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 assay-specific product description and with analysis software version Coffalyser digitalMLPA 2.5.0 or higher.

NXtec products are for Research Use Only (RUO).

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1 INTRODUCTION

Copy number variations are an important source of genetic variation in human DNA and play a role in a wide range of disorders. digitalMLPA is a semi-quantitative technique, that combines the robustness and simplicity of SALSA® MLPA® with the high throughput of Next-Generation Sequencing (NGS) platforms. It is used to determine the relative copy number of 500-2000 DNA sequences in a single multiplex PCR-based reaction, followed by Illumina-based sequencing for amplicon quantification. Probes for the detection and quantification of specific (point) mutations can also be included. In this workflow, NGS is used to determine read counts of each probe amplicon, rather than to analyse the sample DNA sequence itself. Due to this targeted approach, very high read depth can be achieved while using only limited sequencing capacity. Combined with the use of a single PCR primer pair to amplify all ligated probes, digitalMLPA is a highly robust method.

Each product description provides application-specific information, the recommended protocol, and guidance for interpreting results.

MRC Holland offers NXtec reagent kits, NXtec barcode plates and application-specific NXtec probemixes. Data analysis is performed with the free of charge Coffalyser digitalMLPA™ software. For more information contact info@mrcholland.com.

2 NXtec ASSAY COMPONENTS

A NXtec reaction requires at least three items: a NXtec Reagent Kit, a NXtec probemix and one or more NXtec barcode plates. For item-specific details, see the relevant 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. The workflow starts with denaturation of the sample DNA (see Figure 1). Next, a mixture of digitalMLPA probes is added; each probe consists of at least two oligonucleotides. After hybridisation of the oligos to the sample DNA is complete, all hybridised probes are ligated and amplified simultaneously using a universal PCR primer pair. This generates a set of PCR amplicons in which each amplicon represents a specific probe. The amplicons are then sequenced on an NGS platform and analysed using Coffalyser digitalMLPA.

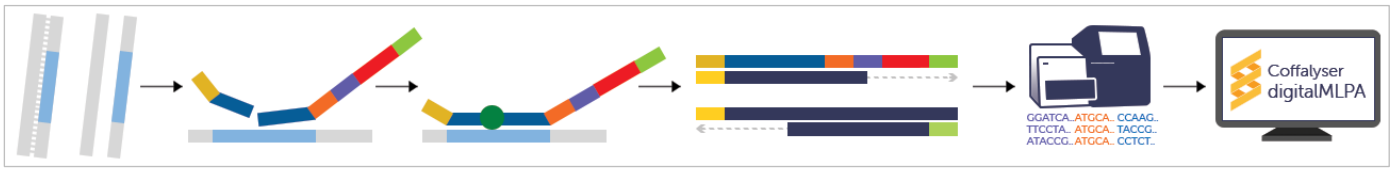


Figure 1. digitalMLPA workflow.

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 certain Illumina sequencers, see section 7.4).
- Illumina sequencer equipment, Illumina sequencer flow cell and reagents.

4.2 SAMPLE REQUIREMENTS

- **Use 20-250 ng (40-100 ng is optimal)[1] of human DNA in a total volume of 4 µl[2]** (unless stated otherwise in the assay-specific product description). If necessary, DNA samples can be concentrated by ethanol precipitation. Glycogen (Roche 901393) can be used as a carrier. For more information see this support article.
- MRC Holland recommends 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)
- 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 REFERENCE & CONTROL SAMPLE SELECTION

Before selecting reference samples, make sure you are using Coffalyser digitalMLPA 2.5.0 or higher. Earlier versions may require a different selection strategy. Email info@mrcholland.com for more information. **Always check the assay-specific product description for application-specific guidance**.

[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.

- REFERENCE SAMPLES. The use of dedicated reference samples is recommended for most experiments. For some assays, it is possible to omit dedicated reference samples and rely on all test samples for inter-normalisation, for example in cases when a large number of test samples are available and copy number changes are expected to be rare. Please refer to the assay-specific product description for detailed instructions on reference sample selection.
  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, if Y-chromosome target probes are present in the probemix and male test samples are included, make sure at least three male reference samples are added to the experiment. Reference samples should be distributed evenly over the sample plate. Reference samples should be defined as type '*Reference*' in Coffalyser digitalMLPA.
  Using test samples for normalisation. For applications in which aberrations 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. In these experiments, all samples should be defined as '*Test*' samples in Coffalyser digitalMLPA.
- SELECTING REFERENCE SAMPLES. Reference samples are DNA samples obtained from individuals expected to have a normal copy number across all probe-targeted regions. 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).
- 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. It is not recommended to include no-DNA controls in digitalMLPA experiments. During digitalMLPA data analysis, nonspecific reads are removed by the software, while SNV probe pairs present in all digitalMLPA assays[4] enable detection of sample contamination. In addition, inclusion of no-DNA controls may contribute to index-hopping. 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 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.

