MRC Holland

digitalMLPA™ General Protocol

for use with Illumina sequencing platforms

Instructions For Use

**digital Multiplex Ligation-dependent Probe Amplification (digitalMLPA)
General Protocol for the detection and quantification of DNA sequences**

This protocol contains information that is essential for obtaining reliable digitalMLPA results. It must be read in its entirety and used in combination with the appropriate digitalMLPA probemix-specific product description.

digitalMLPA products are for Research Use Only (RUO).
A separate protocol exists for MS-digitalMLPA: the simultaneous detection of copy number changes, methylation changes AND specific point mutations in a single reaction.

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1. INTRODUCTION

Copy Number Variations (CNVs) are a prominent source of genetic variation in human DNA and play a role in a wide range of disorders. digitalMLPA is a semi-quantitative technique, based on the well-established (conventional) MLPA method, that is used to determine the relative copy number of up to 1000 DNA sequences in a single multiplex PCR-based reaction, followed by Illumina based sequencing for amplicon quantification. In addition to copy number analysis, probes for the detection and quantification of specific (point) mutations can also be included. NextGen Sequencing is used to determine read numbers of each digitalMLPA probe amplicon, not for sequence analysis of sample DNA.

digitalMLPA requires low sequence read coverage and uses a single PCR primer pair to amplify all ligated probes, making the method very robust.

Each digitalMLPA product description contains information on the intended use, the recommended protocol, and the interpretation of results for that product. Some applications are intended as stand-alone assays, while other applications are intended to complement DNA sequencing.

MRC Holland offers digitalMLPA reagents, digitalMLPA Barcode Solutions plates and application-specific digitalMLPA probemixes. In addition, for data analysis, MRC Holland provides free digitalMLPA data analysis software: Coffalyser digitalMLPA.

2. digitalMLPA ASSAY COMPONENTS & STORAGE CONDITIONS

To perform a digitalMLPA reaction, three items are required: (1) a SALSA digitalMLPA probemix, (2) a SALSA digitalMLPA reagent kit (DRK), and (3) a SALSA Barcode Solutions plate (two different plates of 96 barcode solutions are available). Currently digitalMLPA Barcode Solutions plates and reagent kits are only compatible with Illumina instruments.

2.1 digitalMLPA REAGENT KIT ITEM NUMBERS

Item no.	Description
DRK01-IL	SALSA digitalMLPA reagent kit – 100 rxn – Contains P5/P7 PCR primers for Illumina sequencers
DRK05-IL	SALSA digitalMLPA reagent kit – 500 rxn – Contains P5/P7 PCR primers for Illumina sequencers
DRK20-IL	SALSA digitalMLPA reagent kit – 2000 rxn – Contains P5/P7 PCR primers for Illumina sequencers
BP01-IL	SALSA Barcode Solutions plate 01 – 768 rxn – Illumina sequencers; Barcode solutions 1-96 (20 µl each)
BP02-IL	SALSA Barcode Solutions plate 02 – 768 rxn – Illumina sequencers; Barcode solutions 97-192 (20 µl each)

2.2 digitalMLPA REAGENT KIT COMPONENTS

	Reagent kit component	Volumes		Ingredients[1]
		DRK01	DRK05	
1	SALSA MLPA Buffer (yellow cap)	180 µl	5×180 µl	KCl, Tris-HCl, EDTA, PEG-6000, DTT, oligonucleotides
2	SALSA Ligase-65 (green cap)	115 µl	5×115 µl	Glycerol, EDTA, DTT, KCl, Tris-HCl, non-ionic detergent, Ligase-65 enzyme (bacterial origin)
3	SALSA Ligase Buffer A (transparent cap)	360 µl	5×360 µl	Coenzyme NAD (bacterial origin)
4	SALSA Ligase Buffer C (pink cap)	360 µl	5×360 µl	Tris-HCl, MgCl$_2$, non-ionic detergent
5	SALSA PCR Primer Mix P5P7 (brown cap)	240 µl	5×240 µl	Synthetic oligonucleotides, some with FAM fluorescent dye, dNTPs, salts, buffer, non-ionic detergent
6	SALSA Polymerase (light orange cap)	115 µl	5×115 µl	Glycerol, non-ionic detergents, EDTA, DTT, KCl, Tris-HCl, Polymerase enzyme (bacterial origin)

Items 1, 2, and 3 are identical to the conventional MLPA reagents.

2.3 APPLICATION-SPECIFIC digitalMLPA PROBEMIX

	Application-specific digitalMLPA probemix	Volumes (R=number of reactions)	Ingredients[1]
1	Probemix (grey cap)	40 µl (25R), 80 µl (50R), 160 µl (100R)	Synthetic oligonucleotides, Tris-HCl, EDTA, DTT

2.4 BARCODE SOLUTIONS PLATE

	SALSA Barcode Solutions plate	Volumes (R=number of reactions)	Ingredients[1]
1	BP01	96×20 µl (768R)	Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA, DTT
2	BP02	96×20 µl (768R)	Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA, DTT

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[1] None of the ingredients are derived from humans, animals, or pathogenic bacteria. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. **A Safety Data Sheet (SDS) is not required for these products**: none of the preparations contain dangerous substances (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and amendments) at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments). If spills occur, clean with water and follow appropriate site procedures.

2.5 STORAGE AND SHELF LIFE

SALSA digitalMLPA probemixes, SALSA digitalMLPA reagent kits, and SALSA Barcode Solutions plates must be stored directly upon arrival between -25°C and -15°C, shielded from light and in the original packaging. When stored under the recommended conditions, a shelf life of at least 1 year is guaranteed, also after opening. See the labels on each vial for the exact expiry date of each reagent. Reagents, Barcode Solutions plates and probemixes should not be exposed to more than 25 freeze-thaw cycles. While the Barcode Solutions plates are in use, they can be stored at 4°C for a period of 2 to 3 months.

2.6 PACKAGING LABELS

![icon]	Manufacturer		![icon]	Store at
LOT	Lot Number		![icon]	Keep away from heat or direct sunlight
![icon]	Use by		REF	Catalogue Number
![icon]	Number of Tests		![icon]	Read instructions before use
RUO	Research Use Only		IVD	In Vitro Diagnostics

3. digitalMLPA ASSAY PRINCIPLE

digitalMLPA, like conventional MLPA, is based on the sample DNA-dependent generation of ligated probe products, followed by PCR amplification of ligated probes by a single PCR primer pair (Schouten JP et al. (2002) *Nucleic Acids Res.* 30:e57, Figure 1). In digitalMLPA, up to 1000 different probes can be included in a probemix and used in a single reaction.
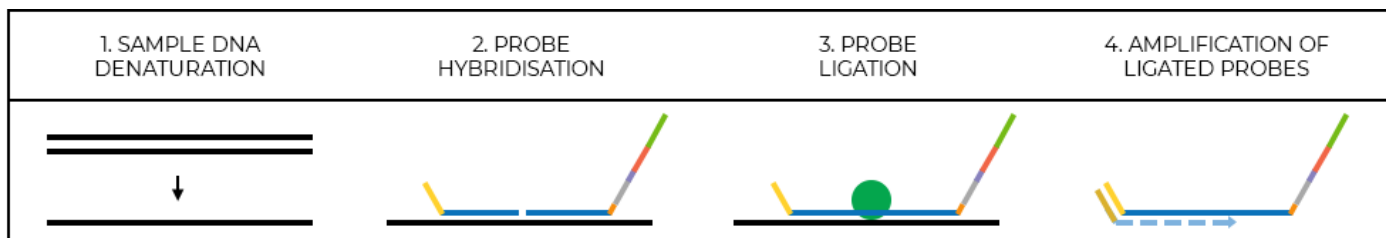
Figure 1. digitalMLPA reaction

Data analysis by Coffalyser digitalMLPA first determines the *absolute* read number for each probe by assigning each read to a sample using the unique barcode and to a specific probe by the unique probe sequence. Next, the absolute read counts of a sample are converted into relative values (intra ratios) by normalising target probe read counts against the read counts of every reference probe in the sample (intra-normalisation). This is done for every probe and every sample. In the next step, called inter-normalisation, Coffalyser digitalMLPA compares the relative probe values of each sample to those of the reference samples, resulting in inter ratios (Figure 2).

