digitalMLPA™ NXtec™ Protocol

for use with Illumina sequencing platforms

Instructions For Use

Protocol for the detection and quantification of DNA sequences

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

NXtec products are for Research Use Only (RUO).

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1 INTRODUCTION

Copy Number Variations 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, which combines the robustness and simplicity of SALSA® MLPA® with the high throughput of Next-Generation Sequencing (NGS) platforms. digitalMLPA is used to determine the relative copy number of over 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. NGS is used to determine read counts of each 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.

To perform a digitalMLPA experiment, an application-specific NXtec probemix, a NXtec Reagent Kit and one or multiple NXtec barcode plates are needed.

Each probemix has a probemix-specific product description that contains information on the application, the recommended protocol, and the interpretation of results for that product.

MRC Holland offers NXtec Reagent Kits, NXtec barcode plates and application-specific NXtec probemixes. In addition, for data analysis, MRC Holland provides free digitalMLPA data analysis software: Coffalyser digitalMLPA™. For more information see support.mrcholland.com or contact info@mrcholland.com.

2 NXtec ASSAY COMPONENTS

To perform a NXtec reaction, at least three items are required: a NXtec Reagent Kit, a NXtec probemix and one or multiple NXtec barcode plates. For more information see the item-specific product descriptions at www.mrcholland.com.

2.1 PACKAGING LABELS

RUO	Research Use Only

3 digitalMLPA PRINCIPLE

digitalMLPA (Benard-Slagter et al., 2017) is a semi-quantitative technique based on the amplification of hundreds of probes, each detecting a specific DNA sequence. In the digitalMLPA technique, the sample DNA needs to be denatured to enable probe binding (see Figure 1 below). Each probe consists of two oligonucleotides binding to adjacent sample DNA sequences. When hybridisation of all oligos to the sample DNA is complete, all probes are ligated and amplified simultaneously using a universal PCR primer pair. This results in a set of PCR amplicons where each amplicon represents a certain probe. These amplicons are then sequenced with an Illumina sequencing platform and analysed using Coffalyser digitalMLPA.

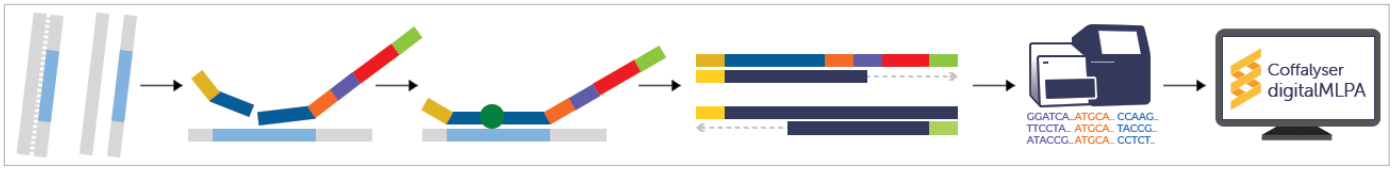

Figure 1. digitalMLPA workflow.

A more in depth explanation of the digitalMLPA technology can be found at www.mrcholland.com.

4 NXtec 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 7.4).
- Illumina sequencer equipment, Illumina sequencer flow cell and reagents.

4.2 SAMPLE TREATMENT

- **For each reaction, use a total quantity of 20-250 ng (40-100 ng is optimal)[1] of human DNA in a 4 µl volume[2]** (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 see this support article.
- 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 $TE_{0.1}$).
- 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.

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

- 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 this support article). 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 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.
  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 this support article in our help centre. 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 formalin-fixed paraffin-embedded (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. 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[3] 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 experiment is not essential. Coffalyser digitalMLPA removes almost all reads from nonspecific amplicons during data analysis. 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. In case of contamination of the no-DNA reaction with human DNA, this will also be detected based on the result of 39 SNP-specific probes with a high minor allele frequency in all populations[4]. More information can be found in this support article.
- POSITIVE CONTROL SAMPLES. Inclusion of positive control samples is recommended when available. MRC Holland does not provide positive samples. Information on commercially available positive samples is available

[3] Promega DNA is a mixture of DNA from different individuals. When using Promega DNA, “Pooled DNA” should be selected as sample type in Coffalyser digitalMLPA, as the software will otherwise provide a warning that the sample contains multiple DNA samples (“contamination”).
[4] For some probemixes the contamination check is not performed, as detection of sample DNA contamination is not always possible, e.g. in tumour derived samples. Please consult the probemix-specific product description for more information.

