemix require special consideration for result interpretation.

Probe type	**Expected inter ratios**	
Mutation-specific (5 probes, *MSH2, MITF, PMS2, CHEK2*)	normal samples	<2% of the average read number of all probes
	mutant samples	determine by testing known positive controls
Wild type-specific (3 probes, *PMS2, POLE, BRCA2*)	normal samples	0.80 < ratio < 1.20
	heterozygous mutation	0.40 < ratio < 0.65
	homozygous mutation	ratio = 0
Probes that detect both *PMS2* and *PMS2CL* exons 12-15	normal samples (4 copies)	0.85 < ratio < 1.15
	two copies	0.40 < ratio < 0.65
	three copies	0.65 < ratio < 0.85
	five copies	1.15 < ratio < 1.35
	six copies	1.35 < ratio < 1.65

General notes on digitalMLPA interpretation:

- Arranging probes according to chromosomal location facilitates interpretation of the results. 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. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe read count, in particular for probes located in or near a GC-rich region. The use of an alternative DNA extraction method may resolve such cases. Control probes are present in all digitalMLPA probemixes that provide a warning for incomplete DNA denaturation.
- False positive duplication results: Contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe read count (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 digitalMLPA are pathogenic. 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. In some genes, intragenic deletions are known that result in very mild, or no disease (Schwartz et al. 2007). Duplications that include the first or last exon of a gene (e.g. exons 1-3) might in some cases not result in inactivation of that gene copy.
- Copy number changes detected by flanking probes are unlikely to have any relation to the condition tested for.

D001-C1 probemix specific notes:

- A hereditary predisposition to cancer due to gene defects in one of the genes covered by D001 Hereditary Cancer Panel 1 probemix should in most cases be regarded as an autosomal dominant disorder. Inactivation of a single copy of one of these genes is thus expected to result in an increased chance/risk on developing cancer. For certain genes, the increased chance/risk on cancer development for heterozygous mutations is only modest. Examples are *CHEK2* and *MUTYH*. The *MUTYH* gene is generally regarded as an autosomal recessive gene.
- A heterozygous deletion of one or more exons present in the major transcript variant of that gene is expected to result in a hereditary predisposition to cancer. The clinical significance of a deletion of a probe sequence located upstream of exon 1, downstream of the last exon, or within the 3' UTR of a gene, is not always clear. For genes with multiple transcript variants, the clinical consequence of a deletion/duplication of an exon that is not present in the major transcript variant is not always clear (e.g. *APC* exon 1 and 2). In certain cases, such probes are included as there is some evidence for deletions in that region being clinically relevant (e.g. *APC* exon 1; *MSH6* upstream region). In other cases, such probes have been included in order to delineate the extent of deletions/duplications and to aid in designing primers for confirmation tests.
- 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. For most genes, duplication of the complete gene is not expected to result in disease.
- For certain genes, such as *PMS2*, *CHEK2*, *BMPR1A* and *PTEN*, pseudogenes exist that are almost identical to the actual gene. In several cases, probes for such genes detect a 1 nt difference between gene and pseudogene. In such cases, an apparent duplication detected by a single probe can be the result of a clinically non-significant one nucleotide sequence change in one of these pseudogenes.
- For two genes, *SMAD4* and *NBN*, the presence of a pseudogene has been reported which is present in less than 1% of individuals tested (Mancini et al. 2015; Millson et al. 2015). These pseudogenes are probably not clinically significant and are not present yet in the human reference sequence. The presence of this (processed) pseudogene will result in a heterozygous duplication detected by some, but not all, probes for that gene.
- The D001-C1 Probe Information File contains information on individual probes that is essential for interpretation of results.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the genes covered by the D001 probemix are small (point) mutations, most of which will not be detected by using this probemix.
- digitalMLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect most copy number neutral inversions or translocations. Even when digitalMLPA 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe read count 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 of multiple consecutive probes detected with the D001 Hereditary Cancer Panel 1 probemix should be verified by another method when possible. Conventional MLPA probemixes are available for many genes in the D001-C1 Hereditary Cancer Panel 1. Most of these conventional MLPA probemixes contain probes with a different ligation site that can be used for initial confirmation of results (see section 'Related conventional SALSA MLPA probemixes' in this product description). Alternatively, copy number changes can be confirmed by another independent technique such as long range PCR, qPCR, array CGH, FISH or Southern blotting.

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.

Mutation database

We strongly encourage users to deposit positive results in the Leiden Open Variation Database (http://www.lovd.nl/3.0/home). 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 SNPs and unusual results to MRC Holland: info@mrcholland.com.