Region	Exon	Probe ratio	As expected	Δ Reference
	Exon …	1	✔	equal
	Exon 3	1	✔	equal
	Exon 4	1	✔	equal
GENE3	Exon 5	1	✔	equal
	Exon 6	1.5	Too high	2 stdev
	Exon 7	1.5	Too high	2 stdev
	Exon 8	1.5	Too high	2 stdev
	Exon …	1.5	Too high	2 stdev

Figure 2. Example Coffalyser digitalMLPA data output visually depicting the inter ratios of genes in a sample and highlighting an aberrant region found in the sample.

4.   ASSAY SETUP INSTRUCTIONS

4.1   MATERIALS REQUIRED BUT NOT PROVIDED

- Ultrapure water
- $TE_{0.1}$ (10 mM Tris-HCl pH 8.0 + 0.1 mM EDTA)
- Calibrated thermocycler with heated lid (99-105°C) and standard laboratory equipment
- 0.2 ml PCR tubes, strips or plates
- LoBind tubes (for library dilution)
- NaOH solution (for denaturation of samples before loading on the Illumina sequencers, see section 7.6.
- Illumina sequencer equipment, Illumina sequencer flow cell and reagents
- Coffalyser digitalMLPA analysis software and User Manual (freely downloadable at www.mrcholland.com).

4.2   SAMPLE TREATMENT

- **For each digitalMLPA reaction, use a total quantity of 20-250 ng[2] (40-100 ng is optimal) of human DNA in a 4 µl[3] volume** (unless stated otherwise in the probemix-specific product description). If necessary, DNA samples can be concentrated by ethanol precipitation. Glycogen (Roche 901393) can be used as a carrier in ethanol precipitations. For more information visit support.mrcholland.com.
- DNA preparations should contain 5-10 mM Tris buffer with a pH of 8.0-8.5 to prevent depurination during the initial denaturation step at 98°C. For example, dissolve and dilute sample DNA in 5-10 mM TE pH 8.0-8.5 (preferred $TE_{0.1}$: 10 mM Tris-HCl pH 8.0-8.5 + 0.1 mM EDTA).
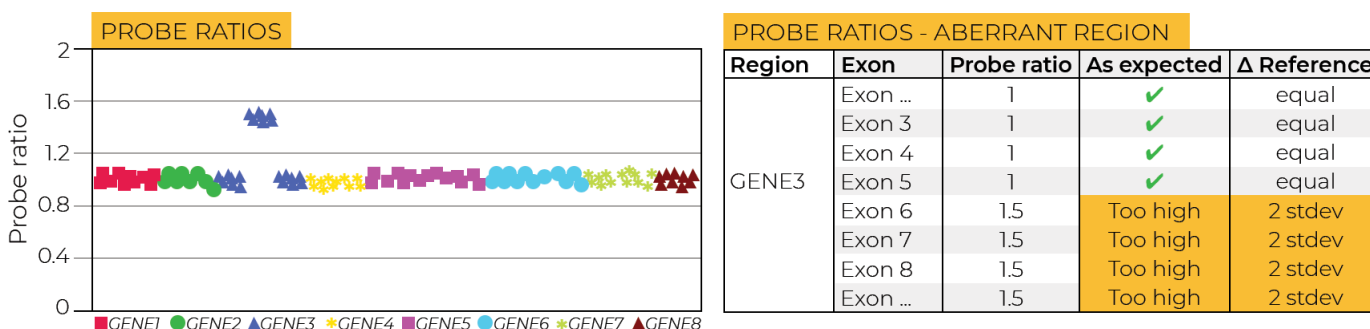- Contaminants remaining after DNA extraction, including NaCl or KCl (>60 mM) and other salts, phenol, ethanol, heparin, EDTA (>4 mM) and Fe, may influence digitalMLPA performance. To minimise their effect, ensure the extraction method, tissue type, DNA concentration and treatment are as identical as possible in test and reference samples. Do not concentrate DNA by evaporation or SpeedVac; this leads to high EDTA and salt concentrations.
- Extraction methods should not leave a high concentration of contaminants. Do not use QIAGEN M6, M48 and M96 systems, as they leave too much salt. For QIAGEN EZ1, use the QIAGEN Supplementary Protocol: Purification of genomic DNA from whole blood, optimized for use in MRC-Holland MLPA® assays, using EZ1® DNA Blood Kits (see support.mrcholland.com). MRC Holland has tested and can recommend the following extraction methods:

  - QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)
  - Promega Wizard Genomic DNA Purification Kit (manual)
  - Salting out (manual)

- Heparinised blood can only be used when the sample has undergone a purification method to remove the heparin contamination (e.g. Nucleospin gDNA Clean-up XS).
- An RNAse treatment step is only essential when examining genes that are highly expressed in the sample tissue studied. Examples are *HBA* and *HBB* genes in blood-derived samples and mitochondrial ribosomal RNA genes (all tissues).
- DNA from whole genome amplification reactions (WGA) is not suitable for digitalMLPA due to amplification bias.
- In case of doubts about DNA purity: a) only use 20 ng of sample DNA; b) clean contaminated samples by ethanol precipitation or with silica-based clean-up kits.

4.3   SELECTING REFERENCE & OTHER CONTROL SAMPLES

- REFERENCE SAMPLES. The use of dedicated reference samples is recommended for most digitalMLPA experiments. In certain situations, e.g., when a large number of samples are used and copy number changes are expected to be (very) rare, it is possible to omit dedicated reference samples and to rely on all test samples for inter-normalisation.
  Using dedicated reference samples. Multiple reference samples are needed to estimate the reproducibility of each probe within an experiment. The minimum number of different reference samples that should be used is three. When testing >21 samples: include one additional reference sample per seven additional test samples. Furthermore, X- and Y-chromosome target probes are analysed using reference samples of the same gender as the test sample, and sufficient (≥3) reference samples should be present per gender. Reference samples should be distributed randomly over the sample plate. Reference samples should be indicated as type "Reference" in Coffalyser digitalMLPA.

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[2] Optical density (260 nm) measurements often overestimate the DNA concentration e.g. due to contamination with RNA.
[3] Never use more than 4 µl sample DNA per reaction. Using more than 4 µl DNA reduces the probe and salt concentration in the digitalMLPA reaction, which reduces the hybridisation speed and the stability of the binding of MLPA probes to the sample DNA.

