Oncomine™ BRCA Research Assay

Manual Library Preparation Using the Ion Library Equalizer™ Kit

Catalog Number A32840

Pub. No. MAN0015993 **Rev.** B.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Oncomine™ BRCA Research Assay User Guide* (Pub. No. MAN0014634). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Note: See the *Oncomine™ BRCA Research Assay User Guide* (Pub. No. MAN0014634) for procedures to quantify libraries by qPCR, or using the Qubit™ 2.0 or 3.0 Fluorometer, or the Agilent™ 2100 Bioanalyzer™ instrument.

- Amplify the targets 1
- Combine the target amplification reactions 1
- Partially digest primers 2
- Ligate adapters to the amplicons and purify 2
- Equalize the library 3
- Combine Oncomine™ BRCA Research Assay barcoded libraries for sequencing on the same Ion chip 4
- Guidelines for templating and sequencing 4
- Limited product warranty 4

Amplify the targets

1. For each sample, add components to 2 adjacent wells of a 96-well plate using a low volume pipettor, as described in the following table.

IMPORTANT! Ion AmpliSeq™ Sample ID Panel primer pairs are included in the Oncomine™ BRCA Research Assay. Do not add additional Ion AmpliSeq™ Sample ID Panel primers to target amplification reactions.

Component	Volume per well (Pool 1)	Volume per well (Pool 2)
5X Ion AmpliSeq™ HiFi Mix (red cap)	2 µL	2 µL
Oncomine™ BRCA Pool 1 (blue cap)	2 µL	—
Oncomine™ BRCA Pool 2 (red cap)	—	2 µL
DNA, 10 ng	≤6 µL	≤6 µL
Nuclease-free Water	to 10 µL	to 10 µL

Note: Add 10 ng of control DNA to 2 wells if running a control.

2. Mix thoroughly by pipetting up and down, then seal the plate with MicroAmp™ Adhesive Film. Alternatively, the plate can be vortexed after sealing, followed by centrifugation at 100 × *g* for 30 seconds to collect volume at the bottom of the wells.

3. Place a MicroAmp™ Compression Pad on the plate, load into the thermal cycler, then run the following program:

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 minutes
Cycle (18 cycles for non-FFPE DNA, 21 cycles for FFPE DNA)	Denature	99°C	15 seconds
	Anneal and Extend	60°C	4 minutes
Hold	—	10°C	Hold (16 hours maximum)

STOPPING POINT Amplification products can be stored at 10°C overnight (12–16 hours) in the thermal cycler. For longer-term storage, store at –20°C.

Combine the target amplification reactions

1. Centrifuge the plate at 100 × *g* for 30 seconds in a plate centrifuge to collect contents at the bottom of the wells.

2. Carefully remove the plate seal from the plate.

3. Combine the two 10-µL target amplification reactions for each sample by transferring them to an empty well of a new column in the plate, or in a new plate.

Partially digest primers

Note: FuPa Reagent is viscous. Flick to mix, then pulse-centrifuge. To avoid carrying over excess enzyme, do not submerge the whole tip in the FuPa Reagent solution. Aspirate solution from the surface.

1. Add 2 µL of FuPa Reagent (brown cap) to each amplified sample. The total volume of each reaction is now ~22 µL.
2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge at 100 × *g* for 30 seconds to collect droplets.
3. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 minutes
55°C	10 minutes
60°C	20 minutes
10°C	Hold (for up to 1 hour)

4. Centrifuge the plate at 100 × *g* for 30 seconds before proceeding to the next step.

Ligate adapters to the amplicons and purify

Combine and dilute the adapters

For each Ion Xpress™ Barcode X chosen, prepare a mix of Ion P1 Adapter and Ion Xpress™ Barcode X at a final dilution of 1:4 for each adapter. Store diluted adapters at –20°C.

Combine the volumes indicated in the following table and use 2 µL of this barcode adapter mix in step 1 below. Scale volumes as necessary.

Component	Volume
Ion P1 Adapter	2 µL
Ion Xpress™ Barcode X[1]	2 µL
Nuclease-free Water	4 µL
Total	**8 µL**

[1] X = barcode chosen

Perform the ligation reaction

1. Centrifuge the plate at 100 × *g* for 30 seconds in a plate centrifuge to collect contents at the bottom of the wells.
2. Carefully remove the plate seal, then add the following components in the order indicated to each well containing digested sample. After adding each component, mix by setting a pipette to 15 µL, then pipetting up and down slowly 5 times.

   Note: Do not make a master mix of these components.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Ion Xpress™ Barcode Adaptor/P1 Adaptor mix[1]	2 µL
3	DNA Ligase (blue cap)	2 µL
—	Total volume (including ~22 µL of digested amplicons)	~30 µL

[1] Prepared in "Combine and dilute the adaptors".

3. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge at 100 × *g* for 30 seconds to collect droplets.
4. Place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 24 hours)

STOPPING POINT Samples can be stored at –20°C.

5. Centrifuge the plate at 100 × *g* for 30 seconds before proceeding to the next step.

Purify the library

1. Prepare fresh 70% ethanol: Combine 230 µL of 100% ethanol with 100 µL of Nuclease-free Water per sample.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension thoroughly with the DNA.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a DynaMag™-96 Side Magnet, then incubate for 2 minutes. Carefully remove and discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol, move the plate side-to-side in the two positions of the magnet to wash the beads, then remove and discard the supernatant without disturbing the pellet.

6. Repeat step 5 for a second wash.

7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.

   Note: While the beads dry, prepare the library amplification mix described in step 1 of "Amplify the library" on page 3.

Equalize the library

Before you begin

Warm all the reagents in the Ion Library Equalizer™ Kit to room temperature. Vortex and centrifuge all reagents before use.