Table 2. digitalMLPA Probemix D001-C1 Hereditary Cancer Panel 1

Chromosomal position (hg38)	Gene	NM sequence and LRG (if available) [a]	# probes / # exons in gene	Gene length (kb)	Remarks
1p34.1	***MUTYH***	NM_001128425.1 LRG_220	16/16	11.2	
2p21	***EPCAM***	NM_002354.2 LRG_215	5/9	17.7	Exon 7-9 covered; three probes between *EPCAM* & *MSH2*
2p21	***MSH2***	NM_000251.2 LRG_218	28/16	80.1	Includes two mutation-specific probes for recurrent 10 Mb 2p inversion; two copy number probes were added in the C1 version
2p16.3	***MSH6***	NM_000179.2 LRG_219	19/10	23.9	
2q35	***BARD1***	NM_000465.4 LRG_297	15/11	84.1	
3p22.2	***MLH1***	NM_000249.3 LRG_216	24/19	57.5	
3p21.1	***BAP1***	NM_004656.2 LRG_529	16/17	9.0	One probe was added in the C1 version
3p13	***MITF***	NM_000248.4	1 probe		Mutation-specific probe for c.952G>A (p.E318K)
5q22.2	***APC***	NM_000038.4 NM_001127510.1 NM_001127511.1 LRG_130	34/18	138.7	
7p22.1	***PMS2***	NM_000535.5 LRG_161	29/15	35.9	Includes one wildtype-specific and one mutation-specific probe for the intron 7 SVA insertion
8q21.3	***NBN***	NM_002485.4 LRG_158	18/16	51.3	
9p21.3	***CDKN2A***	NM_058195.3 NM_000077.4 LRG_011	13/4	26.7	
10q23.2	***BMPR1A***	NM_004329.3 LRG_298	18/13	168.5	
10q23.31	***PTEN***	NM_000314.4 LRG_311	23/9	105.3	Includes three probes in the upstream region (*KLLN* gene)
11q22.3	***ATM***	NM_000051.3 LRG_135	66/63	146.3	
12q14.1	***CDK4***	NM_000075.2 LRG_490	9/8	4.2	
12q24.33	***POLE***	NM_006231.3 LRG_789	4/49		Includes one wildtype-specific probe for c.1270C>G mutation

Chromosomal position (hg38)	Gene	NM sequence and LRG (if available) [(a)]	# probes / # exons in gene	Gene length (kb)	Remarks
					(p.Leu424Val); three probes were added in the C1 version
13q13.1	***BRCA2***	NM_000059.3 LRG_293	42/27	84.2	Includes one wildtype-specific probe for exon 3 ALU insertion c.156_157
15q13.3	***SCG5 GREM1***	NM_001144757.2 NM_013372.6	6 probes	Region covered ~68 kb	Detects recurrent 40-kb duplication
16p12.2	***PALB2***	NM_024675.3 LRG_308	20/13	38.2	Includes one flanking probe in the *DCTN5* gene; two probes were added in the C1 version
16q22.1	***CDH1***	NM_004360.3 LRG_301	20/16	98.2	
17p13.1	***TP53***	NM_000546.5 LRG_321	14/11	19.1	
17q12	***RAD51D***	NM_002878.3 LRG_516	11/10	20.1	
17q21.31	***BRCA1***	NM_007294.3 LRG_292	44/23	81.2	Exon numbering different from LRG/NG sequence; six probes were added in the C1 version
17q22	***RAD51C***	NM_058216.3 LRG_314	11/9	41.3	Includes one flanking probe in the *TEX14* gene
17q23.2	***BRIP1***	NM_032043.2 LRG_300	23/20	184.4	Includes one flanking probe in the *INTS2* gene; one probe was added in the C1 version
18q21.2	***SMAD4***	NM_005359.5 LRG_318	17/12	54.8	
19p13.3	***STK11***	NM_000455.4 LRG_319	17/10	22.6	One probe was removed and one probe was added in the C1 version
22q12.1	***CHEK2***	NM_007194.3 LRG_302	21/15	54.1	Includes one mutation-specific probe for 1100delC mutation And one flanking probe in the *HSCB* gene; three probes were added in the C1 version

(a) NM sequence and LRG: The exon numbering and NM_sequence used have been retrieved on 04/2020. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date. Exon numbering used here may differ from literature.

Extended information on the target genes of the D001-C1 Hereditary Cancel Panel 1 probemix according to chromosomal position:
A comprehensive guide about most of the genes included in this panel and the cancer types associated with them can be found in the Myriad MyRisk Hereditary Cancer Clinical Handbook.

