PIPETMAX®: Automated Library Prep for BRCA MASTR™ Dx Diagnostic Assay

APPLICATION NOTE 1003

APPLICATION BENEFITS

Physicians need test kits to identify all of the genetic mutations of a condition at once so that they can initiate the right—personalized—treatment. BRCA MASTR™ Dx is a simple, robust, and complete molecular diagnostic assay for the identification of mutations in individuals with increased risk for breast, ovarian, and/or related cancers.

SOLUTIONS

PIPETMAX®, a small, personal liquid handling system, performs all liquid handling steps for the BRCA MASTR™ Dx kit to create and purify libraries for next generation sequencing (NGS). The results show that using PIPETMAX achieves consistent and reproducible results for the amplification, pooling, and cleanup of samples.

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INTRODUCTION

Multiplicom BRCA MASTR™ Dx

The BRCA MASTR Dx, developed by Multiplicom, is a simple, robust and complete molecular diagnostic assay. The assay is used for the identification of mutations in the coding regions of the BRCA1 and BRCA2 genes in individuals with increased risk for breast, ovarian, and/or related cancers.[1]

In the first step, all coding regions of BRCA1 and BRCA2 are amplified in five 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 using the Multiplicom MID Dx for Illumina MiSeq kit (Figure 2).

The resulting tagged amplicons are mixed per individual according a predefined assay-specific mixing scheme. Each amplicon library, containing 93 different amplicons, is purified to remove small residual DNA fragments using the Agencourt® AMPure® XP beads. After equimolar pooling, the amplicon libraries are sequenced on a MiSeq benchtop sequencer (Illumina, San Diego, California, United States).

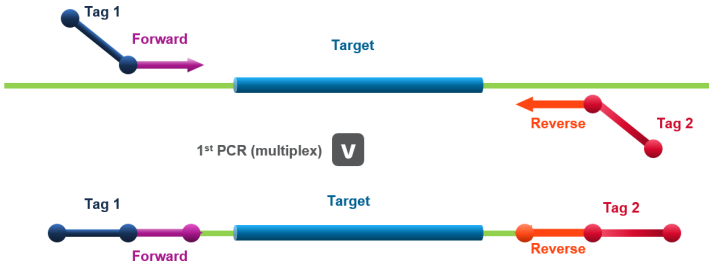

Figure 1
First step: Multiplex PCR using a Multiplicom MASTR assay.

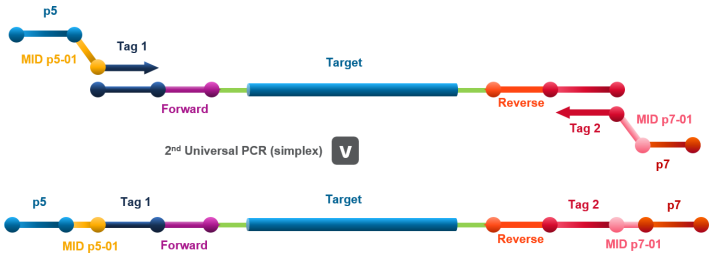

Figure 2
Second step: Universal PCR using a Multiplicom MID Dx kit for Illumina MiSeq sequencing.

Multiplicom
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GILSON®

PIPETMAX®

Automating the protocol on PIPETMAX® eliminates variation between users, reduces the chance for human error, and allows the user to focus on data analysis. All the liquid handling steps needed for the BRCA MASTR™ Dx assay can be performed by PIPETMAX. Since the BRCA MASTR Dx is a five-plex assay, up to sixteen samples per run can be processed. To handle the complete workflow four protocols were created: 1. Multiplex PCR, 2. Universal PCR, 3. Plex Mix, and 4. Purification.

MATERIALS AND METHODS

Samples

Four commercially available genomic DNA samples were processed in duplicate.

Multiplex PCR Procedure

The Multiplex PCR protocol for PIPETMAX prepared and distributed the five Master mixes and then the samples to the reaction plate. Each Master mix consisted of Taq DNA polymerase and a PCR Mix provided in the BRCA MASTR Dx kit. The reaction plate was put in a thermal cycler using the specified cycling profile.

