

# digitalMLPA Protocol Supplement

These instructions are intended for the purpose of offering general guidance in running digitalMLPA reactions on NextSeq 500/550 instruments and combining digitalMLPA reactions with standard Illumina paired-end sequencing runs. The current document is to provide extra guidance and is not intended as an official instruction for use (IFU). In case of conflicting information, the digitalMLPA protocol and other instructions for use take precedence. digitalMLPA products are for Research Use Only (RUO).

Any recommendations or feedback for sequencer loading and procedures can be sent to [info@mrcholland.com](mailto:info@mrcholland.com).

## 1. RUNNING digitalMLPA REACTIONS ON NextSeq 500/550 INSTRUMENTS

Instructions are intended for use with 75 cycle High Output Kit v2.

### 1.1 ITEMS REQUIRED BUT NOT PROVIDED

- Illumina NextSeq 500 or 550 Sequencing System
- Illumina NextSeq 500/550 High Output Kit (75 cycles; Illumina material nr. 20024906)
- Two custom sequencing primers, to be ordered at “100 nmol scale, desalted” from a supplier of choice. These primers cannot be ordered from MRC Holland.
  - Read 1 primer MRC-NS-R1: 5'-TGTGCCAGCAGCATCCAATCTCGCATA-3'
  - Index read primer MRC-NS-IR: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

### 1.2 SEQUENCING PRIMER PREPARATION

- Dissolve the custom sequencing primers in TE buffer to a concentration of 100 µM; these will be referred to as “stock primer solutions” in the remainder of this document. Aliquot and store at -20 °C.
- Before use, thaw the two stock primer solutions and mix by brief vortexing. Prepare a 0.3 µM dilution of each sequencing primer:
  - Mix 6 µl 100 µM MRC-NS-R1 + 2 ml HT1 Hybridization Buffer in a tube labelled “well 7”.
  - Mix 6 µl 100 µM MRC-NS-IR + 2 ml HT1 Hybridization Buffer in a tube labelled “well 9”.
- Thaw sequencing cartridge.
  - Wipe the foil seal of wells 7 and 9 with a low-lint tissue.
  - Pierce the foil seal of wells 7 and 9 with a clean pipette tip.
  - Add the 2 ml of diluted sequencing primers to the respective wells.

### 1.3 NextSeq RUN INSTRUCTIONS (STANDALONE MODE)

- Open the Illumina NextSeq Control Software (NCS): Run setup screen.
- Create Run and select Generate FASTQ.
- Specify custom primers on the run setup screen; Custom read 1 primer; Custom index 1 primer.
- Run Settings:
  - Library prep kit: TruSeqLT.
  - Read type: single read.
  - Read length: 75nt.
  - Index read length: 10nt.
- Under Sample ID, enter a name (can be the experiment name) and add as sequence: NNNNNNNNNN (N10).
- Click Finish once all information is entered.

### 1.4 NextSeq RUN INSTRUCTIONS (BASESPACE OR BASESPACE ONSITE)

- Specify custom primers on the Basespace Prep tab.
- Select the checkbox for R1 and the index read (custom primer for read 1 and custom primer for the index read).
- NOTE: It may be necessary to select the “Edit” icon on the Run Setup screen. From there, select the custom primer option for both read 1 and the index read, click Save.

### 1.5 DEMULTIPLEXING THE digitalMLPA READS

- Demultiplexing is done using Illumina’s Linux-based bcl2fastq Conversion Software. The software converts raw Illumina BCL files into FASTQ files along with performing data demultiplexing.

- Download and learn more [here](#).
- Additional information is available [here](#).
- If needed, email MRC Holland at [info@mrcholland.com](mailto:info@mrcholland.com) for barcode sequence files and help with data demultiplexing.

## 1.6 COFFALYSER digitalMLPA

- Select barcode lot that includes the phrase “Illumina demultiplexed” e.g. 03-xxx-yymmdd; Illumina demultiplexed FastQ (e.g. NextSeq 75 nt reads).
- Follow all other steps as described in the Coffalyser digitalMLPA User Manual.