MUTYH: Inactivation of the *MUTYH* gene results primarily in an increased risk of colorectal and small bowel cancer (MUTYH-associated Polyposis syndrome; MAP). In contrast to e.g. *APC*, MUTYH-associated polyposis is regarded as an autosomal recessive syndrome. 16 probes cover all 16 *MUTYH* exons in NM_001128425.1, except for exon 10 which is at very short distance (0.1 kb) of exons 9 and 11. Most ligation sites are close to those of the probes in MLPA probemix P378 MUTYH. More information is available at www.ncbi.nlm.nih.gov/books/NBK107219/.

EPCAM: Heterozygous deletions that include the *EPCAM* transcription stop site in exon 9 are known to result in Lynch syndrome (formerly known as HNPCC) due to methylation and inactivation of the adjacent *MSH2* gene (PMID 19098912). For this reason, only the last three *EPCAM* exons are covered (by four probes). Furthermore, three probes are included that cover the 15 kb region between *EPCAM* and *MSH2*. These three probes are included only to delineate the extent of deletions/duplications. Most ligation sites are close to those of the probes in MLPA probemix P003 MLH1/MSH2 and P072 MSH6-MUTYH. More information is available at www.ncbi.nlm.nih.gov/books/NBK1211/.

MSH2: The inactivation of one copy of the *MSH2* gene results primarily in an increased risk of colorectal, endometrial, gastric and ovarian cancer (Lynch syndrome). The *MSH2* gene is covered by a total of 23 probes covering all 16 *MSH2* exons in NM_000251.2. Next to these 23 probes, two probes are included that only generate read counts when the recurrent 10 Mb inversion that disrupts *MSH2* is present (PMIDs 24114314, 12203789 and 18335504). These probes do not generate read counts on normal samples. Furthermore, three probes flanking the *MSH2* gene are included. Most ligation sites are close to those of the probes in MLPA probemix P003 MLH1/MSH2. For confirmation of results, MLPA probemix P248 MLH1-MSH2 Confirmation can be used. More information is available at www.ncbi.nlm.nih.gov/books/NBK1211/.

MSH6: The inactivation of one copy of the *MSH6* gene results primarily in an increased risk of colorectal and endometrial cancer (Lynch syndrome). 17 probes cover all ten *MSH6* exons in NM_000179.2. Two additional probes located 5-6 kb upstream of *MSH6* cover a putative regulatory region (PMID 15942939). The long exon 4 (2544 nt) is covered by three probes. Most ligation sites are close to those of the probes in MLPA probemix P072 MSH6-MUTYH. More information is available at www.ncbi.nlm.nih.gov/books/NBK1211/.

BARD1: Inactivation of one copy of the *BARD1* gene results primarily in an increased risk of breast cancer. 15 probes cover all 11 *BARD1* exons and upstream region in NM_000465.4 No conventional MLPA probemix is available for the *BARD1* gene. More information is available at PMIDs 20077502, 21344236 and 20842729.

MLH1: The inactivation of one copy of the *MLH1* gene results primarily in an increased risk of colorectal, endometrial, gastric and ovarian cancer (Lynch syndrome). 24 probes detect the 19 *MLH1* exons in NM_000249.3. Most ligation sites are close to those of the probes in MLPA probemix P003 MLH1/MSH2. The MLPA P248 MLH1-MSH2 Confirmation probemix can be used for confirmation of results. More information is available at www.ncbi.nlm.nih.gov/books/NBK1211/.

BAP1: The inactivation of one copy of the *BAP1* gene results in an increased risk of melanomas (*BAP1* tumour predisposition syndrome). 16 probes cover the 17 *BAP1* exons in NM_004656.2, with the exception of exon 2, which is very close to adjacent exons. Most ligation sites are close to those of the probes in MLPA probemix P417 BAP1. More information is available at https://www.ncbi.nlm.nih.gov/books/NBK390611/ and PMIDs 24243779, 24187051, 23977234, 23849051 and 23684012.

MITF: Only one probe for the *MITF* gene is included. This probe is specific for the recurrent c.952G>A mutation (p.E318K; rs149617956), which has been reported as a cause for a predisposition to melanoma (PMIDs 22080950, 22012259 and 24406078). The probe will give read counts only when the mutation is present.