Universal PCR Procedure

The Universal PCR protocol was designed to work with the MID Dx for Illumina MiSeq kit that contains 12 p5 primers and 16 p7 primers. A variable was created to allow the end user to select the combination of primers for each sample. During the run, PIPETMAX distributed the Master mix and the selected primers to the reaction plate. Next, the samples were transferred to the reaction plate, which was then placed in a thermal cycler using the specified cycling profile.

Plex Mix Procedure

After the Universal PCR, the five plexes of each sample were pooled into a PCR plate using the Plex Mix protocol. The volume ratio of the different plexes was fixed in the PIPETMAX protocol according to the assay specifications.

Purification Procedure

The PCR plate was positioned on top of the magnetic bead separator rack (Figure 3). For the Purification protocol, a custom magnetic bead separator rack was used. The magnets in this rack can be automatically engaged or disengaged by PIPETMAX to enable a good interaction with the beads for binding and elution.

Figure 3
Gilson magnetic bead separator, SPL-2294G-HDW with SPL-2294E-HDW.

The purification was performed using Agencourt® AMPure® XP beads. The beads were resuspended before they were distributed and mixed with the samples. After incubation, the magnets were moved to the engaged position. The magnetic bead pellet was allowed to form, and then the supernatant was removed. The pellet was washed twice with 70% ethanol and allowed to dry. The magnets were moved to the disengaged position and the pellets were resuspended in water. After another incubation, the magnets were put in the engaged position to enable formation of the bead pellets, and then the eluted samples were transferred to the final result plate.

Quality Control

After each protocol, the amplification patterns of all samples were determined using the GeneScan™ module on an ABI 3500XL Genetic Analyzer. Patterns were evaluated with MAQ-S software.[2]

Sequencing

Purified libraries were equimolarly pooled and prepared for sequencing with MiSeq according to the standard Illumina guidelines using the MiSeq Reagent Kit v2 and 250bp paired-end reads.

RESULTS AND DISCUSSION

Amplification Performance

All amplicons associated with the BRCA MASTR™ Dx assay were present in the analysis (Figure 4). All samples show the same amplicon pattern while the negative control show no amplification.

Purification Performance

After the Purification procedure, the GeneScan data confirmed that the primer dimer impurities were removed while all amplicons remained present in the sample (Figure 5). This was confirmed by the sequencing data where only 0.85% [0.55%–1.28%] of the reads originated from primer dimer.

The Purification procedure was assessed by examining the final concentration of the samples after completion of the protocol (Table 1). The data from PIPETMAX® are reproducible and comparable with the results of a manual purification method.

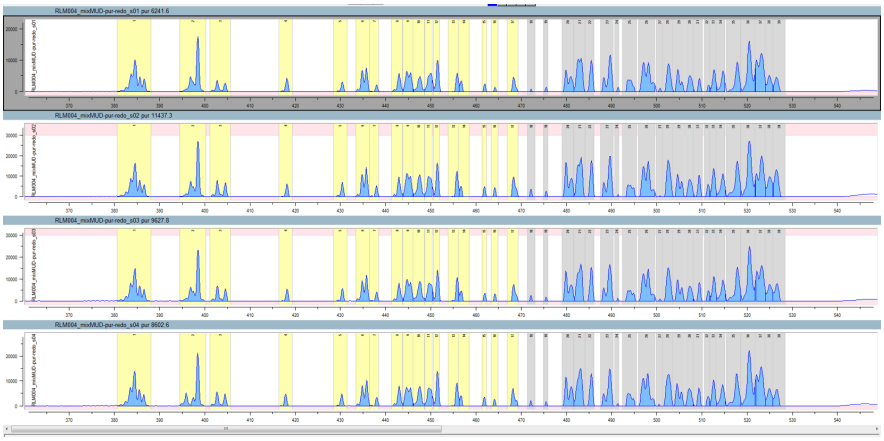

Figure 4
Similar MAQ profiles after Plex Mix procedure.