here. 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 NXtec REACTION - DNA DETECTION/QUANTIFICATION

NOTES TO READ BEFORE YOU START

- Use a calibrated thermocycler with heated lid (99-105°C).
- Accurate pipetting is critical.
- Always vortex thawed buffers, PCR Primer Mix P5P7 and probemix, followed by 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 plate by placing it at room temperature (RT). 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 plates in one experiment, make sure that the first two digits of the lot number are identical, e.g. "03-". Always use only one barcode solution per sample. The barcode plate lot should be compatible with the probemix, as indicated in the latest version of the probemix-specific product description.
- 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 and polymerase) at room temperature 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 the reactions. Use multi-channel pipettes to avoid excessive evaporation.
- After the PCR reaction, do not open tubes in the room with the thermocycler. To avoid contamination, use different micropipettes for performing the reactions and for handling the PCR products.
- Illumina sequencing requires sufficient variation in the first nucleotides of the reads for proper cluster detection. digitalMLPA reads start with the barcode sequence. These barcode sequences are distributed over the barcode plate in such a way that each row or column provides sufficient variation in each nucleotide read. However, to further enhance base diversity and improve the overall sequencing run performance, it is recommended to include a PhiX spike-in in the sequencing library. This spike-in can be performed according to Illumina's *System Denature and Dilute Libraries Guides* for your particular instrument using Illumina PhiX Control.

6 NXtec PROTOCOL

For printing, see the last page of this document for a one-page version of the protocol.

6.1 THERMOCYCLER PROGRAM FOR digitalMLPA

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*	

6.2 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) 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 6.1).
- Cool to 25°C before removing tubes from thermocycler.

6.3 HYBRIDISATION REACTION (DAY 1)

- Prepare hybridisation master mix. For each reaction, mix: 1.5 µl MLPA Buffer + 1.5 µl NXtec probemix. Mix well by pipetting or vortexing.
- After DNA denaturation, add 3 µl hybridisation master mix to each reaction (at step 2 of the thermocycler program). Mix well by pipetting gently up and down.
- Check that all contents are at the bottom of the tubes. If necessary, spin down briefly.
- Continue thermocycler program with steps 3-4. Hybridisation at 60°C should be for 16 to 20 hrs.

6.4 LIGATION REACTION (DAY 2)

- Prepare ligase master mix. For each reaction, mix: 25 µl ultrapure water + 3 µl Ligase Buffer **A** + 3 µl Ligase Buffer **C**, then add 1 µl Ligase-65 enzyme. 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.[6]

6.5 PCR REACTION (DAY 2)

- Prepare polymerase master mix. For each reaction, mix: 7 µl ultrapure water + 2 µl PCR Primer Mix P5P7, then add 1 µl Polymerase. Mix well by pipetting gently up and down.
- At step 9, add 10 µl polymerase master mix to each tube. Mix well by pipetting gently up and down and continue the thermocycler program with steps 10-12.
- PCR product can be stored at 4°C for one week. For longer periods, store between -25°C and -15°C.

7 AMPLICON QUANTIFICATION BY ILLUMINA SEQUENCERS

NOTES TO READ BEFORE YOU START

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

7.1 READ DEPTH

A median read count of at least 600[7] for all reference probes in each reaction is recommended for optimal results. The maximum number of samples that can be combined into a single sequencing run depends on the number of probes in the probemix(es) used, the capacity of the Illumina reagent kit used, and the number of barcode solutions that are available. As an example, in case of a probemix containing a total of 600 probes: 600 probes x 600 reads = 360,000 reads are needed for each sample. When using an Illumina kit with a total capacity of 25,000,000 reads, 69 samples could be included in one sequencer run. The number of samples that can be combined in a single run can also be determined using this calculator.

[5] To minimise the chance of carry-over, we recommend to use different barcode solutions between subsequent NGS runs.
[6] When the tubes are taken to a separate lab for the PCR, pre-heat the thermocycler for the PCR e.g. set at 95°C for 1 sec followed by 20°C paused. Minimise the transfer time (e.g. <5 min), place the tubes in the thermocycler and continue with step 9.
[7] The absolute minimum required read depth is 400. To avoid potential warnings for ***median total reads*** and ***read depth*** during analysis with Coffalyser digitalMLPA, it is recommended to use 600 as required minimum read depth. Determining the optimal value for your system may require some optimisation.