APC: Inactivation of one copy of the *APC* gene results primarily in an increased risk of colorectal and small bowel cancer (Familial Adenomatous Polyposis; FAP). 34 probes cover the 18 *APC* exons in NM_000038.4, NM_001127510.1 and NM_001127511.1. There are three probes included for the non-coding exon 1 (PMID 25243319). Exons 2 and 3 are also non-coding and are covered by two probes each. The long exon 18 is covered by ten probes. MLPA probemix P043 APC can be used for the confirmation of most findings. More information is available at www.ncbi.nlm.nih.gov/books/NBK1345/.

PMS2: The inactivation of one copy of the *PMS2* gene results primarily in an increased risk of colorectal and endometrial cancer (Lynch syndrome). 28 probes cover the 15 *PMS2* exons in NM_000535.5. Next to these, one probe is present that is specific for the presence of a intron 7 2-kb SVA repeat insertion, as described in PMID 22461402. The presence of this SVA insertion will also reduce the number of reads of exon 8 probe S017606 by ~50%.

PMS2 analysis is complicated, as there are no functional differences in exons 12, 13, 14 and 15 between *PMS2* and one of its pseudogenes. Therefore, of the 28 probes, seven probes (two probes for each exon, with the exception of exon 13) target exons 12-15 of *both PMS2* and its pseudogene. As each of these probes detects a sequence that is present in four (rather than two) copies per cell in normal individuals,

a deletion or duplication of one copy will result in a probe ratio (dosage quotient) of 0.75 or 1.25, respectively, rather than the usual 0.5 or 1.5 ratio expected for *diploid* probe targets. For deletions/duplications affecting only exons 12-15 it is not possible to conclude *where* the copy number change resides based on results obtained with this probemix. Although most changes appear to be in *PMS2* itself and not in its pseudogene (PMID 23012243), additional experiments such as long range PCR or RNA analysis will be required.

Most ligation sites are close to the probes in MLPA probemix P008 PMS2. Please note that for several PMS2 probes, there is only one nucleotide difference between the *PMS2* sequence detected by the probe and a sequence in one of the *PMS2* pseudogenes. In such cases, an apparent duplication detected by a single probe can be the result of a clinically non-significant one nucleotide sequence change in one of these pseudogenes. More information is available at www.ncbi.nlm.nih.gov/books/NBK1211/.

NBN: Inactivation of one copy of the *NBN* gene results primarily in an increased risk of breast cancer. 18 probes cover all 16 *NBN* exons in NM_002485.4. Please note that a novel *NBN* processed pseudogene might be present in a small part of the population (< 1:1000 individuals; Mancini et al., Myriad poster presented at ACMG2015). The presence of this pseudogene might result in an apparent duplication of many NBN probes. This new pseudogene is not present in the human reference genome and is probably clinically non-significant. No conventional MLPA probemix is available for the *NBN* gene. More information on the *NBN* gene is available at PMIDs 16770759 and 21514219, and at www.ncbi.nlm.nih.gov/books/NBK1176/.

CDKN2A: Inactivation of one copy of the *CDKN2A* gene results primarily in an increased risk of pancreatic cancer and melanomas. 13 probes cover the four *CDKN2A* exons in NM_000077.4 (p16INK4A) and NM_058195.3 (p14ARF). Two probes are present for an additional exon located between exon 3 and 4 in transcript NM_001195132.1 (p16-gamma). Most ligation sites are close to those of the probes in MLPA probemix P419 CDKN2A/2B-CDK4. More information is available at PMIDs 16234564, 10506626 and 10956390.

BMPR1A: The inactivation of one copy of the *BMPR1A* gene results in an increased risk of gastric and colorectal cancer (Juvenile Polyposis Syndrome; JPS). 18 probes cover all 13 *BMPR1A* exons in NM_004329.3. Analysis of this gene is complicated due to the existence of several closely related pseudogenes. Exons 1 and 2 are non-coding and are each covered by two probes. Two probes are, in a putative promoter region located in intron 2 (PMID20843829). Please note that for several *BMPR1A* probes, there is only one nucleotide difference between the *BMPR1A* sequence detected by the probe and a sequence in one of the *BMPR1A* pseudogenes. In that case, an apparent duplication detected by a single probe can be the result of a clinically non-significant one nucleotide sequence change in one of these pseudogenes. MLPA probemix P158 Juvenile polyposis syndrome (JPS) can be used for the confirmation of most findings. More information is available at www.ncbi.nlm.nih.gov/books/NBK1469/.

