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Performance Characteristics

drMID Dx for Illumina NGS systems

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1. TEST PRINCIPLE

The MASTR assays enable multiplex PCR amplification of all required target regions of the gene(s) of interest in a limited number of PCR reactions. The recommended amount of Deoxyribonucleic acid (DNA) for each multiplex PCR reaction is between 20 and 50 ng of purified genomic DNA from the sample to be analyzed as template. Next, the resulting amplicons are barcoded, pooled and sequenced using a NGS instrument according to the manufacturer's instructions. The resulting sequence reads are subsequently analyzed to identify variant positions compared with the reference sequence of the targeted gene(s). Comparing those variants with public and/or private databases and analyzing the predicted change on the protein level will allow the identification of mutations associated with health and disease. Moreover, a number of MASTR assays enable Copy Number Variant (CNV) analysis directly from NGS data.

MASTR assays serve as front end amplification for sequence analysis on all commercially available bench top NGS platforms. The technology is based on "target amplification". The principle of the MASTR assays relies on two key technologies: multiplex PCR amplification and Next-Generation Sequencing (the detection method).

In the first step, all target regions of the gene of interest are amplified in separate multiplex PCR amplification reactions (number of multiplex reactions is defined per MASTR assay) per individual, using a hot-start DNA polymerase (Figure 1). The resulting amplicons of each multiplex are diluted 1,000 fold.

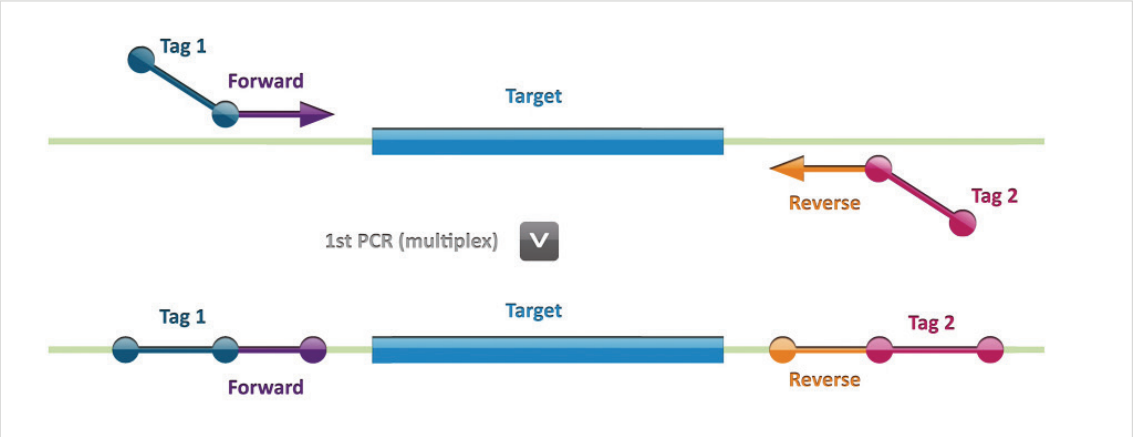

Figure 1. First step: Multiplex PCR Amplification

For a detailed workflow of this first, multiplex PCR step, please refer to the Instructions for Use (IFU) of the specific MASTR assay.

In the second step, a second round of Universal PCR is performed enabling tagging of all amplicons with specific MIDs and p5 and p7 adaptors required for Illumina MiSeq Sequencing (Figure 2).

