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by Thermo Fisher Scientific

Prepare Ion AmpliSeq™ Libraries using the Tecan Freedom EVO® NGS Workstation

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Protocol information

Revision history

Revision	Date	Description
C.0	2 February 2014	• Added RNA workflow • Added "Fill volume" columns to worktable set up tables
B.0	10 October 2014	Corrected callouts in images and corresponding tables in the following sections: • "Purify, elute, and amplify the library" on page 22 • "Add Equalizer™ beads and wash" on page 25 • "Amplify the library" on page 30
A.0	29 August 2014	• First release

Description

This user bulletin describes how to prepare Ion AmpliSeq™ libraries using the Tecan Freedom EVO® NGS Workstation. The workflow for library preparation is similar to that of the Ion AmpliSeq™ library kits, with additional steps to set up the Tecan Freedom EVO® NGS Workstation, import and run the scripts. For more information, refer to the *Ion AmpliSeq™ DNA and RNA Library Preparation Guide* (Pub. no. MAN0006735).

Procedure overview

Amplify target regions from DNA and treat the resulting amplicons with FuPa Reagent to partially digest the primers and phosphorylate the amplicons. Next, ligate the amplicons to adapters with barcodes and purify them. Normalize or quantify libraries and combine them (optional) prior to template preparation and sequencing.

Note: The automated method described here is not compatible with Ion AmpliSeq™ Exome Kits.

The following kits are used in this automation protocol:

- **Ion AmpliSeq™ Library Kit 2.0—96LV or 384LV** (Cat. no. 4480441 or 4480442): One or more kits are required for preparing libraries using the automated platform to accommodate for higher dead volume requirements. Each kit contains reagents for the rapid preparation of either 96 or 384 libraries containing 12–24,576 amplicons per reaction. Each kit uses a 96-well plate-based protocol for easy sample handling and tracking, and for compatibility with automation and high-throughput laboratories.
- **Ion Library Equalizer™ Kit** (Cat. no. 4482298): This kit provides an optional streamlined method for normalizing library concentration without the need for quantitation.
- **Ion Xpress™ Barcode Adapters** (Cat. no. 4474517): One or more kits are required for preparing barcoded libraries. Each kit includes reagents sufficient for preparing up to 40 Ion AmpliSeq™ libraries per barcode (40 × 16 libraries).

Ion kits used in this protocol

Ion AmpliSeq™ Library Kit 2.0—96LV or 384LV

The Ion AmpliSeq™ Library Kit 2.0—96LV or 384LV (Cat. no. 4480441 or 4480442) provides reagents for preparing 96 and 384 libraries, respectively. If preparing the maximum number of libraries per kit, multiple kits may be required to accommodate for higher dead volume requirements associated with the automated platform.

Ion AmpliSeq™ Library Kit 2.0—96LV or 384LV (Cat. no. 4480441 or 4480442)					
		Number of tubes			
Component	**Cap color**	**Cat. no. 4480441**	**Cat. no. 4480442**	**Volume per tube**	**Storage**
5X Ion AmpliSeq™ HiFi Mix	Red	1 tube	4 tubes	384 µL	–30°C to –10°C
FuPa Reagent	Brown	1 tube	4 tubes	192 µL	
Switch Solution	Yellow	1 tube	4 tubes	384 µL	
DNA Ligase	Blue	1 tube	4 tubes	192 µL	
Ion AmpliSeq™ Adapters	Green	1 tube	4 tubes	192 µL	
Platinum® PCR SuperMix HiFi	Black	3 tubes	12 tubes	1.6 mL	
Library Amplification Primer Mix	White	1 tube	4 tubes	192 µL	
Low TE	Clear	1 tube	4 tubes	12 mL	Room temp (15°C to 30°C)

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit contains reagents for 96 reactions.