PTEN: Inactivation of one copy of the *PTEN* gene results primarily in an increased risk of breast, endometrial and thyroid cancer (PTEN Hamartoma Tumor Syndrome; PHTS). 23 probes cover all nine *PTEN* exons in NM_000314.4. Of these 23, three probes are included for the single exon *KLLN* gene that overlaps with *PTEN* exon 1. Please note that for several PTEN probes, there is only one nucleotide difference between the *PTEN* gene and the *PTEN* pseudogene. In such cases, an apparent duplication detected by a single probe could be the result of a clinically non-significant one nucleotide sequence change in the pseudogene. Ligation sites of the PTEN probes are close to the probes in MLPA probemix P225 PTEN. More information is available at PMID 18972196 and www.ncbi.nlm.nih.gov/books/NBK1488/.

ATM: Inactivation of one copy of the *ATM* gene results in an increased risk of breast cancer. 66 probes cover all 63 *ATM* exons in NM_000051.3. We use the LRG exon numbering which defines 63 exons. Exon 1 is non-coding and is covered by two probes. During validation experiments on the D001 probemix, we noticed in two samples a deletion of exons 62 and 63, while in several (unrelated) samples these two exons were duplicated. Please note that duplications that include the first or last exons of a gene might not disrupt that gene and might not be clinically significant. The LaBreche et al. Myriad poster

at ACMG 2017 indeed mentions that duplication of exons 62-63 is probably not associated with an increased risk of hereditary breast cancer based on 188 observations. MLPA probemix P041 ATM-1 and P042 ATM-2 can be used for confirmation of most findings. More information is available at www.ncbi.nlm.nih.gov/books/NBK26468/ and at PMIDs 16998505 and 22585167.

CDK4: Inactivation of a *CDK4* gene copy results primarily in an increased risk of melanomas. Nine probes cover all eight *CDK4* exons in NM_000075.2. Exon 1 is a non-coding region and is covered by two probes. Most ligation sites are close to those of the probes in probemix P419 CDKN2A/2B-CDK4. More information on *CDK4* is available at PMIDs 17047042 and 10861313.

POLE: One probe is included that is specific for the wildtype sequence at the recurrent c.1270C>G mutation (p.Leu424Val), which has been reported as a cause for a predisposition to colorectal adenomas and carcinomas (PMIDs 23447401, 24509466, 24501277, 25529843, 25124163 and 25370038). A 50% reduced read count for this probe can either be due to this mutation or to a deletion of the sequence detected by this probe. During validation experiments on the D001 probemix, we observed a duplication of this probe in three different samples. One sample was further tested and showed a complete *POLE* gene duplication. The clinical significance of this result is not clear. To evaluate whether duplications of the wildtype-specific probe are caused by (partial) duplications of the *POLE* gene, three additional probes are included targeting exon 2, 15 and 46.

BRCA2: The inactivation of one copy of the *BRCA2* gene results primarily in an increased risk of breast, ovarian, prostate and pancreatic cancer (hereditary breast and ovarian cancer; HBOC). 42 probes cover the 27 *BRCA2* exons in the NM_000059.3 transcript. Two probes target the non-coding exon 1. The long exon 11 is covered by nine probes. For exon 3, one extra probe is included that generates decreased read counts in samples harbouring either an exon 3 deletion or the c.156_157insAlu exon 3 mutation. For most probes, the ligation site is close to that of the probes in the P045 BRCA2/CHEK2 and P090 BRCA2 conventional MLPA probemixes. MLPA probemix P077 BRCA2 Confirmation can be used for the confirmation of most results. More information is available at www.ncbi.nlm.nih.gov/books/NBK1247/.

SCG5, GREM1: A recurrent 40-kb duplication in *GREM1* has been described to result in an increased risk of colorectal cancer. Shorter duplication regions have also been described. The presence of this duplication leads to an increased expression of the *BMP* antagonist *GREM1* and results in hereditary mixed polyposis syndrome (PMID 22561515). Six probes are included that are located within (five probes) or just outside (one probe) this recurrent duplication region. Most ligation sites are close to those of the probes in probemix P378 MUTYH. More information is available at www.ncbi.nlm.nih.gov/books/NBK1469/.

PALB2: The inactivation of one copy of the *PALB2* gene results in an increased risk of breast cancer. 19 probes cover the 13 *PALB2* exons in NM_024675.3. In addition, one probe is present for the *DCTN5* gene that is less than 2 kb upstream of *PALB2*. This probe is included only to delineate the extent of deletions/duplications. MLPA probemix P260 PALB2-RAD50-RAD51C-RAD51D can be used for confirmation of results. More information is available at PMID 21285249, 17287723, 17200668, 19264984 and 20412113.