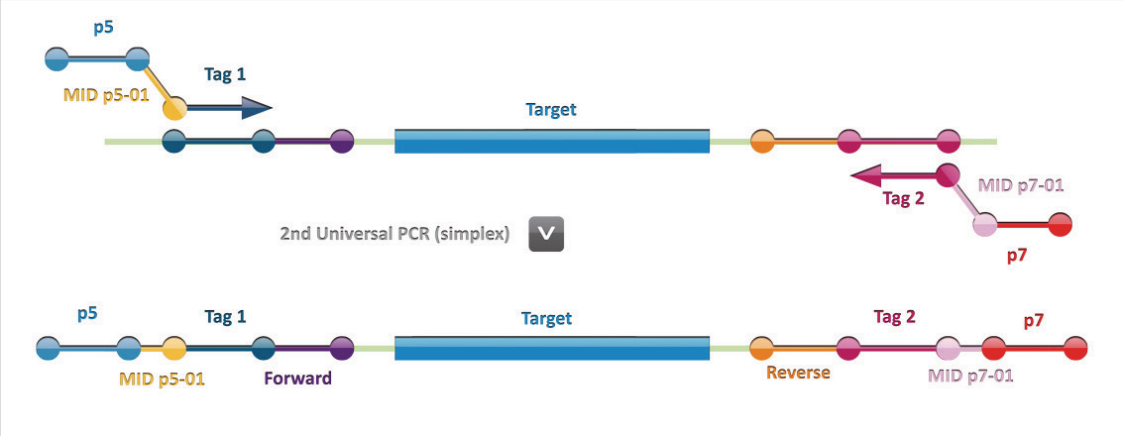

Figure 2. Second step: Universal PCR

The resulting tagged amplicons are mixed per individual applying a predefined assay specific mixing scheme. Each amplicon library is subsequently purified from small residual DNA fragments and the DNA concentration determined.

Next, these purified and individually tagged amplicon libraries are pooled equimolar, resulting in an amplicon pool or sequencing sample, which is then further processed using custom sequencing primers and one of the Reagents assays provided by Illumina Inc., containing all necessary consumables and reagent Cartridge for sequencing the amplicon pool or sequencing sample on the Illumina MiSeq. The position of the custom sequencing primers for Read1, Read2 and the Index Read 1 is indicated in Figure 3.

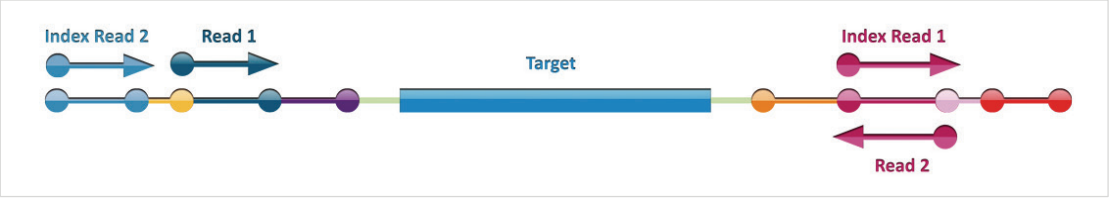

Figure 3. Illumina MiSeq Sequencing run

During the sequencing run, different sequence reads are generated on the same clusters (see Figure 3):

- Read 1: Tag 1 for insert
- Index Read 1: reverse complement Tag 2 for the p7 MID
- Index Read 2: grafted p5 for the p5 MID
- Read 2: Tag 2 for insert

Once NGS data is generated, refer to software of choice for data analysis.

2. PERFORMANCE CHARACTERISTICS

The performance characteristics of the specific MASTR workflow include the performance of the drMID Dx for Illumina NGS systems assays. In addition, specific performance characteristics (2.2.1 Failure rate and 2.2.2 Cross-Reactivity) of the drMID Dx for Illumina NGS systems assay were determined in combination with BRCA MASTR Plus Dx.

The intended use of the drMID Dx for Illumina NGS systems assay is the incorporation of MID identifiers and adaptor sequences to the MASTR amplicons, enabling next-generation sequencing (NGS) in two directions (dual read) on all Illumina platforms (MiSeq, NextSeq and MiniSeq instruments). The drMID Dx for Illumina NGS systems assays are not stand-alone products and should be combined with specific MASTR (Plus) assays.

2.1. Study Design

Two genetic centers (study sites SS1 and SS2) performed the BRCA MASTR Plus Dx workflow. Table summarizes the study design.