Ion Library Equalizer™ Kit (Cat. no. 4482298)				
Component	**Cap color**	**Quantity**	**Volume per tube**	**Storage**
Ion Library Equalizer™ Primers	Pink	1 tube	200 µL	2°C to 8°C
Ion Library Equalizer™ Capture	Purple	1 tube	1 mL	
Ion Library Equalizer™ Elution Buffer Switch Solution	Clear	1 bottle	10 mL	
Ion Library Equalizer™ Beads	Orange	1 tube	300 µL	
Ion Library Equalizer™ Wash Buffer	Clear	1 bottle	35 mL	Room temp (15°C to 30°C)

Ion Xpress™ Barcode Adapters

One or more Ion Xpress™ Adapters Kits are required for preparing barcoded libraries. Each kit includes reagents sufficient for preparing up to 40 Ion AmpliSeq™ libraries per barcode (40 × 16 libraries). Substitute Ion Xpress™ Adapters for standard Ion AmpliSeq™ Adapters as described in this user guide.

Ion Xpress™ Barcode Adapters (Various Cat. nos.—Each kit includes 16 individually numbered barcodes)				
Component	**Cap color**	**Quantity**	**Volume per tube**	**Storage**
Ion Xpress™ P1 Adapter	Violet	1 tube	320 µL	–30ºC to –10ºC
Ion Xpress™ Barcode X	White	16 tubes (1 per barcode)	20 µL each	

Required materials and equipment

Unless otherwise specified, all materials are available from Life Technologies (**www.lifetechnologies.com**). MLS: Fisher Scientific (**www.fisherscientific.com**) or major laboratory supplier.

Description	Supplier	Catalog no.	Quantity
Tecan Freedom EVO® NGS Workstation	Tecan	See www.tecan.com/NGS	1
Tecan Pure LiHa Disposable Tips, Filtered, 50, 200, and 1000 µL	Tecan	30057813, 30057815, and 30057817	1
Disposable Reagent Troughs, 25 mL and 100 mL	Tecan	10613102 and 10613048	1
Eppendorf® Deepwell Plates, 500 µL	Eppendorf	951031801	1
MicroAmp® EnduraPlate™ Optical 96-well Reaction Plates with Barcode	Life Technologies	4483354	1
MicroAmp® Splash-Free 96-well Base	Life Technologies	4312063	1
One of the following: • GeneAmp® PCR System 9700 or Dual 96-well Thermal Cycler • AB® 2720 Thermal Cycler • Veriti® 96-well Thermal Cycler • ProFlex™ 96-Well PCR System	Life Technologies	See web product pages	1
96-well plate centrifuge	MLS	Various	1
(RNA only) SuperScript® VILO™ cDNA Synthesis Kit	Life Technologies	11754-050	50 reactions
MicroAmp® Adhesive Film	Life Technologies	4306311	1
MicroAmp® Compression Pad	Life Technologies	4312639	1 set
Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1
DynaMag™ Side Magnet, or other plate magnet	Life Technologies	12331D	1
Nuclease-Free Water	Life Technologies	AM9932	1000 mL
Absolute ethanol	MLS	N/A	~15 mL
(Optional) RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	Life Technologies	AM1975	40 preps
(Optional) MagMAX™ FFPE Total Nucleic Acid Isolation Kit	Life Technologies	4463365	96 preps
(Optional) PureLink® Genomic DNA Mini Kit	Life Technologies	K182000	10 preps
(Recommended for DNA quantitation) TaqMan® RNase P Detection Reagents Kit	Life Technologies	4316831	1

Description	Supplier	Catalog no.	Quantity
(Optional for library quantitation) If you are not using the Ion Library Equalizer™ Kit (Cat. no. 4482298) for library normalization, select one of the following:			
Qubit® 2.0 Fluorometer and Qubit® dsDNA HS Assay Kit	Life Technologies	Q32866, Q32851/ Q32854	1
Agilent® 2100 Bioanalyzer® and Agilent® High Sensitivity DNA Kit	Agilent	G2939AA, 5067- 4626	

Input DNA requirements

DNA isolation and quantitation

See "Required materials and equipment" on page 5 for recommended kits for isolating DNA.

