CE IVD human

Multiplicom
A part of **Agilent Technologies**

Performance Characteristics

FMF MASTR Dx with drMID Dx for Illumina NGS systems

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1. TEST PRINCIPLE

Multiplicom's FMF MASTR Dx assay is designed to PCR amplify all coding regions of *MEFV* using two multiplexed primer mixes. The recommended amount of DNA for each multiplex PCR reaction is between 20 and 50 ng of purified genomic DNA. Next, the resulting amplicons are barcoded using the drMID Dx for Illumina NGS systems kit, and mixed per sample. Finally, the resulting amplicon libraries are pooled and sequenced using the Illumina MiSeq® 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 *MEFV* gene. Comparing the variants with those from the Human Gene Mutation Database (HGMD®) Professional and analyzing the predicted change on the protein level will allow the identification of mutations associated with Familial Mediterranean Fever. In addition, the FMF MASTR Dx contains two amplicons located on chromosome X and Y respectively that allow gender determination.

FMF MASTR Dx assay serves as front-end amplification for sequence analysis on the Illumina MiSeq®instrument. 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 coding regions of *MEFV* and two amplicons located on the X and Y chromosome are amplified in two separate multiplex PCR amplification reactions per individual, using a hot-start DNA polymerase (Figure 1). The resulting amplicons of each multiplex are diluted 1,000 fold. In the second step, a Universal PCR is performed enabling tagging of the amplicons with specific MIDs and adaptors required for sequencing with the Illumina MiSeq® instrument. The resulting tagged amplicons are mixed per individual applying a predefined assay-specific mixing scheme. Each amplicon library, containing 23 different amplicons, is subsequently purified from small residual DNA fragments and the DNA concentration determined. Next, these purified and individually tagged amplicon libraries are pooled equimolarly, resulting in an amplicon pool or sequencing sample, which is then further processed by bridge amplification followed by sequencing on the Illumina MiSeq® instrument according to the manufacturer's instructions.

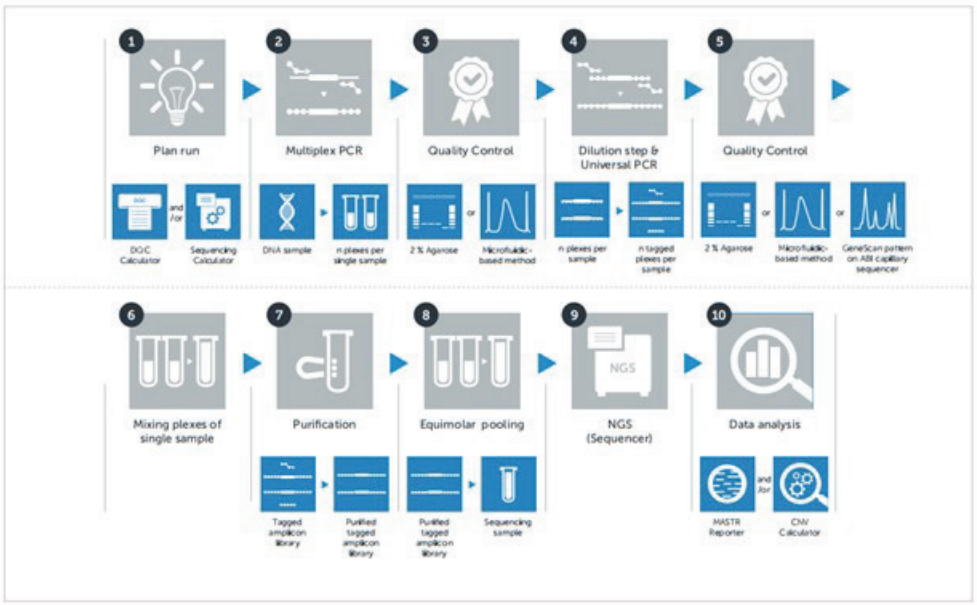

Figure 1.: Graphic representation of the MASTR workflow

2. PERFORMANCE CHARACTERISTICS: FMF MASTR DX AND MID DX FOR ILLUMINA MISEQ® ON GERMLINE DNA EXTRACTED FROM BLOOD

The performance evaluation study (PES) intended to validate the combined performance claims of CFTR MASTR Dx and MID Dx for Illumina MiSeq under the anticipated conditions of use. The drMID Dx for Illumina NGS systems kit contains the same components as the MID Dx for Illumina MiSeq kit, with the addition of an Index 2 primer. This extra primer is included to allow reading of the second index sequence (required for Illumina NextSeq and MiniSeq).