CDH1: Inactivation of one copy of the *CDH1* gene results in an increased risk of gastric and breast cancer (hereditary diffuse gastric cancer; HDGC). 20 probes cover all 16 *CDH1* exons in NM_004360.3. MLPA probemix P083 CDH1 can be used for the confirmation of most findings. More information is available at www.ncbi.nlm.nih.gov/books/NBK1139/.

TP53: Inactivation of one copy of the *TP53* gene results in a strongly increased risk for various types of cancer (Li-Fraumeni Syndrome; LFS). 14 probes cover all but one of the 11 *TP53* exons in NM_000546.5. No probe is present for exon 9, which is present only in certain transcript variants and is located at very short distance from exons 8 and 10. Exon 1 is non-coding and is covered by two probes. Most ligation sites are close to those of the probes in probemix P056 TP53. More information is available at www.ncbi.nlm.nih.gov/books/NBK1311/.

RAD51D: Inactivation of one copy of the *RAD51D* gene results primarily in an increased risk of ovarian cancer. 11 probes cover all ten *RAD51D* exons in NM_002878.3. The P260 PALB2-RAD50-RAD51C-RAD51D MLPA probemix can be used for confirmation of most findings. More information is available at PMIDs 23372765, 22986143 and 22538716.

BRCA1: The inactivation of one copy of the *BRCA1* gene results in an increased risk of breast, ovarian, prostate and pancreatic cancer (hereditary breast and ovarian cancer; HBOC). The D001 probemix contains 44 probes for the *BRCA1* genomic region. These cover each exon in the *BRCA1* NM_007294.3 transcript.

Exon 1 is a non-coding region and is covered by two probes (exon 1a and exon 1b; exon 1b has an alternative transcription start site at 200 nt distance from exon 1a). Two probes at 4.6 kb and 0.7 kb upstream of exon 1 are included only to delineate the extent of deletions/duplications. The long exon 11 (3426 nt) is covered by eight probes. Exon 13, which is frequently deleted or duplicated, is covered by three probes. Deletions of exons 1a, 1b and 2 are relatively frequent (PMID 19405878), though lower probe read counts for these exons should be treated with caution. The presence of salt in the DNA sample can lead to an incomplete DNA denaturation of the CG rich exon 1a, 1b and 2 genomic region, resulting in reduced read counts for these probes. Please note that for several BRCA1 probes, there is only one nucleotide difference between the *BRCA1* gene and the *BRCA1* pseudogene. In such cases, an apparent duplication detected by a single probe could be the result of a clinically non-significant one nucleotide sequence change in the pseudogene.

The BRCA1 probe ligation sites are close to those of the probes in conventional MLPA probemix P002 BRCA1. MLPA probemix P087 BRCA1 Confirmation can be used for the confirmation of results. More information is available at www.ncbi.nlm.nih.gov/books/NBK1247/. A list of more than 65 publications describing the use of MLPA for *BRCA1* gene analysis can be found in the P002 BRCA1 MLPA product description.

Please note that MRC Holland uses the traditional *BRCA1* exon numbering, which lacks exon 4. We have not (yet) adopted the new exon numbering used by LRG and in the GenBank NG_005905.2 sequence.

RAD51C: Inactivation of one copy of the *RAD51C* gene results primarily in an increased risk of ovarian cancer. Ten probes cover all nine *RAD51C* exons in NM_058216.3. In addition, one probe for the *TEX14* gene, which is located upstream of *RAD51C* exon 1, is included only for delineation of the extent of any deletions/duplications found. MLPA probemix P260 PALB2-RAD50-RAD51C-RAD51D can be used for the confirmation of results. More information about *RAD51C* is available at PMIDs 20400964, 22538716, 21616938 and 20400963.

BRIP1: Inactivation of one copy of the *BRIP1* gene results in an increased risk of ovarian cancer. 22 probes cover all 20 *BRIP1* exons in NM_032043.2. In addition, one probe for the *INTS2* gene (located upstream of *BRIP1*) is included only to enable the delineation of the extent of any deletions/duplications found. Exon 1 is non-coding. MLPA probemix P240 BRIP1/CHEK1 can be used for confirmation of results. More information is available at PMIDs 21964575 and 17033622 and at www.ncbi.nlm.nih.gov/books/NBK1401/.