Using test samples for normalisation. For applications in which copy number changes are rare, no separate reference samples are required when sufficient samples from unrelated families are tested and the chance is negligible that the majority of the samples tested have the same mutation/copy number change. The exact number of samples that should be included depends on the application and on the experiment set up, but in general a larger population is better. More information on determining the number of samples needed, can be found in our help centre: https://www.mrcholland.com/r/digitalmlpa-number-of-samples. In such cases, all samples tested should be indicated as "test" samples in Coffalyser digitalMLPA.

- SELECTING REFERENCE SAMPLES. Reference samples are DNA samples, obtained from healthy individuals, with a normal copy number for sequences detected by the target and reference probes. They should be as similar as possible to the test samples in all other aspects, including extraction method and sample source (see section 4.2). For FFPE tissue, use reference samples derived from similarly treated, healthy tissue if possible. Please note that not all probemixes are suitable for use with DNA from all sources (e.g. formalin-fixed paraffin-embedded (FFPE) tissue). Always consult the probemix-specific product description for suitable DNA sources.

- COMMERCIAL DNA. In case of doubts about sample quality, include one or more commercial DNA samples for comparison (recommended: Promega Cat. Nr G1471 male, G1521 female DNA[4] or cell line-derived DNA from Coriell.org). The commercial DNA should only be used as a control to check sample quality and cannot be used as a reference sample.

- NO DNA CONTROL. Inclusion of a No DNA control reaction in each digitalMLPA experiment is not essential. Almost all reads from non-specific amplicons are removed during data analysis as reads are only counted when a correct RPO, LPO and barcode sequence are present. The absence of a substantial number of reads linked to unused barcode sequences indicates the absence of contamination from previous experiments. The 78 SNP-specific control probes included in each digitalMLPA probemix will indicate if there is contamination of sample DNA or of the reagents used with another DNA sample. When reads containing a correct LPO and RPO are obtained in a No DNA reaction, these are usually derived from a very small number of ligation events. Such rare non-specific amplicons are outcompeted in normal digitalMLPA reactions by the large number of specific ligation events.

- POSITIVE CONTROL SAMPLES. Inclusion of positive control samples is recommended when available. MRC Holland does not provide positive samples. A list of commercially available positive samples is available on support.mrcholland.com. When using DNA from cell lines as a positive control sample, please note that cell lines may have acquired additional copy number changes, including gains or losses of complete chromosomes.

- ALIQUOT PRECIOUS REFERENCE/CONTROL SAMPLES. Store these aliquots at -20°C. Contamination with microorganisms or moulds can deteriorate samples that are stored at 4°C for an extended period of time.

5. digitalMLPA REACTION - DNA DETECTION/QUANTIFICATION

NOTES TO READ BEFORE YOU START

- Use a calibrated thermocycler with heated lid (99-105°C).
- Always vortex thawed buffers, primer mix and probemix, followed by centrifugation a brief spin before use. MLPA buffer is typically frozen at -20°C but may remain liquid due to its high salt concentration.
- Thaw the Barcode Solutions plate by placing it at room temperature. After thawing, spin the plate at 1000 rpm for a few seconds, then mix the barcode solutions by gently swirling the plate using a rocking plate or microplate shaker, followed by brief centrifugation of the plate for a few seconds at 1000 rpm.
- When using barcode solutions from multiple Barcode Solutions plates in one experiment, make sure that the first two digits of the lot are identical, e.g. "03-". N.B. Within one sample in an experiment, only one Barcode Solution from one plate can be used, it is not possible to combine barcode solutions from multiple plates in one sample in one experiment.
- Enzyme solutions contain 50% glycerol and remain liquid at -20°C. Master mixes containing enzymes should be mixed thoroughly by gently pipetting up and down. Insufficient mixing can result in unreliable results. When preparing master mixes, always add enzymes last. Never vortex enzymes or solutions containing enzymes as enzyme inactivation can occur.
- To minimise sample-to-sample variation, prepare sufficiently large volumes of master mixes (5-10% volume surplus).
- Prepare master mixes (Ligase-65 and polymerase) at room temperature (RT) right before use. When prepared >1 hr before use, store master mixes on ice or at 4°C. Master mixes should be warmed to RT before addition to digitalMLPA reactions. Use multi-channel pipettes to avoid excessive evaporation.

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[4] Promega DNA is a mixture of DNA from different individuals. When using Promega DNA, "Pooled DNA" should be selected as sample type. as Coffalyser digitalMLPA will otherwise provide a warning that the sample contains multiple DNA samples ("contamination").

- Illumina sequencing requires sufficient variation in the first nucleotides of the reads for proper cluster detection. Except for 75 nt runs with custom primers, digitalMLPA reads start with the barcode sequence. These barcode sequences are distributed over the Barcode Solutions plate in such a way that each row or column provides sufficient variation in each nucleotide read. When only digitalMLPA reactions are loaded on a flow cell, and the experiment consists of less than 8 reactions, a PhiX spike-in is recommended. This spike-in can be performed according to Illumina's *System Denature and Dilute Libraries Guides* for your particular instrument using Illumina PhiX Control v3, FC-110-3001.

6. digitalMLPA PROTOCOL – IN BRIEF

1. DNA DENATURATION

    - Mix 4 µl DNA sample and 2 µl barcode solution. Use a different barcode solution for each reaction!
    - Heat this mixture for 10 minutes at 98°C.

2. HYBRIDISATION OF PROBES TO SAMPLE DNA

    - Cool down to room temperature, open the tubes.
    - Add 3 µl Hybridisation master mix*.
    - Incubate 1 minute at 95°C and hybridise at 60°C for 16-20 hours.

3. LIGATION OF HYBRIDISED PROBES

    - Lower thermocycler temperature to 48°C; open tubes (in the thermocycler!).
    - Add 32 µl Ligase master mix*; incubate 30 minutes at 48°C.
    - Heat inactivate the Ligase enzyme: 5 minutes at 98°C, followed by 20 minutes at 65°C.

4. PCR AMPLIFICATION OF LIGATED PROBES

    - Cool down to room temperature; remove tubes from thermocycler; open tubes.
    - Add 10 µl Polymerase master mix* at room temperature.
    - Return tubes to thermocycler and start PCR (65°C 1 minute, 45 x {95°C 30 seconds, 65°C 40 seconds, 72°C 90 seconds}, 15°C pause).

5. MIX A PORTION OF ALL digitalMLPA PCR REACTIONS

    - When the PCR program has been completed, mix 5 µl of each reaction in a LoBind tube.

        NOTE: When experiments with different digitalMLPA probemixes are combined in one run, the relative amount used of each PCR reaction will depend on the number of probes in each reaction (see section 8.1).

6. PREPARE MIXED digitalMLPA PRODUCTS FOR LIBRARY HANDLING BEFORE SEQUENCER LOADING

    - **See section 7.6** for sequencer specific dilutions (use LoBind tubes!). Example for MiSeq: Dilute 4.5 µl of the digitalMLPA PCR product mix with 95.5 µl water.

        o Denature with NaOH: 5 µl **diluted** digitalMLPA PCR mix + 5 µl 0.2 M NaOH.
        o Incubate 5 minutes at room temperature.
        o Add 990 µl cold Illumina HT1 buffer.

7. LOAD ON SEQUENCER

    - Start sequencer run to generate single reads of 110 nt or longer.