2.1. Study setup

The combined performance characteristics of the FMF MASTR Dx and the MID Dx for Illumina MiSeq® kits were assessed in one independent medical genetic laboratory. In total 56 independent DNA samples were amplified, sequenced and analyzed; as described in the corresponding IFUs. The laboratory extracted, amplified and sequenced 37 DNA samples selected amongst clinical samples. DNA was extracted by either MagNa Pure LC 2.0 or the MagNa Pure Compact system (Roche). A reference method, such as Sanger sequencing or NGS was performed for these samples. In addition, 19 CEPH samples were processed and sequenced for which reference data was retrieved from the 1000 Genomes database. In total, 553 SNVs and one indel were included in the performance study.
Thermal cyclers used by the laboratories: GeneAmp® PCR System 9700 (Applied Biosystems®, Life Technologies™). Quality control was performed by agarose gel electrophoresis
One Illumina MiSeq® run was performed with the Illumina MiSeq® Reagent Nano Kit v2. This resulted in the generation of 234,192 sequenced bases, of which 222,631 bases had been determined by one of the reference methods. Software for data analysis included the SeqNext module v4.1.2 of the Sequence Pilot software (JSI medical systems, Kippenheim, Germany) and the Sophia DDM application with pipeline ILL1MR1G3 (Sophia Genetics, Ecublens, Switzerland).

2.2. On target read pair counts

The total number of filter passed read pairs per sample was mapped to the human genome and the on target read pairs counted. The percentage of on-target, or amplicon derived, read pairs are represented in Table 1.

2.3. Uniformity of amplification

For each sample the number of read pairs per amplicon were counted and normalized to the average read pairs per amplicon. Combining all normalized data showed that 99.4 % of the total read pairs fall within 20 % of the mean number of read pair counts (Table 1).

2.4. Accuracy, sensitivity & specificity

These parameters are calculated using the following definitions:

- True positive (TP): variant bases present both in the NGS and the reference dataset
- True negative (TN): non variant bases present both in the NGS and the reference dataset
- False positive (FP): variant bases present in the NGS dataset, absent in the reference dataset
- False negative (FN): variant bases present in the reference dataset, absent in the NGS dataset
- Accuracy = (TP + TN) / (TP + TN + FP + FN)
- Sensitivity = TP / (TP + FP)
- Specificity = TN / (TN + FN)

No regions were excluded due to insufficient coverage. The observed values and their 95 % confidence intervals (CI) for these three parameters analysed with Sequence Pilot and Sophia DDM are presented in Table 1.

Table 1. Performance characteristics FMF MASTR Dx and MID Dx for Illumina MiSeq® on germline DNA extracted from blood.

Parameter	Observed [95 % CI] (SeqNext)	Observed [95 % CI] (Sophia DDM)
On target read pair counts	94.6 % (range 87.6 %-97.6 %)	
Uniformity of amplification	99.4 %	
Analytical sensitivity*	100 % [≥ 99.459 %]	100 % [≥ 99.459 %]
Analytical specificity*	100 % [≥ 99.999 %]	100 % [≥ 99.999 %]
Accuracy*	100 % [≥ 99.999 %]	100 % [≥ 99.999 %]

** Excluding pure changes in length of homopolymer stretches of 10bp or longer.*

3. DISCLAIMER

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

4. LIST OF ABBREVIATIONS

CE:	The CE symbol certifies that a product complies with the European standards.
CI:	Confidence Interval
DNA:	Deoxyribonucleic acid
FMF:	Familial Mediterranean Fever
FN:	False Negative
FP:	False Positive
IFU:	Instructions For Use
IVD:	For In Vitro Diagnostic Use
MASTR Dx:	Multiplex Amplification of Specific Target for Resequencing for Diagnostics
MEFV:	Mediterranean Fever
MID:	Molecular Identifier
NGS:	Next-Generation Sequencing
PCR:	Polymerase Chain Reaction
Plex:	Set of MASTR Dx-derived amplicons
TN:	True Negative
TP:	True Positive

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