## 1.7 ADDITIONAL NOTES

- The minimum length required by Coffalyser digitalMLPA for read 1 is 67 nt. Combined with a separate barcode read of 10 nt, this means at least 77 sequencing cycles are required. The NextSeq 75 nt reagent kit contains sufficient reagents for 92 sequencing cycles (see more information [here](#)).
- More information on NextSeq custom primer use can be found [here](#).

## 2. COMBINING digitalMLPA REACTIONS AND STANDARD PAIRED-END SEQUENCING REACTIONS

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Instructions are intended for use with standard Illumina paired-end sequencing runs.

### 2.1 RUN CONDITIONS

- First read:
  - Must use the standard Illumina Rd1 primer.
  - Must have a length of at least 110 cycles.

### 2.2 SEQUENCING MIX PREPARATION

- Prepare a digitalMLPA library according to the guidelines in the digitalMLPA General Protocol specified for the sequencer you are using. Perform all steps including the denaturation of the library (if specified) and specified dilution(s). No library concentration measurement is needed. The final digitalMLPA library concentration should not be adjusted to match the concentration of other NGS libraries.
- Prepare your other sequencing library according to your standard protocol. Denature the library (if required) and dilute the library to the normal end sequencing concentration. Do not adjust the concentration of your library to match the concentration of the digitalMLPA library.
- Based on the sequencing capacity of the kit you are using determine the percentage of reads that needs to be allocated for the digitalMLPA reactions. This will be based on the number of reads needed per digitalMLPA reaction and equates to a minimum of 400 reads per probe (see digitalMLPA General Protocol for more details). NOTE: The percentage of reads that need to be allocated for each library should be based only on the first strand read if using paired end sequencing.
- Combine the two libraries in proportion to the percentage of reads required for each library<sup>1</sup>.
- Load the prepared mixture on the sequencer.
  - For example: Sequencing on the MiSeq using a v3 reagent kit of 150 cycles (25 million SE reads) of a digitalMLPA library containing 25 digitalMLPA reactions where a probemix containing 700 probes was used.
    - Calculate the number of reads needed for the digitalMLPA library
      - 25 samples (700 probes x 400 reads) = 7,000,000 reads
    - Calculate the percentage of first reads this equates to for the chosen kit
      - 7,000,000 reads for digitalMLPA library/ 25,000,000 total reads for the kit = 28% by volume of the denatured library loaded should be the digitalMLPA library.
    - For the MiSeq 600µl of the (combined) denatured library needs to be loaded for sequencing.
      - 28% (600µl) = 168 µl of the digitalMLPA library should be combined with the other sequencing library(s) for a total of 600µl.

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<sup>1</sup> Optimisation may be required when combining digitalMLPA and other NGS libraries, we therefore recommend that you do not base your calculations on the max capacity of the cartridge in your first experiment.

### 2.3 FASTQ FILE FOR ANALYSIS OF digitalMLPA READS

- When paired-end sequencing is performed digitalMLPA reads will be placed in an “Undetermined” FASTQ file. This is because digitalMLPA amplicon clusters do not generate second reads or index reads. This FASTQ file is typically named “Undetermined\_S0\_L001\_R1\_001.fastq.gz” (L001 = Lane, R1 = Read 1).
- Coffalyser digitalMLPA only counts reads that start with a correct digitalMLPA barcode, followed by the digitalMLPA read identifier sequence. Some failed reads from your sequencing experiment may also be in the “Undetermined” FASTQ file, these reads will be discarded by Coffalyser digitalMLPA and will not influence digitalMLPA results.

### 2.4 ADDITIONAL NOTES

- Using a protocol with index reads will not affect the sequencing of digitalMLPA libraries.
- When combining digitalMLPA libraries with other sequencing libraries, it is not possible to use the NextSeq 500/550 75nt reagent kit protocol.
- If there is any doubt about digitalMLPA read presence in a FASTQ file containing paired-end reads please contact MRC Holland at [info@mrcholland.com](mailto:info@mrcholland.com).