7.2 POOLING A digitalMLPA EXPERIMENT

In order to pool the different reactions within one experiment, combine equal amounts (minimum 5 µl) of each PCR reaction in a single tube. Each reaction must have a unique barcode solution.

7.3 COMBINING MULTIPLE digitalMLPA EXPERIMENTS IN ONE SEQUENCING RUN

When combining multiple digitalMLPA experiments in one sequencing run, they have to be mixed proportionally. In addition, a unique barcode solution must have been used for every sample that will be in the library regardless of the probemix used.

1. For each experiment, mix 5 µl of each PCR reaction in a vial as described in paragraph 7.2 (resulting in a separate vial per experiment).
2. For each of these PCR-product pools, calculate the required amount as follows:

$$amount\ in\ \mu l = \frac{number\ of\ probes\ in\ the\ probemix\ \times\ number\ of\ samples\ in\ the\ experiment}{1000}$$

   The number of probes is specified in the probemix-specific product description.
3. Mix the calculated amounts of the PCR-product pools into a single vial.
4. Use this mixture as a starting point for the library preparation as described in section 7.4.

More information can be found in this support article.

7.4 LIBRARY PREPARATION

The optimum library concentration is dependent on the Illumina instrument and kit type used. Please consult the Illumina website for more information. Examples of instrument reagent kits used and subsequent dilutions at MRC Holland can be found in Table 1. Please note that these may require optimisation.

Table 1. Examples of instrument reagent kits used and subsequent dilutions at MRC Holland

NextSeq 1000
NextSeq 1000/2000 P1 Reagent kit (v3) Illumina: catalog #20050264 (300 cycles) 100M reads
NextSeq 1000/2000 P1 Reagent kit (v3) Illumina: catalog #20074933 (100 cycles) 100M reads
NextSeq 1000/2000 P2 Reagent kit (v3) Illumina: catalog #20046811 (100 cycles) 400M reads
Please note that this dilution is to be used in combination with the NextSeq "onboard denature and dilute". ○ Mix 10 µl of combined PCR product with 990 µl RSB with Tween 20. This results in a 2 nM library. ○ Mix 7.8 µl of the 2 nM library with 16.2 µl RSB with Tween 20. This results in a 650 pM library. ○ Load 20 µl of the 650 pM library in the Library reservoir of the NextSeq reagent cartridge.
MiSeq
MiSeq Reagent kit (v3) Illumina: catalog #MS-102-3001 (150 cycles) 25M reads
○ Mix 5 µl of combined PCR product with 95 µl ultrapure water. This results in a 10 nM library. ○ Mix 5 µl of the 10 nM library with 5 µl of NaOH (0.2 M)[8]. Incubate for 5 minutes at room temperature. This results in a 5 nM library. ○ Mix 10 µl of the 5 nM library with 990 µl pre-chilled HT1 buffer. This results in a 50 pM library. ○ Load 600 µl of the 50 pM library in the Load Samples reservoir of the MiSeq reagent cartridge.
MiniSeq
MiniSeq Reagent Kit Illumina: catalog #FC-420-1002 (150 cycles) 25M reads
○ Mix 5 µl of combined PCR product with 75 µl ultrapure water. This results in a 12.5 nM library. ○ Mix 4 µl of the 12.5 nM library with 251 µl ultrapure water. This results in a 200 pM library. ○ Mix 5 µl of the 200 pM library with 5 µl of NaOH (0.1 M)[8]. Incubate for 5 minutes at room temperature. This results in a 100 pM library.

[8] For 0.2 M NaOH (MiSeq): 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.

- Mix 10 µl of the 100 pM library with 990 µl pre-chilled HT1 buffer. This results in a 1 pM library.
- Load 500 µl of the 1 pM library in the Load Library here reservoir of the MiniSeq reagent cartridge.

For questions about the use of different sequencing kits or library setups visit the support portal.

7.5 ILLUMINA SEQUENCER RUN SHEET

When setting up the sequencing run in the Illumina Local Run Manager or in the Illumina BaseSpace® Analysis Environment (basespace.illumina.com):

- Make sure a FASTQ file is generated, other important parameters are:
  - Single read
  - No index reads
  - Read 1: at least 110 nt[9]
  - Read 2: 0 nt
  - Adapter trimming should be disabled

It is also possible to import our digitalMLPA Library Prep Kit template, more information can be found in this support article.

7.6 digitalMLPA READS

Each digitalMLPA read contains a 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.