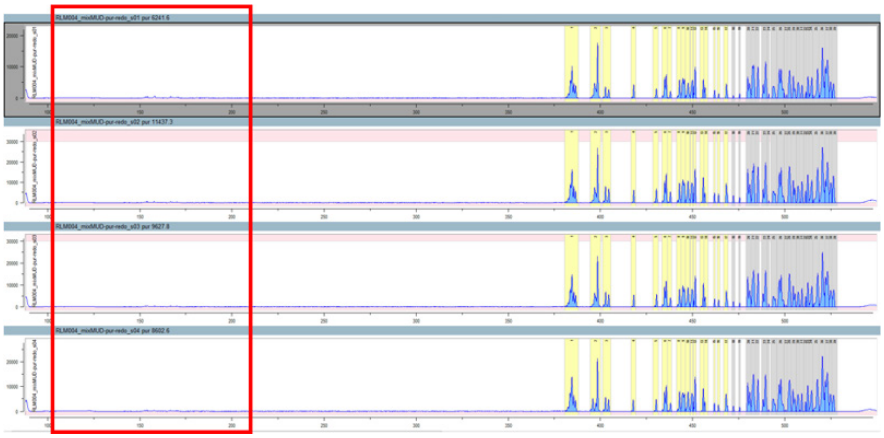

Figure 5
MAQ profile after purification. Red Box indicates possible primer dimer region.

Table 1:
Concentration of samples following Purification with Agencourt® AMPure® XP beads. Comparison of samples prepared either manually or on PIPETMAX® using a magnetic bead separator.

	Average Concentration (SD) ng/µL	
	PIPETMAX	Manual
gDNA s01	30.26 (1.27)	36.99 (5.01)
gDNA s02	46.31 (1.88)	39.15 (4.65)
gDNA s03	34.85 (0.40)	40.63 (4.36)
gDNA s04	32.81 (0.23)	32.40 (0.55)

Sequencing Results

All passed filter reads per sample were mapped to the BRCA1 and BRCA2 genes using a bioinformatics pipeline developed by Multiplicom. For each sample, the number of read pairs per amplicon were counted and normalized. Combining all normalized data showed that 100% of the amplicons have a coverage higher than 0.2x of the average coverage, which demonstrates uniformity of amplification across all samples (Table 2).

Table 2

Characteristics, including observed data from this experiment and reported data from extensive performance evaluation study.

Parameter	Observed	Reported
On target read counts	99.86%	98.40%
Amplification uniformity	100%	99.71%
Analytical Sensitivity*	100%	100%
Analytical Sensitivity*	100%	99.99997%
Accuracy*	100%	99.99997%
Repeatability*	100%	100%

Variant calling was performed on the data with a bioinformatics pipeline developed by Multiplicom and compared to the reference NGS dataset for each sample. Across the 4 samples, all 68 homo- and heterozygous (coding and non-coding) variants were reported correctly with no extra variants called. Resulting observed analytical parameters are shown in Table 2 next to the reported characteristics of the CE-IVD labeled manual method.

Variant calling performed on the second set of samples produced the same outcome, which demonstrated a 100% intra-experiment repeatability (Table 2). All reported characteristics in this section conform to the CE-IVD characteristics of the BRCA MASTR™ Dx kit.

DISCLAIMER

The use of PIPETMAX® for running the BRCA MASTR Dx kit is not covered by the CE-IVD label of this kit.

CONCLUSIONS AND BENEFITS

- Amplification and sequencing performance of the BRCA MASTR Dx is consistent between the automated method with PIPETMAX and the CE-IVD manual method.
- The automated method with PIPETMAX is robust and repeatable.

REFERENCES

1. Additional BRCA MASTR Dx information: http://www.multiplicom.com/product/brca-mastr-dx-0
2. MAQ-S software v1.4.0 (or higher), more info available via http://www.multiplicom.com/support-tools/maq-s

ORDERING INFORMATION

Items from Gilson

Description	Part Number
PIPETMAX	32100001
MAX8x200 Pipette Head	FC10021
MAX8x20 Pipette Head	FC10022
Code 425 Rack	32000197
Magnetic Bead Separator Base	SPL-2294G-HDW
Magnet insert for magnetic bead separator	SPL-2294E-HDW
DF200 Filter Tips	F172503
DF30 Filter Tips	F172303

Items from Multiplicom

Description	Part Number
BRCA MASTR Dx (8 rxn / 40 rxn)	MR-2012.008 / MR-2012.040
MID Dx 1-48 for Illumina MiSeq®	ML-0204.240
MID Dx 49-96 for Illumina MiSeq®	ML-0205.240