We recommend the TaqMan® RNase P Detection Reagents Kit (Cat. no. 4316831) for quantitating amplifiable human genomic DNA (see http://ioncommunity.lifetechnologies.com/docs/DOC-7431). The Qubit® dsDNA HS Assay Kit (Cat. no. Q32851 or Q32854) may also be used. Methods such as densitometry (e.g., NanoDrop® Spectrophotometers) are not recommended, because they do not discriminate between DNA and RNA and thus are extremely sensitive to small fragments of hydrolyzed RNA. This can lead to gross overestimation of the concentration of sample DNA, underseeding of the target amplification reaction, low quality libraries, and low library yields.

Amount of DNA needed

Each target amplification reaction requires 10 ng of 2 ng/µL genomic DNA (gDNA) or DNA from FFPE (at least 8 µL for each reaction).

Note: Be sure to use an FFPE-compatible Ion AmpliSeq™ panel for DNA isolated from FFPE tissue. Standard designs with longer amplicons may perform poorly with DNA from FFPE tissue.

Input RNA requirements

Amount needed and quantitation

In general, the library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance. See "Required materials and equipment" on page 5 for kits recommended for isolating RNA. Each reverse transcription reaction requires 10 ng of DNase-treated RNA (≥ 1.4 ng/µL), prepared from normal or FFPE tissue.

We recommend the Qubit® RNA HS Assay Kit (Cat. no. Q32855) for quantitating RNA.

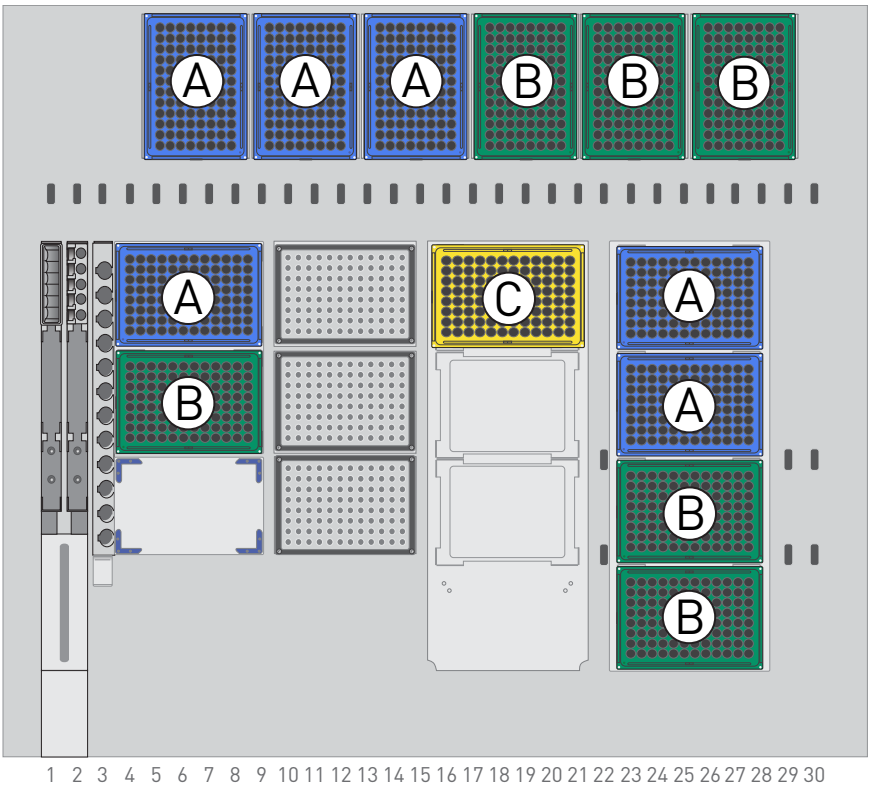
Using the Tecan Freedom EVO® NGS Workstation

Import the script file

1. Download and extract the AmpliSeq_DNA_RNA.exd.zip from the Ion Community (**http://ioncommunity.lifetechnologies.com/docs/DOC-9141**).
2. Open the Tecan Export Import tool
3. Select **File > Load > Importfile**.
4. Select "AmpliSeq_DNA_RNA.exd" and select **Open**.
5. Select **Import All** to import the scripts.