8. ANALYSE RESULTS WITH COFFALYSER digitalMLPA

    - Load FASTQ files into Coffalyser digitalMLPA.

* Master mixes:

- Hybridisation master mix: 1.5 µl digitalMLPA probemix + 1.5 µl MLPA Buffer
- Ligase master mix: 25 µl ultrapure water + 3 µl Ligase Buffer **A** + 3 µl Ligase Buffer **C** + 1 µl Ligase-65
- Polymerase master mix: 7 µl ultrapure water + 2 µl PCR Primermix **P5P7** + 1 µl polymerase.

7. digitalMLPA PROTOCOL

7.1 THERMOCYCLER PROGRAM FOR digitalMLPA REACTIONS

DNA denaturation			
1.	98°C	*10 minutes*	
2.	25°C	*pause*	
Hybridisation reaction			
3.	95°C	*1 minute*	
4.	60°C	*pause*	*16-20 hours*
Ligation reaction			
5.	48°C	*pause*	
6.	48°C	*30 minutes*	
7.	98°C	*5 minutes*	
8.	65°C	*20 minutes*	
9.	20°C	*pause*	
PCR reaction			
10.	65°C	*1 minute*	
11.	45 cycles	• 95°C *30 seconds*	
		• 65°C *40 seconds*	
		• 72°C *90 seconds*	
12.	15°C	*pause*	

7.2 DNA DENATURATION (DAY 1)

- Label 0.2 ml tubes, strips or plates.
- Add 4 µl DNA sample (20-250 ng; 40-100 ng is optimal) or TE (No DNA control) to each tube.
- Add 2 µl barcode solution to each tube. Use a unique barcode solution for each reaction that will be combined in one run on the Illumina flow cell or lane[5].
- Place closed tubes in thermocycler; start digitalMLPA thermocycler program steps 1-2 (see section 7.1).
- Ensure samples are at 25°C before removing tubes from thermocycler.

7.3 HYBRIDISATION REACTION (DAY 1)

- Prepare Hybridisation master mix. For each reaction, mix: 1.5 µl MLPA Buffer (yellow cap) + 1.5 µl probemix (grey cap). Mix well by pipetting or vortexing.
- After DNA denaturation, add 3 µl Hybridisation master mix to each reaction. Accurate pipetting is critical. Mix well by pipetting gently up and down.
- Check that all contents are at the bottom of the tubes. If necessary, spin down briefly before placing the tubes in the thermocycler.
- Continue thermocycler program with steps 3-4. Hybridisation at 60°C should be for at least 16 hrs.

7.4 LIGATION REACTION (DAY 2)

- Prepare Ligase master mix. For each reaction, mix: 25 µl ultrapure water + 3 µl Ligase Buffer **A** (transparent cap) + 3 µl Ligase Buffer **C** (pink cap), then add 1 µl Ligase-65 enzyme (green cap). Mix well by pipetting gently up and down.
- Continue the thermocycler program with step 5.
- When the samples are at 48°C and **while the samples are IN the thermocycler**, add 32 µl Ligase master mix to each tube. Mix well by pipetting gently up and down.
- Continue the thermocycler program with steps 6-9.

7.5 PCR REACTION (DAY 2)

- Prepare Polymerase master mix. For each reaction, mix: 7 µl ultrapure water + 2 µl PCR Primermix P5P7 (brown cap), then add 1 µl SALSA Polymerase (light orange cap). Mix well by pipetting gently up and down.
- **At room temperature,** add 10 µl Polymerase master mix to each tube. Mix well by pipetting gently up and down. Immediately place the tubes in the thermocycler and continue the thermocycler program with steps 10-12.
- After the PCR reaction, do not open tubes in the room with the thermocycler. To avoid contamination, use different micropipettes for performing digitalMLPA reactions and for handling digitalMLPA PCR products.

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[5] To minimise the chance of carry-over, we recommend to use different barcodes between subsequent NGS runs.

- PCR product can be stored at 4°C for at least 1 week. For longer periods, store between -25°C and -15°C.

7.6 PREPARATION OF THE digitalMLPA LIBRARY FOR SEQUENCING (DAY 2)

- **Use LoBind tubes for each step of library preparation.**
- No post-PCR clean-up is necessary for digitalMLPA.
- No quantification of library or quality check on Tapestations or other devices is required.
- Each reaction in the library must have a unique barcode solution.

7.6.1 iSeq INSTRUMENT (DAY 2)

- o Combine 5 µl of each PCR reaction in a single tube (each reaction must have a unique barcode solution)[6]. Mix by brief vortexing.
- o Dilute 4 µl[7] of this mixture with 196 µl of ultrapure water. Mix well. This results in a 4 nM amplicon library.
- o Mix 5 µl of this 4 nM library with 995 µl of ultrapure water. Mix well. This results in a 20 pM amplicon library.
- o Load 20 µl of the amplicon library (20 pM) at the bottom of the position indicating "library" (orange) of the iSeq reagent tray and start the iSeq run.

7.6.2 MiSeq INSTRUMENT (DAY 2)

- o Combine 5 µl of each PCR reaction in a single tube (each reaction must have a unique barcode solution)[6]. Mix by brief vortexing.
- o Dilute 4.5 µl[7] of this mixture with 95.5 µl of ultrapure water. Mix well. This results in a 9 nM amplicon library.
- o Mix 5 µl of the 9 nM amplicon library with 5 µl of 0.2 M NaOH[8].
- o Mix well and incubate at room temperature for 5 minutes.
- o Add 990 µl of pre-chilled Illumina HT1 Buffer. Place on ice until loading on the MiSeq reagent tray.
- o Load 600 µl of the denatured library (50 pM) in position 17 (orange) of the MiSeq reagent tray (v3; 150 cycle kit; Illumina MS-102-3001) and start the MiSeq run immediately.

7.6.3 MiniSeq INSTRUMENT (DAY 2)

- o Combine 5 µl of each PCR reaction in a single tube (each reaction must have a unique barcode solution)[6]. Mix by brief vortexing.
- o Dilute 5 µl[7] of this mixture with 75 µl of ultrapure water. Mix well. This results in a 12.5 nM amplicon library.
- o Mix 4 µl of this 12.5 nM library with 251 µl of ultrapure water. Mix well. This results in a 200 pM amplicon library.
- o Mix 5 µl of the 200 pM library with 5 µl of 0.1 M NaOH[8].
- o Mix well and incubate at room temperature for 5 minutes.
- o Add 990 µl of pre-chilled Illumina HT1 Buffer. This is a 1 pM denatured library. Place on ice until loading on the MiniSeq reagent tray.
- o Load 500 µl of the 1 pM denatured library in position 16 (orange) of the MiniSeq reagent tray (150 cycle kit; Illumina FC-420-1002) and start the MiniSeq run immediately.

7.6.4 NextSeq INSTRUMENT (DAY 2)

- o Combine 5 µl of each PCR reaction in a single tube (each reaction must have a unique barcode solution)[6]. Mix by brief vortexing.
- o Dilute 5 µl[7] of this mixture with 95 µl of ultrapure water. Mix well. This results in a 10 nM amplicon library.
- o Mix 5 µl of the 10 nM amplicon library with 5 µl of 0.2 M NaOH[8].
- o Mix well and incubate at room temperature for 5 minutes.
- o Add 490 µl of pre-chilled Illumina HT1 Buffer. Place on ice. This is a 100 pM denatured library.
- o Mix 75 µl of the 100 pM denatured library with 1425 µl of pre-chilled HT1 Buffer. This is a 5 pM denatured library. Place on ice until loading on the NextSeq reagent tray.
- o Load 1300 µl of the 5 pM denatured library in position 10 (orange) of the NextSeq reagent tray (Mid Output 150 cycles; Illumina FC-404-2001) and start the NextSeq run immediately.