7.7 SEQUENCING RUN QUALITY

We recommend to check the quality of your sequencing run in BaseSpace or in the Illumina Sequence Analysis Viewer Software prior to data analysis using Coffalyser digitalMLPA.

8 COFFALYSER digitalMLPA FOR digitalMLPA DATA ANALYSIS

Coffalyser digitalMLPA 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 www.mrcholland.com. Coffalyser digitalMLPA does not require powerful hardware.

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 9.2). Comparative analysis is subsequently performed and result reports are generated.

Coffalyser digitalMLPA also generates one output file (Coffa file) per sample/barcode that can be used to quickly reanalyse existing data. In case different digitalMLPA experiments were combined in one sequencer run, this allows for separate storage of the results of each digitalMLPA experiment. It also prevents (privacy) problems when files are sent to MRC Holland for troubleshooting or when central sequencing facilities provide results to different departments. More information is provided in the Coffalyser digitalMLPA User Manual.

9 QUALITY CONTROL AND TROUBLESHOOTING

Coffalyser digitalMLPA carries out quality control checks through analysis of the control probes that are included in every NXtec probemix.

Quality control of NXtec reactions:

1. Dyes are included in the NXtec barcode plates to identify sample mix up and double use of barcodes via unique column or row colour patterns. These dyes are also visible after the PCR and have no effect on the results. For more information on barcode plate dye patterns, see the NXtec barcode plate-specific product descriptions at www.mrcholland.com.

[9] A read length of 110 nt can be generated using a 100-cycles reagent kit, as no index is required and the reagents for the index can be used to get to the required 110 nt.

2. Quality control probes and SNP-specific probes are present in each NXtec probemix. These control probes are described in section 9.1. Coffalyser digitalMLPA issues warnings when insufficient reads are present, when sample DNA appears to be contaminated with DNA from another individual (see below), when overall reaction conditions differ too much from the reaction conditions at MRC Holland, when sample DNA quality or reaction conditions for an individual reaction differ too much from the median values obtained on the reference reactions, or when data normalisation suffers from variation.
3. For troubleshooting purposes, quality control of the PCR reactions by capillary electrophoresis is possible. More information can be found in this support article.

9.1 QUALITY CONTROL PROBES PRESENT IN NXtec PROBEMIXES

Table 2. Quality control probes

Name / function	Explanation
Probemix & barcode plate lot identification	The NXtec probemix and lot used, as well as the NXtec barcode plate and lot used, are automatically recognised for each reaction (barcode) by Coffalyser digitalMLPA using these probes.
Sample DNA gender identification	These X-chromosome and Y-chromosome specific probes are included for sample sex identification. For certain digitalMLPA products, sex determination is essential for a correct interpretation of results. For most applications, Coffalyser digitalMLPA 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.
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 and therefore not performed. Please check the probemix-specific product description for details. 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	These control probes detect a sequence near exceptionally GC-rich regions, which are not completely denatured at 98°C when too much salt is present in the sample, thereby reducing read counts for affected probes. 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 generally 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	These control probes detect sample DNA fragmentation. Coffalyser digitalMLPA will provide a warning when sample DNA is extensively fragmented or when sample DNA fragmentation is considerably different between the test and reference samples. Most digitalMLPA probes detect a sample DNA sequence of 55-100 nt in length. Therefore, fragmented DNA samples can be used in most cases (see probemix-specific product description). However, test samples should always be as similar as possible to the reference samples used in the same experiment.
Hybridisation Tm	These 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	These 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.

Name / function	Explanation
Ligation start temperature	These control probes provide a warning for a room temperature start of the ligation reaction. Probe specificity is reduced when this occurs and reliable results cannot be guaranteed.
Ligase activity / Polymerase activity / DNA contamination	These 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	These control probes provide a warning when simultaneous ligase and polymerase activity occurs, e.g. due to incomplete ligase inactivation. This can result in false positive signals for certain mutation-specific probes.
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.

9.2 TROUBLESHOOTING BASED ON WARNINGS OF COFFALYSER digitalMLPA

In two Coffalyser digitalMLPA reports, a table is present outlining the detailed quality checks for the sample. This table is divided into two categories: (1) Reaction analysis, which 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. (2) Comparative analysis, which focuses on the quality checks from the comparison of the test sample to the reference samples. Only data that meets the quality requirements is suitable for result interpretation. Table 3 lists the warnings that may appear in the quality check table, with options for solving the indicated problem.