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, then briefly spin before use. MLPA Buffer is usually stored at -20°C but may remain liquid due to its high salt concentration.
- Thaw the barcode plate at room temperature (RT). Then spin briefly at 1000 rpm (50-200 rcf), mix the barcode solutions by gentle swirling on a rocking plate or microplate shaker, and spin again briefly.
- If you use barcode solutions from multiple barcode plates in one experiment, make sure the first two digits of the lot number are identical (e.g. '*03-*'). Use only one barcode solution per sample and confirm that the barcode plate lot is compatible with the probemix, as stated in the assay-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 enzyme-containing solutions as this may reduce activity.
- 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 PCR products.

[3] Promega Human Genomic DNA is a mixture of DNA from different individuals. When using Promega DNA, '*Pooled DNA*' should be ticked in Coffalyser digitalMLPA, as the software will otherwise provide a warning that the sample contains multiple DNA samples ('*DNA 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 assay-specific product description for more information.

- 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 the 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 the ligase master mix. For each reaction, mix: 25 µl ultrapure water + 3 µl Ligase Buffer **A** + 3 µl Ligase Buffer **C** + 1 µl Ligase-65 enzyme (added last). Mix well by pipetting gently up and down.
- Continue the thermocycler program with step 5.
- When the samples reach 48°C, add 32 µl ligase master mix to each tube **while the samples remain IN the thermocycler**. Mix well by pipetting gently up and down.

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

- Continue the thermocycler program with steps 6-9.[6]

6.5 PCR REACTION (DAY 2)

- Prepare the polymerase master mix. For each reaction, mix: 7 µl ultrapure water + 2 µl PCR Primer Mix P5P7 + 1 µl Polymerase (added last). Mix well by pipetting gently up and down.
- When the thermocycler is at 20°C (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.
- Store PCR products between -25°C and -15°C.

7 AMPLICON QUANTIFICATION ON ILLUMINA SEQUENCERS

NOTES TO READ BEFORE YOU START

- **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 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 for all reference probes in each reaction is recommended for optimal results[7] (unless stated otherwise in the assay-specific product description). 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 estimated number of samples can be determined using this calculator.

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, mix them proportionally. In addition, every sample in the final library must have a unique barcode solution, 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 assay-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

digitalMLPA is compatible with Illumina standard and XLEAP-SBS chemistries. The optimum library concentration is dependent on the Illumina instrument and kit type used. Please consult the Illumina website for more information.

[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 pause. 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 (unless stated otherwise in the assay-specific product description). To avoid potential warnings related to 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 optimisation.

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 (standard SBS v3) Illumina: catalog #20050264 (300 cycles) 100M reads
NextSeq 1000/2000 P1 Reagent kit (standard SBS v3) Illumina: catalog #20074933 (100 cycles) 100M reads
NextSeq 1000/2000 P2 Reagent kit (standard SBS v3) Illumina: catalog #20046811 (100 cycles) 400M reads
NextSeq 1000/2000 P1 Reagents kit (XLEAP-SBS) Illumina: catalog #20100983 (100 cycles) 100M reads
NextSeq 1000/2000 P2 Reagents kit (XLEAP-SBS) Illumina: catalog #20100987 (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.

For questions about the use of different sequencing kits or library setups, visit the support portal or email info@mrcholland.com.

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 in Local Run Manager (more information in this support article) or using BaseSpace (more information in this support article).

7.6 digitalMLPA READS

Each digitalMLPA read contains a digitalMLPA read identifier sequence, which is used to identify reads belonging to digitalMLPA reactions within a FASTQ file. The FASTQ file from a run can be directly analysed using 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

Use Coffalyser digitalMLPA for data analysis. The Coffalyser digitalMLPA User Manual provides step-by-step guidance and can be downloaded together with the software through your MRC Holland account at www.mrcholland.com.

[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.

[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.

