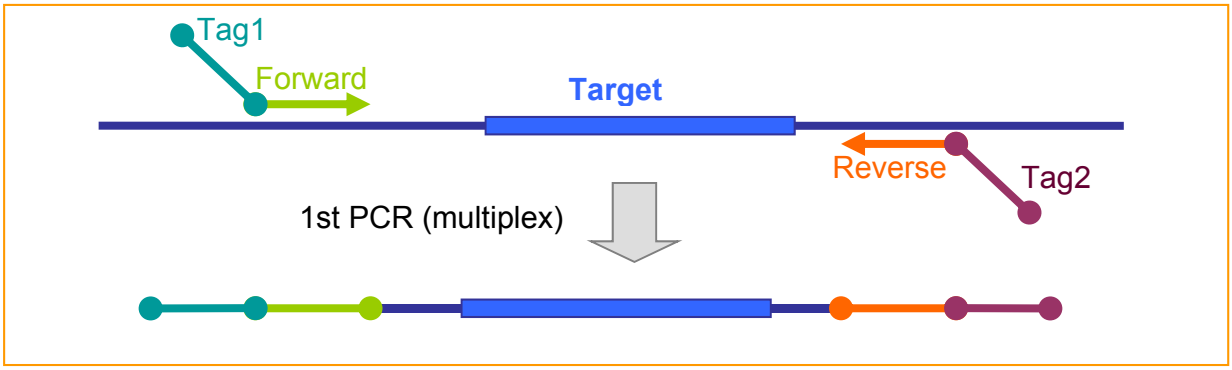
(1) Multiplex PCR, Amplification of Specific Targets

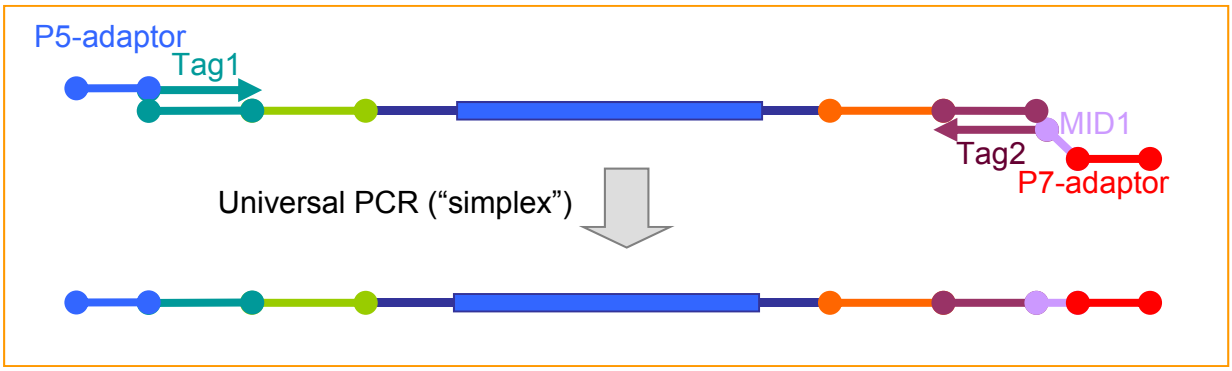


Template: 50 ng of human genomic DNA
Necessary kit: BRCA MASTR Dx Assay

- Cat. Nr. MR-2012.008 or MR-2012.040
- Contents:
  - PCR Mix (BRCA plex1 – BRCA plex5)
  - Taq DNA polymerase

Number of reactions: 5 per DNA sample
Next step: (2) Universal PCR

(2) Universal PCR, Addition of MIDs and MiSeq adaptor sequences



Template : 1/1000 dilution of (1) Multiplex PCR product
Necessary kit: MiSeq MID kit

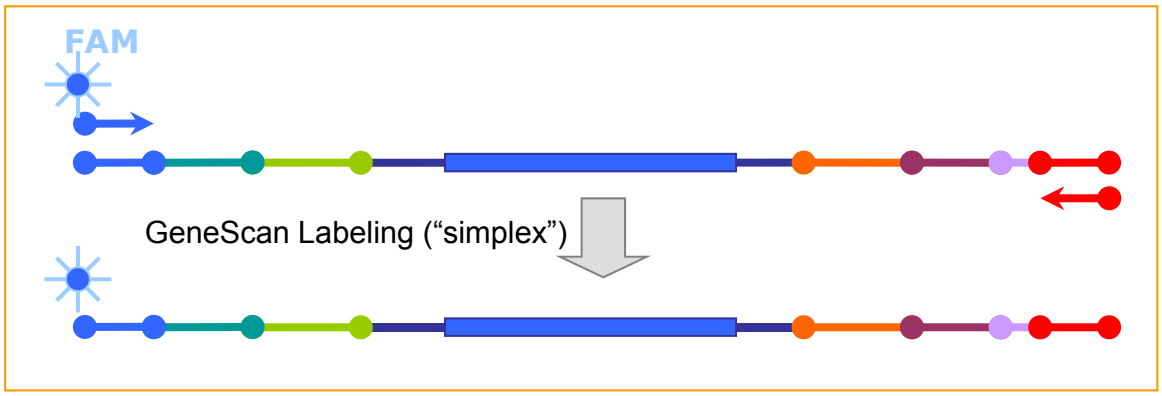
- Cat. Nr. ML-0200.384 ML-0201.384

- Consumables used:
  - Universal PCR Mix
  - MiSeq MID Primer Mix
  - Taq DNA polymerase