Table 3. Troubleshooting based on warnings in Coffalyser digitalMLPA reports

Warning	Category	Possible solution(s)
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.
Read depth	Reaction analysis	When the DNA input amount is sufficient, i.e. median distinct reads is close to the median total reads of 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. Moreover, by including a PhiX spike-in in the sequencing library, the risk of sequencing errors associated with low-diversity can be mitigated, thereby improving the overall sequencing run performance and increasing read depth.
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. To check read quality, we recommend using Illumina's BaseSpace® Analysis Environment or Illumina's Sequence Analysis Viewer Software.
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. Furthermore, check the read quality of the NGS run as reads with many errors cannot be assigned to probes and end up as unrecognised. To check read quality we recommend using Illumina's BaseSpace® Analysis Environment or Illumina's Sequence Analysis Viewer Software.

Warning	Category	Possible solution(s)
DNA contamination [a]	Reaction analysis	Among the possible causes and solutions are: - Contamination of the DNA sample with another DNA sample: collect a new DNA sample. - A recent blood transfusion: collect a new DNA sample. - Double usage of a barcode or contamination of a barcode solution with another barcode solution: check the barcode colour coding in the NXtec barcode plate and in the PCR tubes used in the experiment. - Copy number changes in target regions of the sample identification probes: not possible to solve this problem. - Lab equipment and/or reagents contaminated: if many samples get this warning, clean all lab equipment and/or start using new reagents.
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 might target a region with a copy number loss. Some females possess part of the Y chromosome which is translocated to another chromosome (PMID12205122).
Sample DNA denaturation	Reaction analysis & Comparative analysis	Warning in all samples: ensure the thermocycler used is properly calibrated and the thermocycler program is correct. A warning in all samples can also be due to e.g. the use of a suboptimal DNA extraction method for all samples or all samples having suffered from evaporation (and thus having high salt concentrations). 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 above). 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 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 hybridisation. A low value 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 master mix to the reaction. An increased value for both types of control probes can be due to evaporation (see 'Hybridisation Tm' section above).

Warning	Category	Possible solution(s)
Ligation start temperature	Reaction analysis	Ensure the ligase master mix 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 master mix (by pipetting up and down) before use at room temperature. Do not vortex. Do not preheat the ligase master mix 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 master mix (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[10]	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. More information can be found in this support article.
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 (see probemix-specific product description). 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 problems with reaction conditions are not solved by following the above recommendations, please contact info@mrcholland.com for further troubleshooting assistance.

10 INTERPRETATION OF RESULTS

Each product description and probe information file contains essential information for correct result interpretation!

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

- Small changes (e.g. SNVs, small indels) in the sequence targeted by a probe can cause false positive results. Sequence changes 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. Deviations detected by NXtec products should be confirmed, and single-probe deviations always require confirmation. Sequencing of the target region is recommended. Please contact MRC Holland for more information: info@mrcholland.com.[11]
- 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 SALSA® 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.
- 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/dgv/app/home.

[10] Please note that the reference probe quality check is less reliable in samples that have a very high amplification of a target gene.
[11] 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.

- 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 assays 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.

11 PRECAUTIONS AND WARNINGS

- All NXtec products are for Research Use Only (RUO).
- Always consult the most recent version of the relevant probemix-specific product description AND this digitalMLPA NXtec 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.
- Internal validation of each NXtec application is essential; include at least 16 normal DNA samples in a first experiment in order to determine the variation. Set all samples as Reference in Coffalyser digitalMLPA for data analysis. 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 DNA extraction method etc.
- False positive or negative results can be caused by various factors, including:
  - Use of low quality plastics, as these may leak impurities into the reaction.
  - Sample DNA depurination. FFPE-derived sample DNA in particular can be severely damaged.
  - Contamination with PCR products from previous digitalMLPA experiments.
  - Problems during data normalisation, including the use of incorrect normalisation algorithms or software.

12 LIMITATIONS OF THE PROCEDURE

- The major cause of genetic defects are small (point) mutations, most of which will not be detected by NXtec probemixes.
- digitalMLPA cannot detect any changes that lie outside the target sequence of the probes. 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 NXtec Protocol – Document History

Version dNXP-010 (8 July 2025)

- The name of the products was updated throughout the document from SALSA® digitalMLPA™ to NXtec.
- The technique name was updated throughout the document from SALSA® digitalMLPA™ to digitalMLPA.
- Introduction section updated to explain NXtec products.
- The trademark sentence was updated with NXtec.
- Small minor textual adjustments throughout the document.
- The code of the document was adjusted from dMDP to dNXP.