Coffalyser digitalMLPA automatically recognises and extracts digitalMLPA sequence reads from FASTQ files. For each barcode (sample), it assigns reads to digitalMLPA probes, normalises and compares the read counts per probe, generates result reports and performs extensive quality control checks (see section 9).

9 QUALITY CONTROL AND TROUBLESHOOTING

Quality control of NXtec reactions:

1. NXtec barcode plates contain dyes that help identify sample mix-ups and double barcode use through unique row and column colour patterns. These dyes remain visible after PCR and do not affect the results. For more information on barcode plate dye patterns, see the NXtec barcode plate-specific product descriptions at www.mrcholland.com.
2. Quality control probes and SNV-specific probes are present in each NXtec probemix. These control probes are described in section 9.1. Coffalyser digitalMLPA performs various quality control checks, and issues warnings when for example insufficient reads are present, when sample DNA appears to be contaminated with DNA from another individual 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 reference reactions, or when data normalisation suffers from variation.
3. Coffalyser digitalMLPA extensively assesses the quality of digitalMLPA data. The results of the quality assessment are included in the output files. Only samples that pass the quality checks are suitable for result interpretation. Please visit this support article on our website for extensive explanation on these checks and possible causes and corrective actions for each warning.
4. For troubleshooting PCR reactions can be assessed by capillary electrophoresis. More information is available 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 sex 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 aberrations, and does not report detailed results of the sex control probes.
Sample DNA identifier / Detection of sample DNA contamination	Probes for 39 common SNVs provide a unique sample identifier. Every position can have four different options: 0, 1, 2 or ?, where 0 and 2 are SNVs that are homozygous for allele A or B respectively, while 1 represents a heterozygous SNV. 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 SNVs have a minor allele frequency close to 0.5 in all populations. For probemixes that are used on normal sample DNA, SNV 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 assay-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 or when DNA denaturation is considerably different between the test sample and reference population.
Sample DNA depurination	These control probes detect 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. Coffalyser digitalMLPA provides a warning when sample DNA appears to be extensively depurinated or when sample DNA depurination is considerably different between the test sample and reference population.

Name / function	Explanation
Sample DNA fragment length	These control probes detect sample DNA fragmentation. 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 assay-specific product description). However, test samples should always be as similar as possible to the reference population used in the same experiment. Coffalyser digitalMLPA will provide a warning when sample DNA appears to be extensively fragmented or when sample DNA fragmentation is considerably different between the test sample and reference population.
Hybridisation temperature	These control probes are sensitive to the overnight hybridisation reaction conditions. Coffalyser digitalMLPA will provide a warning when the hybridisation reaction conditions appear to be outside specifications or when the hybridisation reaction conditions are considerably different between the test sample and reference population. *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 overnight hybridisation reaction. Coffalyser digitalMLPA provides a warning if the hybridisation reaction appears to be incomplete or is considerably different between the test sample and reference population. *These control probes can also be used to detect evaporation during the overnight hybridisation reaction, see 'Evaporation' section below.*
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 / Presence of contaminants	These control probes determine the ligase and polymerase activity. A decrease in ligase and/or polymerase activity in certain reactions can be due to 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 or considerably different between the test sample and reference population.
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 temperature probes)	An issue with the concentration of reagents in the overnight hybridisation step can be detected with the *Hybridisation completeness* and *Hybridisation temperature* control probes. Evaporation is most likely to occur during the overnight hybridisation and can be detected by elevated ratios of both the *Hybridisation completeness* and *Hybridisation temperature* control probes, whereas dilution would cause decreased ratios of these probes (ratios are available in the Excel Report generated by Coffalyser digitalMLPA).

10 INTERPRETATION AND CONFRIMATION OF RESULTS

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

Interpretation of results shall only be performed by personnel with adequate knowledge of the digitalMLPA methodology and the application tested for.

General Interpretation Guidelines:

- Deviations detected by digitalMLPA 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.[10]
- Single probe aberrations can be caused by small changes (e.g. SNVs, small indels) within probe target regions or, for probes that rely on a 1 nt difference with a related (pseudo)gene, or homologous sequence, if the sequence variation exists in the related gene or pseudogene.
- In certain cases, analysis of parental samples might be necessary for correct interpretation of results.
- 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.

[10] When designing probes, known SNVs are avoided when possible. However, new SNVs are continuously being discovered. Please notify us when a polymorphism or a frequent pathogenic mutation influences a probe signal.