Number of reactions: 5 per DNA sample
Next step:

- (3) GeneScan Labeling (recommended but optional)
- Mixing of 5 reactions per sample and MiSeq sequencing protocol

(3) GeneScan Labeling (optional)



Template : (2) Universal PCR product
Necessary kit: MiSeq MID kit

- Cat. Nr. ML-0200.384 ML-0201.384

- Consumables used:
  - MiSeq GS-labeling PCR Mix
  - Taq DNA polymerase

Number of reactions: 5 per DNA sample
Next step: fragment analysis on GeneScan

Remark:

For diagnostic purposes, the MiSeq MID kits (RUO) and downstream workflow should first be validated when used in combination with the CE-validated BRCA MASTR Dx.

SHORT PROTOCOL MASTR ASSAY

Research Use Only

Multiplex PCR with amplicon specific primermix:

Preparation of master reaction mix

Remark:

- Prepare one master reaction mix for each Multiplex PCR that is part of the MASTR assay.

1. Remove the PCR mix from the -20°C freezer and allow complete thawing on ice.
2. Vortex thoroughly and centrifuge the vials at 12.000g for 10s before use.
3. Mix together **10µl** PCR mix with **0.075µl** Taq DNA polymerase.
4. Vortex briefly and centrifuge the vials at 12.000g for 10s.

PCR reaction setup

5. For each sample, combine **50ng** genomic DNA with **10µl** of the master reaction mix.
6. Adjust with distilled water to final volume of **15µl**.
7. Vortex briefly and centrifuge the vials at 12.000g for 10s.

PCR cycling profile

98°C – 10 min
95°C – 45 s
60°C – 45 s } x20
68°C – 2 min
72°C – 10 min
4° C (*)

Remark:

- Set the ramp rate of the PCR machine at approx. 1.25°C/s (below 2.5°C/s)

(*) Important notice !
Do not store the amplified Multiplex PCR reactions at 4°C, but continue immediately with the Universal PCR or freeze at -20°C.

Universal PCR with MID incorporation:

Preparation of master reaction mix

Remark:

- Prepare one master reaction mix for each MiSeq MID that will be used
- Use one MiSeq MID Primer Mix for all Multiplex PCR products of 1 individual

1. Remove the Universal PCR mix and MiSeq MID primer mix from the -20°C freezer and allow complete thawing on ice.
2. Vortex thoroughly and centrifuge the vials at 12.000g for 10s before use.
3. Mix together **20µl** Universal PCR mix with **0.125µl** Taq DNA polymerase and **4 µl** of the MiSeq MID-primer mix.
4. Vortex briefly and centrifuge the vials at 12.000g for 10s.

PCR reaction setup

5. For each sample and each Multiplex, dilute the PCR product of the first Multiplex PCR 1/1000 (2-step serial dilution in distilled water) and combine 1µl of this dilution with **24µl** of the master reaction mix.
6. Vortex briefly and centrifuge the vials at 12.000g for 10s.

PCR cycling profile

98°C – 10 min
95°C – 45 s
64°C – 45 s } x20
68°C – 2 min
72°C – 10 min
4° C – ∞

Remark:

- Set the ramp rate of the PCR machine at approx. 1.25°C/s (below 2.5°C/s)

Labeling PCR for check on Genescan (recommended, but optional):

Preparation of master reaction mix

1. Remove the GS-labeling PCR mix from the -20°C freezer and allow complete thawing on ice.
2. Vortex thoroughly and centrifuge the vials at 12.000g for 10s before use.
3. Mix together **13µl** GS-labeling PCR mix with **0.075µl** Taq DNA polymerase.
4. Vortex briefly and centrifuge the vials at 12.000g for 10s.

PCR reaction setup

5. For each sample, combine **2µl of Universal PCR product** with **13µl** of the master reaction mix.
6. Vortex briefly and centrifuge the vials at 12.000g for 10s.

PCR cycling profile

98°C – 10 min
95°C – 45 s
60°C – 45 s } **x5**
68°C – 2 min
72°C – 10 min
4° C – ∞

Fragment Analysis

7. Prepare the size standard mix by combining **10µl** of HiDi-Formamide (not supplied) with **0.3µl** GS500-LIZ size standard (not supplied, other options for the size standard are GS500-ROX or the GS600 standards).
8. For each reaction dispense **10µl** of size standard mix per well into a 96-well plate.
9. Add **2µl** of the labeling PCR-product to a well containing the "Size standard mix".
10. Denature samples at 95°C for 3 minutes and put on ice immediately.
11. Centrifuge the plate at 1.000g for 10s.
12. Load onto the Fragment Analyzer.
13. *Analyze data with MAQ-S analysis software (freely downloadable from www.multiplicom.com*)