Version dMDP-009 (1 May 2025)

- Title page: minor textual adjustment.
- Chapter 2: adjusted heading.
- Chapter 5: added information on PhiX spike-in.
- Chapter 7: adjusted recommended read depth to 600 in text and in footnote 7.
- Chapter 9, Table 3: Added PhiX spike-in as possible solution for read depth warning.

Version dMDP-008 (30 April 2024)

- Title page: Removed reference to a separate MS-digitalMLPA protocol.
- Chapter 1: Removed sentence about intended use of digitalMLPA probemixes.
- Chapter 2:
  - Removed sections on digitalMLPA assay components and storage conditions and moved them to the item-specific product descriptions.
  - Removed standard ISO symbols from packaging labels.

- Chapter 3: rephrased digitalMLPA principle, improved Figure 1, removed Figure 2.
- Chapter 4:
    - removed Coffalyser digitalMLPA from Materials required but not provided.
    - Added a footnote about the contamination check being switched off for certain probemixes.
- Chapter 5:
    - Moved note about accurate pipetting from Chapter 6 to this chapter.
    - Moved warning about opening tubes in the PCR room and about using micropipettes from Chapter 6 to this chapter.
    - Simplified warning about using multiple barcode plates.
    - Added note that barcode plate lot should be compatible with the probemix.
    - Removed reference to 75 nt runs.
- Chapter 6:
    - Added reference to one-page protocol at the end.
    - Removed instruction to add TE for no-DNA controls.
    - Removed indications of tube cap colours.
    - Adjusted instruction on hybridisation time from “at least 16 hrs” to “16 to 20 hrs”.
    - Added footnote with instructions for the situation when PCR is performed in a separate room.
    - Replaced “at room temperature” with “at step 9” in the instruction about adding the polymerase master mix.
    - Removed instruction to place back tubes in thermocycler as there was no instruction to take them out.
- Chapter 7: updated and completely rewrote chapter on Amplicon quantification by Illumina sequencers. Among others:
    - Modified the recommended read depth to at least 500 reads/probe.
    - Adjusted volumes for library preparation of MiSeq instruments.
    - Added new recommendations for the NextSeq 1000 instruments.
    - Removed recommendations for NextSeq 500 and iSeq.
    - Added advice to check sequencing run quality before data analysis.
- Chapter 8: updated text on Coffalyser digitalMLPA , removed list of system requirements.
- Chapter 9:
    - Removed Figure 3.
    - Removed note about MS-digitalMLPA from table (HhaI digestion control probes).
    - Removed advice to reconsider reference sample selection (missing reference probes warning).
    - Added advice to use Illumina software to check sequence quality (sequence quality warning and unrecognised reads warning).
    - Added more possible solutions to DNA contamination warning.
    - Added more possible causes for denaturation warning.
    - Added link to knowledge base article about RSQ (reference sample quality warning).
    - Added information about contamination check being switched off for certain probemixes (Sample DNA identifier probes)
    - Added footnote that the reference probe quality check is less reliable in samples that have a very high amplification of a target gene.
- Chapter 10: rephrased warning about the effect of SNVs. Removed note about pathogenicity of deletions or duplications detected by digitalMLPA.
- Chapter 11:
    - removed warning about the person responsible for result interpretation as it is not relevant in a RUO context.
    - added instruction to set all samples as Reference in Coffalyser digitalMLPA when analysing data of internal validation.
- Added a sentence about trademarks of MRC Holland after Chapter 12.
- Chapter digitalMLPA Workflow – In Brief moved to the end.
- Minor textual corrections, fixed incorrect links.
- Changes implemented in previous document versions removed.

NXtec WORKFLOW – 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 25°C, 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-65 enzyme: 5 minutes at 98°C, followed by 20 minutes at 65°C.
4. PCR AMPLIFICATION OF LIGATED PROBES
   - Cool down to 20°C; 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 NXtec probemixes or probemix versions 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 7.3).
6. PREPARE MIXED digitalMLPA PRODUCTS FOR LIBRARY HANDLING BEFORE SEQUENCER LOADING
   - See section 7.4 for sequencer-specific dilutions (use **LoBind tubes**) and further instructions.
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 NXtec 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 Primer mix P5P7
  - ✓ 1 µl Polymerase

Thermocycler program:

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*	