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[6] When different digitalMLPA probemixes are used in one run, the relative amount used of each PCR reaction depends on the number of probes in each reaction (section 8.1).

[7] These are the dilutions used at MRC Holland for iSeq, MiniSeq, MiSeq and NextSeq runs. Adjust this dilution in order to generate more or decrease the number of clusters. For your first experiment, we recommend to use only 80% of the recommended amount of PCR mix.

[8] For 0.2 M NaOH (MiSeq/NextSeq): dissolve 0.4 gram solid NaOH in 50 ml ultrapure water. For 0.1 M NaOH (MiniSeq): dissolve 0.4 gram solid NaOH in 100 ml ultrapure water. Make fresh NaOH or store single use 50 µl aliquots at -20°C.

8. AMPLICON QUANTIFICATION BY ILLUMINA SEQUENCERS

8.1 NOTES TO READ BEFORE YOU START

- Run conditions, recommended flow cell and dilution factor of the digitalMLPA PCR reactions mix (library concentration) depend on instrument type. Settings given here may require optimisation in order to obtain the highest read count possible.
- When loading only digitalMLPA reactions, we recommend to use 110 nt single reads. No separate index read is required. Instructions for the use of NextSeq 75 cycles High-Output Kit and HiSeq / NovaSeq instruments, and for combining digitalMLPA reactions with other experiments in the same run, are available upon request (contact info@mrcholland.com).
- When preparing the Illumina run sheet with the Local Run Manager, click *Create Run* and select *Generate FASTQ*. In the run settings:
  - o For Library Prep Kit, select Custom (MiSeq) or TruSeq LT (MiniSeq).
  - o For *Index Reads*, select *0*.
  - o For Read Type, select single read and set Read Lengths (Read 1) to 110.
  - o Under *SampleID*, enter a name (can be the experiment name) and click *Save Run*. The status of the run is now *Ready for Sequencing*.
    For more information see: https://www.mrcholland.com/r/digitalmlpa-library-prep-kit-template
- Experiments with different digitalMLPA probemixes can be combined in one run or lane. When mixing PCR reactions, the volume of each reaction used must maintain the correct proportions of digitalMLPA "data points" (number of reactions × number of probes in the probemix, as specified in the Probemix-specific product description) for each probemix used. In addition, a unique barcode must have been used for every sample that will be combined regardless of the probemix used as control probes are identical in all digitalMLPA probemixes.
  - o For example
    - ▪ 20 reactions with probemix A (575 probes) + 56 reactions with probemix B (300 probes).
    - ▪ Prepare a mix containing 5 µl of each PCR reaction of probemix A (**vial 1**; 20 × 575 = **11,5**00 "data points").
    - ▪ Prepare a mix containing 5 µl of each PCR reaction of probemix B (**vial 2**; 56 × 300 = **16,8**00 "data points").
    - ▪ Prepare a mixture containing **11.5** µl of vial 1 + **16.8** µl of vial 2. Use 5 µl of this mixture and dilute with ultrapure water as described in section 7.6.
    - ▪ For this example there are 11,500 + 16,800 = 28,300 (11,500 + 16,800) "data points". A median of at least 400 reads / "data point" is recommended. These 28,300 data points require 28,300 × 400 = 11,320,000 reads.
- digitalMLPA reactions can be combined with sequencing reactions in one run or lane. Each digitalMLPA read contains a 27 nt digitalMLPA read identifier sequence, which is used to distinguish reads belonging to digitalMLPA reactions within a FASTQ file. The FASTQ file from a run can be directly loaded into Coffalyser digitalMLPA.
- When the digitalMLPA reactions are mixed with sequencing or other reactions, either single reads or paired-end reads can be selected. The digitalMLPA clusters will not generate a sequence in the second read or in the separate barcode reads. As a result, digitalMLPA reads will be placed in the FASTQ file with discarded (non-indexed) reads. This FASTQ file can be imported into Coffalyser digitalMLPA.

8.2 FLOW CELL USED & INSTRUMENT SETTINGS

Instrument	Read length	Recommended flow cell /reagent kit *	Initial settings
iSeq	Single reads, 110 nt or more. No separate index read required.	Standard iSeq flow cell	▪ Prepare library as described in section 7.6.1. Combine a maximum of 10 reactions for probemixes with ~600 probes. More reactions can be mixed when fewer probes are present (see section 8.1).
MiSeq	Single reads, 110 nt or more. No separate index read required.	Illumina # MS-102-3001 MiSeq Reagent Kit v3 (150 cycle)[9].	▪ Prepare library as described in section 7.6.2. Combine a maximum of 64 reactions for probemixes with ~600 probes. More reactions can be mixed when fewer probes are present (see section 8.1). ▪ Library prep kit: Custom. Index reads: 0. Select single read and set read length (Read 1) to 110 nt. Enter experiment name under SampleID, save run and start sequencing.

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[9] We strongly recommend not to use the MiSeq Reagent Kit v2 for digitalMLPA.

MiniSeq	Single reads, 110 nt or more. No separate index read required.	Illumina # FC-420-1002 MiniSeq High Output Kit (150 cycle).	▪ Prepare library as described in section 7.6.3. Combine a maximum of 64 reactions for probemixes with ~600 probes. More reactions can be mixed when fewer probes are present (see section 8.1). ▪ Library prep kit: TruSeq LT. Index reads: 0. Select single read and set read length (Read 1) to 110 nt. Enter experiment name under SampleID, save run and start sequencing.
NextSeq 500/550	Single reads, 110 nt or more. No separate index read required.	Illumina NextSeq 500/550 Mid Output Kit v2.5 (150 cycle).	▪ Prepare library as described in section 7.6.4. Combine a maximum of 192 reactions for probemixes with ~600 probes. More reactions can be mixed when fewer probes are present (see section 8.1). ▪ Select protocol "FASTQ Only". Index reads: 0. One single read of minimum 110 nt. Optional: Library prep kit: TruSeqLT.
NextSeq 500/550	Single reads, 75 nt with a separate index read	Illumina NextSeq High Output Kit (75 cycle).	▪ Custom primers are required. Contact MRC Holland for primer sequences and more information (info@mrcholland.com).
HiSeq / NovaSeq / NextSeq 1000/2000	Single reads, 110 nt or more. No separate index read required.	Any flow cell capable of generating 110 nt or more sequences in the first read.	▪ Combine a maximum of 192 reactions. For probemixes with ~600 probes 192 reactions requires approximately 80 million reads. Contact MRC Holland for assistance (info@mrcholland.com).

* If recommended reagent kits are no longer available, please contact Illumina for the current equivalent.

8.3   READ DEPTH REQUIRED FOR OPTIMAL DATA ANALYSIS

The use of 20 ng or more sample DNA in each reaction and a median read number of at least 400 for all reference probes in each reaction are required for optimal results. Within one reaction, all probes targeting unique autosomal sequences will have similar read numbers when testing normal samples (+/-30%). The average read number of all probes will be similar between different reactions (+/-20%), irrespective of the quantity of sample DNA used in the range of 20-250 ng. When using less than 20 ng sample DNA, the average read number of the probes decreases and variation increases.