SMAD4: Inactivation of one copy of the *SMAD4* gene results in an increased risk of gastric and colorectal cancer (Juvenile Polyposis Syndrome; JPS; Hereditary Hemorrhagic Telangietasia; HHT). 17 probes cover all 12 *SMAD4* exons in NM_005359.5. Two of these probes are located in the putative promoter region A (PMID 21421563) located 62 kb upstream of exon 1. Exon 1 is non-coding and is covered by one probe. Please note that a novel *SMAD4* processed pseudogene was described, which can result in an apparent duplication of seven SMAD4 probes that are located almost entirely within exonic sequences (PMID 26165824). This new pseudogene is not present in the human reference genome, is probably clinically non-significant and is thought to be present in ~0.3% of the population. The MLPA probemix P158 Juvenile polyposis syndrome (JPS) can be used for confirmation of most results. More information is available at www.ncbi.nlm.nih.gov/books/NBK1469/.

STK11: Inactivation of one copy of the *STK11* gene results in an increased risk of various types of cancer (Peutz-Jeghers syndrome). 17 probes cover all ten *STK11* exons in NM_000455.4. The last exon, which is covered by two probes, is non-coding but its presence might be required for mRNA stability. Please note that the complete *STK11* gene is located in an exceptionally strong CpG island! The presence of salt in DNA samples can hinder a complete denaturation of the *STK11* gene region, resulting in false positive deletions (or duplications when the reference samples are affected). MLPA probemix P101 STK11 can be used for confirmation of most results. More information is available at www.ncbi.nlm.nih.gov/books/NBK1266/.

CHEK2: The inactivation of one copy of the *CHEK2* gene results in an increased risk for breast, colorectal and prostate cancer. 19 probes cover the 15 *CHEK2* exons in NM_007194.3. Two probes target the non-coding exon 1. In addition, the D001 panel contains one probe specific for the 1100delC mutation and one probe for the *HSCB* gene just upstream of *CHEK2* exon 1. This HSCB probe is included only to delineate the extent of deletions/duplications. Please note that for several CHEK2 probes, there is only one nucleotide difference between the *CHEK2* gene and *CHEK2* pseudogenes. In such cases, an apparent duplication detected by a single probe could be the result of a clinically non-significant one nucleotide sequence change in the pseudogene. The 1100delC specific probe contains a second ligation site to be able to detect this mutation only when it is present in the *CHEK2* gene, not in its pseudogene. Homozygosity for the *CHEK2* 1100delC mutation has been described (PMID 22058428). Most ligation sites are close to those of the probes in MLPA probemix P190 CHEK2. More information is available at PMIDs 18172190, 15122511, 23109706, 1167536 and 17085682.

More information on the location, mutation details and warnings of the probes present in this probemix can be found in the Probe Information File (PIF) available at www.mrcholland.com.

Table 3. Related conventional SALSA MLPA probemixes

<table>
<tr><th>Related probemix</th><th>Gene</th><th>Coverage</th><th>Can be used for confirmation ^</th></tr>
<tr><td>P378 MUTYH</td><td rowspan="3">MUTYH</td><td>MUTYH, GREM1, SCG5</td><td>no</td></tr>
<tr><td>P072 MSH6-MUTYH</td><td>MSH6, EPCAM, MUTYH (partly covered)</td><td>no</td></tr>
<tr><td>P043 APC</td><td>APC, MUTYH (partly covered)</td><td>no</td></tr>
<tr><td>P072 MSH6-MUTYH</td><td rowspan="2">EPCAM</td><td>MSH6, EPCAM, MUTYH</td><td>no</td></tr>
<tr><td>P003 MLH1/MSH2</td><td>MLH1, MSH2, EPCAM</td><td>no</td></tr>
<tr><td>P003 MLH1/MSH2</td><td rowspan="2">MSH2</td><td>MLH1, MSH2, EPCAM</td><td>no</td></tr>
<tr><td>P248 MLH1-MSH2 Confirmation</td><td>MLH1, MSH2</td><td>yes</td></tr>
<tr><td>P072 MSH6-MUTYH</td><td>MSH6</td><td>MSH6, EPCAM, MUTYH</td><td>no</td></tr>
<tr><td>No probemix available</td><td>BARD1</td><td>-</td><td>-</td></tr>
<tr><td>P003 MLH1/MSH2</td><td rowspan="2">MLH1</td><td>MLH1, MSH2, EPCAM</td><td>no</td></tr>
<tr><td>P248 MLH1-MSH2 Confirmation</td><td>MLH1, MSH2</td><td>yes</td></tr>
<tr><td>P417 BAP1</td><td>BAP1</td><td>BAP1</td><td>no</td></tr>
<tr><td>P419 CDKN2A/2B-CDK4</td><td>MITF</td><td>CDKN2A, CDKN2B, CDK4, MITF E318K</td><td>no</td></tr>
<tr><td>P043 APC</td><td>APC</td><td>APC</td><td>yes</td></tr>
<tr><td>P008 PMS2</td><td>PMS2</td><td>PMS2</td><td>no</td></tr>
<tr><td>P494 NBN</td><td>NBN</td><td>NBN</td><td>no</td></tr>
<tr><td>P419 CDKN2A/2B-CDK4</td><td>CDKN2A</td><td>CDKN2A, CDKN2B, CDK4, MITF E318K</td><td>no</td></tr>
<tr><td>P158 JPS</td><td>BMPR1A</td><td>BMPR1A, SMAD4</td><td>yes</td></tr>
<tr><td>P225 PTEN</td><td>PTEN</td><td>PTEN</td><td>no</td></tr>
<tr><td>P041 ATM-1, P042 ATM-2</td><td rowspan="2">ATM</td><td>ATM</td><td>yes</td></tr>
<tr><td>P190 CHEK2</td><td>CHEK2, ATM (partly covered), TP53</td><td>no</td></tr>
</table>