Table 1. Design of study to determine failure rate and cross-reactivity of drMID Dx for Illumina NGS systems

SS1 library		SS2 library
3 proficiency samples in triplicate 13 clinical samples (1 in duplicate)		3 proficiency samples 19 clinical samples (1 in duplicate)
MiSeq	NextSeq	MiniSeq
Illumina MiSeq Reagent assay v3 600 cycles, run at 2x251bp 1 library/run 5 % PhiX	Illumina NextSeq Mid-Output assay 300 cycles, run at 2x151bp 1 library/run 1 % PhiX	Illumina MiniSeq High-Output assay 300 cycles, run at 2x151bp 1 library/run 0.5-2 % PhiX

2.2. Performance characteristics of drMID Dx for Illumina NGS systems

2.2.1. Failure rate

The failure rate is the fraction of sequencing reads that are lost from the analysis.

Calculation of the failure rate was based on the observation that all undetermined reads can be attributed to uniform failure of all MIDs. The ratio between the assay-specific undetermined reads (no PhiX reads) and the assay-specific assigned reads over all samples was determined. Table shows the average failure rate of the drMID Dx for Illumina NGS systems assay on Illumina's MiSeq instrument.

Table 2. Average failure rate of the drMID Dx for Illumina NGS systems on Illumina's MiSeq, NextSeq and MiniSeq

Illumina sequencing instrument	Average failure rate	Range
MiSeq	1.584 %	1.567-1.600 %
NextSeq	2.158 %	2.157-2.158 %
MiniSeq	2.046 %	1.966-2.126 %

2.2.2. Cross-reactivity

The cross-reactivity is the fraction of reads assigned to the wrong sample due to incorrect MID sequences. A MID sequence can deviate from the expected MID for the sample due to sequencing errors or contamination issues (contaminated MID primers, other PCR components, template or carryover contamination).

Starting from the unused MID combination showing the highest number of assay-specific reads, the cross-reactivity was calculated in two analyses. The first assumed that all cross-reacting reads originated from the

MID combination with the lowest number of reads having one of the MID primers in common. The ratio between the cross-reacting reads and the actual reads of the sample with the lowest number of reads having one of the MID primers in common was determined. The second analysis assumed that all cross-reacting reads originated from all MIDs/samples having one of the MID primers in common. The ratio between the cross-reacting reads and the assigned reads over all samples having one of the MID primers in common was determined. Table shows the average cross-reactivity rate of the drMID Dx for Illumina NGS systems assay on Illumina's MiSeq.

Table 3. Average cross-reactivity of the drMID Dx for Illumina NGS systems on Illumina's MiSeq, NextSeq and MiniSeq

Illumina Sequencing instrument	Best case average cross-reactivity	Worst case average cross-reactivity	Range
MiSeq	0.022 %	0.127 %	0.016-0.159 %
NextSeq	0.022 %	0.105 %	0.016-0.159 %
MiniSeq	0.022 %	0.185 %	0.016-0.159 %

3. DISCLAIMER

The purchase of this product enables the purchaser to use it for the amplification of MASTR Dx derived amplicons from a specific MASTR Dx assay. Purchaser has to take into account that information obtained from amplicons generated using this product may not be used in procedures that are protected by valid claims owned and/or controlled by a third party, unless prior written approval of such party has been obtained.

IVD product performance claims apply only when combining MASTR Dx and drMID Dx and when both CE-Marked assays are used according to the specific CE-Marked Instructions For Use.

4. LIST OF ABBREVIATIONS

CE:	The CE symbol certifies that a product complies with the European standards.
CNV:	Copy Number Variation
DNA:	Deoxyribonucleic acid
IFU:	Instructions For Use
IVD:	For In Vitro Diagnostic Use.
MASTR Dx:	Multiplex Amplification of Specific Target for Resequencing for Diagnostics
MID:	Molecular Identifiers
NGS:	Next-Generation Sequencing
OD:	Optical Density
PCR:	Polymerase Chain Reaction
Plex:	Set of MASTR Dx derived amplicons

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