As an example, in case of a digitalMLPA probemix containing a total of ~600 probes, optimal results are obtained when 600 probes × 400 reads = 240,000 correct reads are generated for each sample. When 64 samples are combined on one MiSeq flow cell, 64 × 240,000 = 15,360,000 correct reads are required. This is well within capability of the recommended V3 flow cell and reagents. For probemixes containing fewer probes, more samples can be combined in a single sequencing run.

9.   COFFALYSER digitalMLPA SOFTWARE FOR digitalMLPA DATA ANALYSIS

Coffalyser digitalMLPA software should be used for digitalMLPA data analysis. The Coffalyser digitalMLPA User Manual provides step-by-step instructions on digitalMLPA data analysis. Both software and manual can be downloaded via your MRC Holland account at mrcholland.com. To run Coffalyser digitalMLPA, a Windows 10 (32 or 64 bit) system, Intel i5 (or higher or equivalent), 4 GB RAM, and free hard drive space at least the size of the FASTQ file to be processed is required.

Coffalyser digitalMLPA automatically recognises and extracts digitalMLPA sequence reads from FASTQ files and performs raw data analysis (identification of correct and incorrect ligation amplification products) and extensive quality control checks (see section 10.2). Comparative analysis is subsequently performed and two result reports are generated.

Coffalyser digitalMLPA also generates Coffa files during data analysis, which can also be used for comparative analysis. These Coffa files are relatively small (~1 Mb) files that contain only read count information for a specific barcode. In case different digitalMLPA experiments were combined in one sequencer run, or if digitalMLPA was combined with sequencing experiments, the Coffa files allow for separate storage of the results of each digitalMLPA experiment. It also prevents (privacy) problems when files are send to MRC Holland for trouble shooting or when central sequencing facilities provide results to different departments.

10. digitalMLPA QUALITY CONTROL AND TROUBLESHOOTING

For digitalMLPA data analysis, Coffalyser digitalMLPA software is used. Coffalyser digitalMLPA uses Illumina FASTQ files and assigns reads to specific probes. With the assigned read counts, extensive quality control checks are carried out through analysis of the control probes that are included in every digitalMLPA probemix.

Quality control of digitalMLPA reactions:

1. Dyes are included in the digitalMLPA Barcode Solutions to identify sample mix up and double use of barcodes via unique column or row colour patterns (section 10.1).
2. Quality control probes and SNP specific probes are present in each digitalMLPA probemix, these control probes are described in section 10.2. Coffalyser digitalMLPA provides warnings when: insufficient reads are present, sample DNA appears to be contaminated with DNA from another individual, overall reaction conditions differ too much from the reaction conditions at MRC Holland, sample DNA quality or reaction conditions for an individual reaction differ too much from the median values obtained on the reference reactions, or when the standard deviation of the reference probes is too high.
3. For troubleshooting purposes, quality control of the PCR reactions by capillary electrophoresis is possible, for more information see: https://www.mrcholland.com/r/digitalmlpa-electrophoresis.

10.1 BARCODE SOLUTION DYES

Each row/column of the digitalMLPA Barcode Solutions plate contains a unique dye pattern that can be used to verify which barcodes were used and whether one or more rows/columns of barcode solutions was accidentally used twice. These dyes are also visible after the PCR and have no effect on digitalMLPA results. An example is shown in Figure 3 (for barcode solution information, see the Barcode Solutions plate specific product descriptions).

BP01:

Figure 3. Barcode Solutions plate BP01 (lot 03-xxx-yymmdd). Other Barcode Solutions plates have different orientations of the red, blue and yellow dyes. The barcode numbering used at MRC Holland is indicated.

10.2 QUALITY CONTROL PROBES PRESENT IN digitalMLPA PROBEMIXES

Table 1. digitalMLPA quality control probes

Name / function	Explanation
Probemix lot & Barcode Solutions lot identification	The digitalMLPA probemix and lot used, as well as the Barcode Solutions plate and lot used, are automatically recognised for each reaction (barcode) by Coffalyser digitalMLPA.
Sample DNA gender identification	Three X-chromosome and three Y-chromosome specific probes are included for sample gender identification. For certain digitalMLPA products, gender determination is essential for a correct interpretation of results. For most applications. the Coffalyser software does not provide a warning for X0, XXY, XXX and other sex chromosome abnormalities, and does not report detailed results of the gender control probes.

Name / function	Explanation
Sample DNA identifier / Detection of sample DNA contamination with other samples	Probes for 39 common SNPs provide a unique sample identifier. Every position can have four different options: 0, 1, 2 or ?, where 0 and 2 are SNPs that are homozygous for allele A or B respectively, while 1 represents a heterozygous SNP. If one of these options cannot be determined, this results in a "?". DNA samples from the same individual will have the same sample identifier. The sample identifier is mentioned on the Coffalyser digitalMLPA reports, e.g. 11011.22001.10202.01110.11011.12001.20202.0111 v1. All selected SNPs have a minor allele frequency close to 0.5 in all populations. For probemixes that are used on normal sample DNA, SNP probe results are also used to detect sample DNA contamination (5-50%). A contaminated DNA sample will result in multiple "?" signs in the sample identifier. For tumour derived sample DNA, detection of sample DNA contamination is not always possible. Detection of contamination may also be compromised if samples are contaminated with DNA from close relatives as close relatives may have similar sample identifiers.
Detection of incomplete sample DNA denaturation	Four probes detect a sequence near an exceptionally strong CpG island. Strong CpG islands and exceptionally GC-rich regions are not completely denatured at 98°C when too much salt is present in the sample, reducing read numbers for probes that bind to sequences located within a few kb of the CpG island. Coffalyser digitalMLPA provides a warning when sample DNA denaturation appears to be incomplete.
Sample DNA depurination	Probes with a high pyrimidine content near their ligation sites and probes that consist of three oligonucleotides are in general more affected by depurination. DNA depurination is assessed by comparing depurination sensitive and -insensitive control probes to each other. Coffalyser digitalMLPA provides a warning when sample DNA depurination is considerably different between the test and reference samples.
Sample DNA fragment length	Four control probes detect sample DNA fragmentation. Coffalyser digitalMLPA will provide a warning when DNA fragmentation is considerably different between the test and reference samples. digitalMLPA probes detect a sample DNA sequence of 55-100 nt in length. Therefore, even extensively fragmented DNA samples can be used. However, test samples should always be as similar as possible to the reference samples used in the same experiment.
Hybridisation Tm	Five control probes provide a warning when the hybridisation reaction conditions are outside specifications, e.g. when the thermocycler temperature during the overnight incubation is significantly different from 60°C. These control probes can also be used to detect evaporation during the overnight hybridisation reaction, see 'Evaporation' section below.
Hybridisation completeness	Five control probes determine the extent of the hybridisation reaction. Coffalyser digitalMLPA provides a warning if the hybridisation reaction is not complete. These control probes can also be used to detect evaporation during the overnight hybridisation reaction, see 'Evaporation' section below.
Ligation start temperature	Two control probes provide a warning for a room temperature start of the ligation reaction. Probe specificity is reduced when a room temperature ligation occurs and good results cannot be guaranteed.
Ligase activity / Polymerase activity / DNA contamination	Nine control probes determine the ligase and polymerase activity. A decrease in ligase and/or polymerase activity in certain reactions can be due to sample DNA contamination with salt, heparin, EDTA, phenol or other substances and can increase probe variability. An increase in ligase activity is often due to pipetting less than 3 µl Hybridisation master mix. Coffalyser digitalMLPA provides a warning when the ligase or polymerase activity are outside safe margins.
Incomplete ligase inactivation	Two control probes provide a warning when simultaneous ligase and polymerase activity occurs, e.g. due to incomplete Ligase-65 inactivation. This can result in false positive signals for certain mutation-specific probes.
HhaI digestion	Four control probes indicate if digestion by the HhaI enzyme is complete in MS-digitalMLPA reactions. *These control probes are only relevant when performing MS-digitalMLPA reactions.*