Amplify the library

1. Prepare library amplification mix according to the following table. Prepare a master mix for multiple reactions.

Order of addition	Component	Volume per reaction	Volume for 8 reactions[1]
1	1X Library Amp Mix (black cap)	50 µL	450 µL
2	Equalizer™ Primers (pink cap)	2 µL	18 µL
—	**Total**	**52 µL**	**468 µL**

[1] One additional reaction added as overage to compensate for pipetting error.

2. Remove the plate from the magnet (at step 7 of "Purify the library"), then add 52 µL of library amplification mix to each well containing air-dried beads.

3. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge at 100 × *g* for 30 seconds to collect droplets.

4. Place the plate back on the magnet for 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new plate without disturbing the pellet.

5. Seal the plate with the adhesive film, place a MicroAmp™ Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
Cycling (9 cycles)	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold (for up to 1 hour)

Wash the Equalizer™ Beads *(if not previously performed)*

1. Bring the Equalizer™ Beads to room temperature, then mix thoroughly.

2. For each reaction, pipet 3 µL of beads into a clean 1.5-mL tube, then add 6 µL/reaction of Equalizer™ Wash Buffer.

3. Place the tube in a magnetic rack for 3 minutes or until the solution is clear.

4. Carefully remove the supernatant without disturbing the pellet, then discard.

5. Remove the tube from the magnet, add 6 µL per reaction of Equalizer™ Wash Buffer, then pipet up and down to resuspend.

Add Equalizer™ Capture to the amplified library

1. Carefully remove the seal from the plate, then add exactly 10 µL of Equalizer™ Capture to each library amplification reaction.

   Note: The final equalized library concentration is dependent upon accurate pipetting of the Equalizer™ Capture reagent.

2. Seal the plate with a clear adhesive film, vortex thoroughly, then centrifuge to collect droplets.

3. Incubate at room temperature for 5 minutes.

Add Equalizer™ Beads and wash

1. Mix the washed Equalizer™ Beads by gentle vortexing or pipetting up and down.

2. Add 6 µL of washed beads to each plate well containing the captured library.

3. Set the pipette volume to 40 µL, then pipet the mixture up and down at least 5 times to mix thoroughly.

4. Incubate at room temperature for 5 minutes. Briefly centrifuge the plate to collect all the liquid to the bottom of the plate wells.

5. Place the plate in the magnet, then incubate for 2 minutes or until the solution is clear.

6. Carefully remove the supernatant without disturbing the pellet.

7. Add 150 µL of Equalizer™ Wash Buffer to each reaction.

8. Move the plate side-to-side in the two positions of the magnet to wash the beads.

9. With the plate still in the magnet, carefully remove, then discard the supernatant without disturbing the pellet.

10. Repeat the bead wash as described in steps 7–9.

    Note: Ensure that as much wash buffer as possible is removed without disturbing the pellet.

Elute the equalized libraries

1. Remove the plate from the magnet, then add 100 µL of Equalizer™ Elution Buffer to each pellet.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then centrifuge to collect droplets.

   Note: Centrifuge with sufficient force to collect droplets, but not pellet beads. If beads are pelleted, vortex again and centrifuge more gently.
3. Elute the libraries by incubating in a thermal cycler at 32°C for 5 minutes.
4. Place the plate in the magnet, then incubate at room temperature for 5 minutes or until the solution is clear.

   The supernatant contains the equalized library at ~100 pM, which can be stored with beads for up to 1 month at 4–8°C.

Proceed to "Combine Oncomine™ BRCA Research Assay barcoded libraries for sequencing on the same Ion chip" and template preparation, or store libraries. Store libraries at 4–8°C for up to 1 month, or at –30°C to –10°C for longer-term storage.

Combine Oncomine™ BRCA Research Assay barcoded libraries for sequencing on the same Ion chip

Multiple Oncomine™ BRCA Research Assay libraries can be sequenced on a single Ion 318™ v2 BC or Ion 530™ Chip by combining equal volumes of each barcoded library before template preparation. Recommendations for the maximum number of libraries sequenced per chip are given in the following table:

Ion sequencing chip	Germline libraries sequenced per chip	Somatic libraries sequenced per chip
Ion 318™ v2 BC Chip	32	8
Ion 530™ Chip	32	32

Guidelines for templating and sequencing

- Proceed to template preparation and sequencing using the following kits appropriate to your instrument setup. See the appropriate user guide or guides listed in the table for more information.

Kit	Cat. No.	User Guide
Ion PGM™ Hi-Q™ View OT2 Kit *and* Ion PGM™ Hi-Q™ View Sequencing Kit	A29900 A30044	*Ion PGM™ Hi-Q™ View OT2 Kit User Guide* (Pub. No. MAN0014579) *Ion PGM™ Hi-Q™ View Sequencing Kit User Guide* (Pub. No. MAN0014583)
Ion PGM™ Hi-Q™ View Chef Kit	A29902	*Ion PGM™ Hi-Q™ View Chef Kits User Guide* (Pub. No. MAN0014571)
Ion 520™ & Ion 530™ Kit – Chef	A27757 A30010	*Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0010846)

- See Chapter 3, "Create a Planned Run and analyze results with an Ion Reporter™ workflow", in the *Oncomine™ BRCA Research Assay User Guide* (Pub. No. MAN0014634) for detailed instructions to analyze your results.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Manufacturer: Multiple Life Technologies Corporation manufacturing sites are responsible for manufacturing the products associated with the workflow covered in this guide.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

Revision history: Pub. No.MAN0015993

Revision	Date	Description
B.0	8 June 2017	Minor updates to align with Rev. B.0 of the *Oncomine™ BRCA Research Assay User Guide*
A.0	12 October 2016	New quick reference for manual library preparation using the Oncomine™ BRCA Research Assay.