11 LIMITATIONS OF THE PROCEDURE

- digitalMLPA is limited to detecting aberrations within the probe target regions only (typically 55-90 nucleotides).
- digitalMLPA does not detect copy number neutral inversions, translocations or variation outside the probe target region.
- The major cause of genetic defects are small (point) mutations, most of which will not be detected by NXtec assays.
- Small changes (e.g. SNVs, small indels) within probe target regions – even when located >20 nt from the probe ligation site – may cause reduced probe read counts and false positive results of single probes (by preventing ligation of the probes or by destabilising the binding of a probe oligo to the sample DNA).
- 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.
- Certain copy number aberrations can be due to somatic alterations, including large deletions and duplications of entire chromosomes.
- Not all copy number changes detected by digitalMLPA are pathogenic. MRC Holland cannot provide information whether a specific deletion or duplication will result in disease.

12 PRECAUTIONS AND WARNINGS

General Precautions

- Do not use the product if it is damaged or expired.
- All NXtec products are for Research Use Only (RUO).
- Always consult the most recent version of the relevant assay-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, see the assay-specific product description for instructions. Samples used for validation should be representative of samples used in daily practice, with regard to source and DNA extraction method, etc.
- The person responsible for result interpretation should be aware of the latest scientific knowledge of the application and of any limitations of the digitalMLPA technique that could lead to incorrect results.
- Coffalyser digitalMLPA should be used for data analysis. Use of other software may lead to false results.
- Analytical performance can be compromised by impurities in the DNA sample, incomplete DNA denaturation (e.g. due to salt contamination), the use of insufficient or too much sample DNA, insufficient or unsuitable reference samples, problems with NGS, or other technical errors.

Sample quality precautions

- DNA depurination caused by insufficient buffering capacity of the sample DNA can result in false results. If it is unknown whether sufficient buffer is present, add Tris-HCl: 3.2 µl sample DNA + 0.8 µl 50 mM Tris-HCl pH 8.5. FFPE-derived sample DNA in particular can be severely damaged.
- Extraction methods should not leave a high concentration of contaminants including salts, heparin, EDTA (>4 mM) and iron, as they may influence assay performance.
- Do not use QIAGEN M6, M48 or M96 systems, as they leave too much salt. This can result in apparent deletions, even of several probes recognising adjacent genomic targets. 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).
- 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).
- Do not concentrate DNA by evaporation or SpeedVac; this leads to high EDTA and salt concentrations.

Precautions during execution

- Do not mix different lots of a digitalMLPA probemix.
- Do not use low quality plastics, as these may leak impurities into the reaction.
- Evaporation that increases concentrations of contaminants and salt may occur during overnight hybridisation or when pipetting the ligation master mix. To prevent/reduce evaporation:
    - Use a multi-channel pipette to reduce handling time;
    - Ensure the heated lid works correctly;

  - Increase/decrease the pressure of the heated lid;
  - Try using different reaction tubes;
  - Put a small drop of mineral oil on top of the DNA sample to cover the liquid surface.
- Contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal. Analysis of a second independently collected and isolated DNA sample can exclude these contamination artefacts.

MRC Holland, SALSA, MLPA, digitalMLPA, NXtec, Coffalyser.Net, Coffalyser digitalMLPA, and their logos are trademarks or registered trademarks of MRC Holland BV. All other brands and names herein are the property of their respective owners.

digitalMLPA NXtec Protocol – Document History

Version dNXP-013 (9 June 2026)
- Textual changes throughout the document to improve readability
- Removed chapter 9.2 and troubleshooting table, added a link to the support portal with this information in Chapter 9.
- Chapter 10, 11 and 12 rewritten and restructured for clarity.
- Added rcf to the spinning down of barcode plates.
- Removed option to store PCR products at 4°C, according to Illumina guidelines.

Version dNXP-012 (23 April 2026)
- Removed information on Coffa files from Chapter 8.
- Added information on adapter trimming in Table 3.

Version dNXP-011 (8 December 2025)
- Added references throughout the text to ensure compatibility with software version Coffalyser digitalMLPA 2.5.0 and higher.
- Removed MiniSeq information from Table 1.
- Textual changes to Table 2.
- Replaced Table 3 to ensure compatibility with this newest version of the analysis software.

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. POOL A PORTION OF ALL digitalMLPA PCR REACTIONS
   - When the PCR program is completed, mix 5 µl of each reaction in a single 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. SET UP 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*			