Preparation of amplicon library for MiSeq sequencing
BRCA MASTR Dx assay

Mixing of the 5 Universal PCR products per sample

Combine for each sample in a different 1.5 ml tube the 5 Universal PCR products according to following scheme:

Plex	Volume
Plex1	8.0 µl
Plex2	11.0 µl
Plex3	13.0 µl
Plex4	8.0 µl
Plex5	10.0 µl

Purification of the amplicon libraries

For each sample, purify the obtained mixes using Agencourt® AMPure® XP beads using the following adapted protocol:

1. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.
2. Combine **40 µl amplicon library** with **34 µl Agencourt AMPure XP**
3. Mix reagent and amplicon library thoroughly by pipette mixing 10 times. Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
4. Place the tubes onto a Magnetic Bead Separator for 2 minutes to separate beads from the solution.
5. Aspirate the cleared solution from the reaction plate and discard.
6. Dispense 200 µL of 70% ethanol to each tube and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat this wash step once.
7. Let the pellet dry.
8. Off the Magnetic Bead Separator, add **20 µl** of distilled water to each tube and pipette mix 10 times.
9. Place the tubes onto a Magnetic Bead Separator for 1 minute to separate beads from the solution.
10. Transfer the eluent to a new tube.
11. It is recommended to verify the absence of short fragments by GeneScan analysis or similar. In case residual short fragments or observed repeat the purification protocol described above.

Mixing of amplicon libraries

1. Determine the concentration of each of the obtained amplicon libraries by a spectrophotometric or fluorimetric method.
2. Calculate the concentration of each amplicon library in nM, using the following equation:
   (The average size of the amplicons after Universal PCR is **472 bp**)

$$\text{Sample conc [nM]} = \frac{\text{sample conc [ng/µl]} \times 10^6}{660 \times 472}$$

3. Dilute each amplicon library to the same concentration e.g. 2 nM
4. If multiple amplicon libraries need to be sequenced in the same MiSeq run, mix an equal volume of these diluted amplicon libraries to form an amplicon pool. Take care to mix only amplicon libraries that contain different MiSeq MIDs (incorporated in the Universal PCR step).

MiSeq run set-up

1. A detailed description on how to set up a MiSeq run can be found in the **MiSeq system user guide.** Always use the latest version of the user guide.
2. For MiSeq sequencing of the BRCA MASTR Dx assay use only the MiSeq Reagent Kit v2, 500 Cycles (Catalog # MS-102-2003).
3. Custom sequencing primers and a custom index primer are required for MiSeq based BRCA MASTR Dx sequencing. These 3 primers are provided as a 100 µM solution and can be found in the amplification box of the MiSeq MID kit. The 3 primers are termed:
    - Read1 primermix
    - Index primermix
    - Read2 primermix
4. Add 3.4 µl of the custom primers to the following cartridge position:
    - Read 1 primermix to cartridge position 12;
    - Index primermix to cartridge position 13 and
    - Read 2 primermix to cartridge position 14

    Following guidelines below:
    - With a clean pipette tip, pierce the foil seal covering the port associated with the primer you are loading.
    - Load 3.4 µl of custom primer in the appropriate port on the reagent cartridge. Take care to avoid touching the foil seal as you dispense the primer.
    - With a clean **gel loading pipette tip** for each cartridge position, mix the primermix thoroughly by slowly pipetting 150 µl up and down, at least 5 times.
    - Inspect the bottom of the cartridge to make sure that no bubbles are present. Remove bubbles by tapping the cartridge.
5. Denature and dilute the prepared amplicon pool to a concentration of 6 pM and **spike-in PhiX control at 1%** as described in the MiSeq system user guide under 'Performing a run' to obtain the **Final Sample**.
6. **Pipette 600 µl of the Final Sample** into the **Load Samples** reservoir. Take care to avoid touching the foil seal as you dispense your sample.
7. Create a sample sheet with the Illumina Experiment Manager as follows:
    - Select as Instrument "MiSeq", click Next.
    - Select as Category "Targeted Resequencing", Select as Application "PCR Amplicon", click Next.
    - Fill out the PCR Amplicon Run settings, with these specific settings:
        i. Sample Prep Kit: select "Nextera"
        ii. Index Reads: select "1"
        iii. Read Type: select "Paired End"
        iv. Cycles Read 1: 251
        v. Cycles Read 2: 251
    - Do not change the PCR Amplicon Workflow-Specific Settings: leave the "Custom Primer" unselected as the Custom Primer positions on the cartridge are not used. Click Next.
    - Add rows to the sample sheet. Select Indexes from the list. Ignore eventual warning on diversity of index cycles. Select the correct Manifest file (can be obtained by simple request at customerservice@multiplicom.com). Click Finish and save the sample sheet.
    - Edit the sample sheet and replace Index sequences by the used index sequences (can be obtained by simple request at customerservice@multiplicom.com). When using Excel to edit the sample sheet, extra commas might be added to the definition lines in the Header. Check this be opening the sample sheet in Notepad and eventually correct this by manually deleting the commas.
8. Proceed directly with the run setup steps using the MiSeq Control Software (MCS) interface.