Name / function	Explanation
Evaporation detected by Hybridisation completeness probes and Hybridisation Tm probes	Evaporation of individual reactions can be detected with the Hybridisation completeness and Hybridisation Tm control probes. These probes will provide increased values when excessive evaporation appears to have occurred during the overnight hybridisation.

10.3 COFFALYSER digitalMLPA REPORT WARNING TROUBLESHOOTING

In two Coffalyser digitalMLPA reports, the General Details.pdf and Sample Results 1.pdf, a table is present outlining the detailed quality checks for the sample. This table is divided into two categories: (1) Reaction analysis, this focuses on the quality checks for each individual sample. For control probes/fragments, this is based on comparing intra ratios to what is expected based on internal testing data generated at MRC Holland, and (2) Comparative analysis, this focuses on the quality checks from the comparison of the test sample to the reference samples.

Table 2. Coffalyser digitalMLPA report warning troubleshooting

Coffalyser report Warning	Category	Troubleshooting
Product detection [a]	Reaction analysis	Ensure the correct Product Sheet is defined for your experiment in the Coffalyser Definition File and that probemix lots have not been combined. If your Product Sheet is not present, re-download Coffalyser digitalMLPA from www.mrcholland.com.
Missing reference probes [a]	Reaction analysis	Ensure the correct Product Sheet is defined and all No DNA samples are specified as such in the Coffalyser Definition File. In case this is all correct, reconsider the reference sample selection used in the experiment.
Read depth	Reaction analysis	When the DNA input amount is sufficient, i.e. median distinct reads is close to the median total reads the sequencing run, the sequencing depth can be increased by sequencing an additional amount of the digitalMLPA PCR product. The two FastQ files can be combined in one analysis using Coffalyser digitalMLPA. When the DNA input amount is insufficient, i.e. the median distinct reads is much lower compared to the median total reads, repeating the sequencing run may not improve results.
Unique reads [a]	Reaction analysis	Ensure at least 20 ng of DNA is used as input for each reaction.
Sequence quality	Reaction analysis	Check the Q30 score of the NGS run. When sequencing quality issues arise consult Illumina.
Unrecognised reads	Reaction analysis	Ensure the correct Product Sheet is defined in the Coffalyser Definition File and at least 20 ng of DNA is used as input for each reaction.
DNA contamination [a]	Reaction analysis	Among the possible causes are: - Contamination of the DNA sample with another DNA sample: collect a new DNA sample. - A recent blood transfusion. - Double usage of a barcode or contamination of a barcode solution with another barcode solution: check the barcode colour coding in the Barcode Solutions plate and in the PCR tubes used in the experiment. - Extensive copy number changes in a tumour sample. If many samples present with this warning clean all lab equipment and ensure reagents are not contaminated.
Sample uniformity [a]	Reaction analysis	Ensure your NGS library preparation includes equal amounts of each digitalMLPA reaction when combining them before the sequencing run.
X-presence control [a]	Reaction analysis	One of the X chromosome probes might be affected by a SNP or targets a region with a copy number loss.
Y-presence control [a]	Reaction analysis	The Y chromosome probes might be influenced by Y-loss, or sample contamination of a male sample with female DNA, or v.v. In case only one probe deviates, it might be affected by a SNP or it targets a region with a copy number loss. Some females possess part of the Y chromosome which is translocated to another chromosome (PMID12205122).

Coffalyser report Warning	Category	Troubleshooting
Sample DNA denaturation	Reaction analysis & comparative analysis	Warning in all samples: ensure the thermocycler used is properly calibrated and the thermocycler program is correct. Warning in a single or a few samples: dilute the DNA sample (given that the DNA concentration is sufficient), or perform an extra DNA purification step.
Sample DNA depurination [a]	Reaction analysis & comparative analysis	Ensure DNA samples contain 5-10 mM Tris buffer with a pH of 8.0-8.5 and all samples used in one experiment are from a similar source and treated similarly.
Sample DNA fragment length [a]	Comparative analysis	Ensure all samples used in one experiment are from a similar source and treated similarly.
Hybridisation Tm	Reaction analysis & comparative analysis	Ensure the thermocycler used is properly calibrated, and the amount of salt present in the DNA sample is not too high (see 'Sample DNA denaturation' section below). In case a warning for Hybridisation completeness is also present, it can be an indication of evaporation. Ensure tube caps are properly closed before starting the overnight hybridisation. Test for evaporation by incubating 9 µl H_2O overnight at 60°C; in the morning >5 µl H_2O should remain. To reduce evaporation: 1. check heated lid of the thermocycler; 2. use multi-channel pipettes to reduce handling time; 3. increase/decrease pressure of lid on tubes: ensure tubes are not deformed; 4. try different tubes (e.g. Thermo Fisher AB-0773, AB-0451); 5. use mineral oil (Vapor-lock, Qiagen 981611): add just enough to cover the surface. There is no need to remove the oil. After probemix and polymerase master mix addition, spin down the tubes briefly. After ligase master mix addition, pipet up and down below the oil layer.
Hybridisation completeness	Reaction analysis & comparative analysis	A low value of the hybridisation completeness probes in all reactions can be due to a short hybridisation time at 60°C, e.g. due to power interruption, or can be due to a lower or higher temperature of the thermocycler during o.n. incubation. A low value of the hybridisation completeness probes in a single reaction, in combination with a low value of the hybridisation Tm control probes, can be due to pipetting less than 3 µl hybridisation mastermix to the reaction. An increased value for both types of control probes can be due to evaporation (see 'Hybridisation Tm' section below).
Ligation start temperature	Reaction analysis	Ensure the ligase mastermix is added when samples are in the thermocycler at 48°C.
Ligase activity	Reaction analysis & comparative analysis	Warning in all samples: gently mix the ligase mastermix (by pipetting up and down) before use at room temperature. Do not vortex. Do not preheat the Ligase mastermix and use a multichannel pipet (if available). Warning in a single sample: perform an extra DNA purification or dilute the DNA sample to reduce contaminants.
Ligase inactivation	Reaction analysis	Ensure the thermocycler used is properly calibrated and the thermocycler program is correct.
Polymerase activity	Reaction analysis & comparative analysis	Warning in all samples: gently mix the polymerase mastermix (by pipetting up and down) before use. Do not vortex, and use a multichannel pipet (if available). Warning in a single sample: perform an extra DNA purification or dilute the DNA sample to reduce contaminants.
Reference probe quality	Comparative analysis	Ensure the tissue type used is compatible with the probemix (see probemix-specific Product Description).
Reference sample quality	Comparative analysis	Ensure all samples used in one experiment are from a similar source and treated similarly. Dedicated reference samples should not have any copy number variation in the genes tested.
Detected probes that did not meet the minimum requirements to derive a classification [a]	Comparative analysis	Ensure that enough suitable reference samples are included in the experiment. If not, repeat the experiment including enough suitable dedicated reference samples.
Number of reference samples [a]	Comparative analysis	Repeat the experiment including enough reference samples.