Related probemix	Gene	Coverage	Can be used for confirmation ^
P419 CDKN2A/2B-CDK4	*CDK4*	*CDKN2A, CDKN2B,* ***CDK4****, MITF* E318K	no
No probemix available	*POLE*	-	-
P090 BRCA2 P045 BRCA2/CHEK2	*BRCA2*	***BRCA2*** (identical probes in P090 and P045), *CHEK2* (only in P045)	no
P077 BRCA2 Confirmation		***BRCA2***	**yes**
P378 MUTYH	*SCG5* *GREM1*	*MUTYH,* ***SCG5, GREM1***	no
P260 PALB2-RAD50-RAD51C-RAD51D	*PALB2*	***PALB2****, RAD51C, RAD51D, RAD50*	**yes**
P083 CDH1	*CDH1*	***CDH1***	**yes**
P056 TP53	*TP53*	***TP53***	no
P190 CHEK2		*CHEK2, ATM,* ***TP53*** **(partly covered)**	**yes**
P260 PALB2-RAD50-RAD51C-RAD51D	*RAD51D*	*PALB2, RAD51C,* ***RAD51D****, RAD50*	**yes**
P002 BRCA1	*BRCA1*	***BRCA1***	no
P087 BRCA1 Confirmation		***BRCA1***	**yes**
P260 PALB2-RAD50-RAD51C-RAD51D	*RAD51C*	*PALB2,* ***RAD51C****, RAD51D, RAD50*	**yes**
P240 BRIP1/CHEK1	*BRIP1*	***BRIP1****, CHEK1*	**yes**
P158 JPS	*SMAD4*	*BMPR1A,* ***SMAD4***	**yes**
P101 STK11	*STK11*	***STK11***	**yes**
P190 CHEK2	*CHEK2*	***CHEK2****, (ATM* and *TP53* partly covered)	no
P045 BRCA2/CHEK2		*BRCA2,* ***CHEK2*** **(partly covered)**	no

^ Probemixes can be used for confirmation when ligation sites are different between D001-C1 probes and the probes in the corresponding probemixes. Of note, this statement concerns the majority of the probes in a probemix and does not mean that all probes always have a different ligation site. For more information, please contact info@mrcholland.com.

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D001 product history	
Version	*Modification*
C1	14 target probes adjusted (not in sequence detected), two target probes replaced, one target probe removed and 19 target probes added. Reference probe selection adjusted and an updated set of control probes included.
B1	First version commercially available as research use only (RUO) product.

Implemented changes in the product description
Version C1-04 – 28 June 2023 (03) - Reference to MS-digitalMLPA removed. - Text on CHEK2 1100delC mutation adjusted. *Version C1-03 – 14 April 2023 (03)* - Barcode plates BP03-IL and BP04-IL added under Catalogue numbers. - The list of positive samples tested with D001 (Table 1) was updated. *Version C1-02 – 09 December 2022 (03)* - Minor textual changes. - 'Amplifications' was changed to 'heterozygous/homozygous duplications/triplications' throughout the document. - Related conventional SALSA MLPA probemix P494 NBN was added to Table 3. *Version C1-01* – 21 February 2022 *(03)* - Product description adapted to a new product version (version number changed, changes in Table 2). - The list of positive samples tested with D001 (Table 1) was updated. *Version B1-03 – 03 February 2021 (03)* - Confirmation of results section updated. *Version B1-02 – 15 December 2020 (03)* - NM-sequence *MITF* updated. *Version B1-01 – 13 July 2020 (03)* - Not applicable, new document.

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