[a] These quality checks will only be displayed in the Quality table in the event of a warning or error.

If reaction conditions are not solved by following the above recommendations, please contact info@mrcholland.com for further troubleshooting assistance.

11. INTERPRETATION OF RESULTS

Each probemix-specific product description and Probe Information File contains essential information for correct result interpretation!

To judge whether obtained results are reliable and to interpret digitalMLPA results correctly, a good understanding of the digitalMLPA technique and the application tested for is essential. Keep the following in mind:

- Sequence changes (SNPs, point mutations) in the probe's target DNA sequence may lower the number of reads obtained for a probe signal (in very rare cases even when >20 nt from the ligation site) by preventing ligation or by destabilising the binding of the probe oligonucleotide to the sample DNA[10] and may thereby cause false results resembling a deletion. Certain probes rely on a 1 nt difference with a related (pseudo)gene. A possibly non-significant single nucleotide sequence change in the related (pseudo)gene can result in an apparent duplication of the probe in question.
- Copy number changes detected by a single probe always require confirmation. Sequencing of the probe target sequence may show that a lowered probe read count is caused by a mutation/polymorphism. The finding of a **heterozygous** sequence typically indicates that the sample DNA does contain two different alleles. For many genes, a conventional MLPA probemix with different probes for the gene of interest is available. Long-range PCR and qPCR are often used to confirm (single) exon deletions.
- Not all deletions and duplications detected by digitalMLPA are pathogenic, sufficient knowledge of the application or gene(s) in question and the clinical effect of a found genetic aberration is required. For many genes, exons are described that are only present in certain transcript variants. For some genes, such as DMD, in-frame deletions resulting in mild, or no disease, have been described[11]. A duplication of one or more exons may disrupt that copy of the gene resulting in disease, whereas a complete gene duplication may not be pathogenic. Note that duplications that include the first or the last exon of a gene may leave that gene copy functionally intact and may not be pathogenic!
- In case of poor sample DNA denaturation, even the apparent deletion of *several probes* recognising adjacent genomic targets can be a false positive result! The presence of salt in DNA samples (e.g. >60 mM NaCl) prevents DNA denaturation of GC-rich regions. Always examine results of the denaturation control probes carefully as several DNA extraction methods result in DNA samples with a high salt concentration!
- Germline copy number variations reported in healthy individuals can be found at http://dgv.tcag.ca/.
- Certain copy number aberrations can be due to somatic alterations, including large deletions and duplications of entire chromosomes. Example: trisomy 12 is present in 10-20% of Chronic Leukemia (CLL) cases.
- digitalMLPA tests provide the *average* copy number of the target sequences in the cells from which the DNA sample was extracted. In case several probes targeting adjacent sequences have an unusual value (e.g. 0.75) but do not reach the usual threshold values for a deletion/duplication, mosaicism is a possible cause.
- In certain cases, analysis of parental samples might be necessary for correct interpretation of results.

12. PRECAUTIONS AND WARNINGS

- At this moment, all digitalMLPA products are for Research Use Only (RUO).
- Always consult the most recent version of the relevant probemix-specific product description AND this digitalMLPA General Protocol. Strictly follow this protocol.
- For professional use only. Assay performance is dependent on operator proficiency and adherence to procedural directions. The assay should be performed by professionals trained in molecular techniques. The person responsible for result interpretation should be aware of the latest scientific knowledge of the application in question and any limitations of the digitalMLPA procedure that could lead to incorrect results.
- Internal validation of each digitalMLPA application is essential; include at least 16 normal DNA samples in a first experiment in order to determine the variation. Validation should show a standard deviation <0.10 for each probe (unless the relevant product description or probe information file states otherwise). Samples used for validation should be representative of samples used in daily practice, with regard to source and method of DNA extraction etc.

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[10] When designing probes, known SNPs are avoided when possible. However, new SNPs are continuously being discovered. Please notify us when a polymorphism or a frequent pathogenic mutation influences a probe signal.
[11] Schwartz M. et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat. 28:205.*

- False positive or negative results can be caused by various factors, including:

  o Use of low quality plastics, as these may leak impurities into the MLPA reaction.
  o Sample DNA depurination. In particular formalin-fixed paraffin-embedded (FFPE)-derived sample DNA can be severely damaged.
  o Contamination with PCR products from previous digitalMLPA experiments.
  o Problems during data normalisation, including the use of incorrect normalisation algorithms or software.

13. LIMITATIONS OF THE PROCEDURE

- For most genetic disorders, the major cause of genetic defects are small (point) mutations, most of which will not be detected by digitalMLPA probemixes.
- digitalMLPA cannot detect any changes that lie outside the target sequence of the probes, digitalMLPA probes typically detect a sequence of 55-100 nt, and will not detect copy number neutral inversions or translocations. Even when digitalMLPA does not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.

digitalMLPA General Protocol – Document History
Version dMDP-007 (7 February 2022)

Version dMDP-007 (7 February 2022)
- Chapter on Reference samples updated.
- Chapters on Coffalyser digitalMLPA software updated.
- DRK20-IL kit added to Reagents section.
- Note added to only use one Barcode Solution from one plate within one sample in an experiment (chapter 5) and that a unique barcode must have been used for every sample that will be combined (chapter 8).
- Adjusted instructions for proper mixing of barcode solutions plates.
- Added a footnote to section 7.2 about minimising the chance of carry-over.
- Added information on checking libraries on Tapestations.
- Added a warning not to use the MiSeq Reagent Kit v2 for digitalMLPA (section 8.2).
- Broken cross-references corrected.
- Minor textual corrections.
- Changed font type to new company style.
- Removed www.mrcholland.eu from manufacturer details and footer.

Version dMDP-006 (13 July 2020)
- Company logo changed and company name adjusted.
- Website and email addresses changed.
- Name of analysis software adjusted.
- Name of barcode plate adjusted.
- Figure 1 updated.
- Paragraph on Coffalyser analyses deleted.
- Table on Coffalyser digitalMLPA troubleshooting added.

Version dMDP-005 (15 October 2019)
- Barcode plate example changed.
- Digital MS-MLPA renamed to MS-digitalMLPA.

Version dMDP-004 (27 May 2019)
- Volumes of probemix adjusted.
- Volume of SALSA Polymerase adjusted.
- LoBind tubes added to the materials required but not provided.
- Ingredients of SALSA MLPA buffer and Ligase adjusted.
- Water replaced by ultrapure water throughout the protocol.
- S4 removed from the protocol.
- Illumina run sheet preparation for MiSeq and MiniSeq adjusted.

Version dMDP-003 (13 February 2019)
- Protocol change: digitalMLPA buffer replaced by MLPA buffer, volumes adjusted accordingly.
- Figure 4 adjusted.
- DQ changed from 0.40-0.70 to 0.40-0.65.
- NextSeq reagent tray loading position changed.
- Various textual changes.