Tip handling

A subroutine within all scripts contains a method for tracking and handling 50-µL and 200-µL tips. You only need to replace tips when all six boxes are empty. Alternatively, at the start of a run you can replace all tip boxes and enter a value of "1" when prompted with "New tips boxes?" This will reset the tip count. The 1000-µL tips are handled separately and replaced as necessary.



No.	Tip type	Location(s)
A	200 µL (blue)	Grid 3, Site 1 Grid 22, Sites 1–2 Storage rack positions 1–3
B	50 µL (green)	Grid 3, Site 2 Grid 22, Sites 3–4 Storage rack positions 4–6
C	1000 µL (yellow)	Grid 16, Site 1

Prepare and amplify cDNA targets

Reverse transcribe RNA

If RNA was prepared from FFPE tissue and not previously heat-treated, pre-heat at 80°C for 10 minutes, then cool to room temperature.

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below:

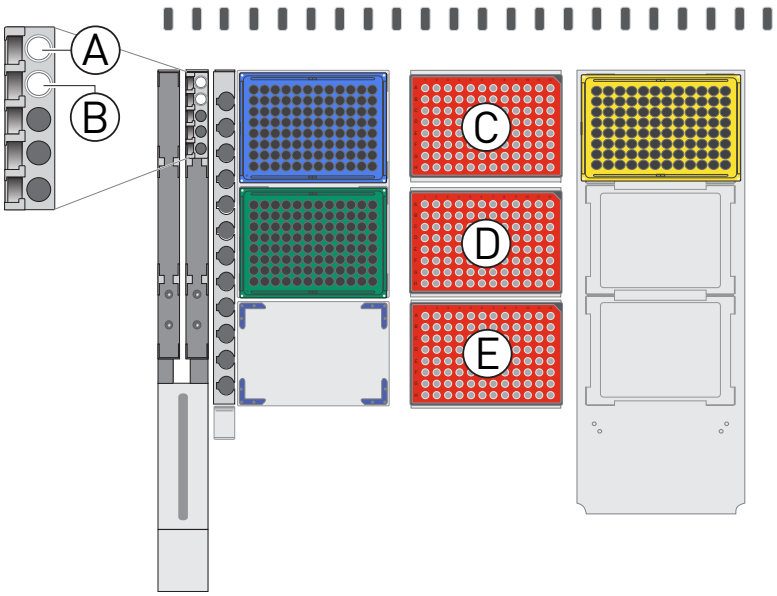


Table 1 Reagent setup summary: Reverse transcribe RNA

No.	Position	Description	Labware	Fill volume[1]
A	Grid 2, Site 1, Well 1 ***(Standard Transfer Only)***	10X SuperScript® III Enzyme Mix	0.5-mL tube	120 µL[2]
B	Grid 2, Site 1, Well 2 ***(Standard Transfer Only)***	5X VILO™ RT Reaction Mix	0.5-mL tube	240 µL[2]
C	Grid 10, Site 1 ***(Fast Transfer Only)***	10X SuperScript® III Enzyme Mix	MicroAmp® EnduraPlate™ reaction plate	10X SuperScript® III Enzyme Mix : 20 µL/well in column 2
		5X VILO™ RT Reaction Mix		5X VILO™ RT Reaction Mix: 40 µL/well in column 4
D	Grid 10, Site 2	RT plate	MicroAmp® EnduraPlate™ reaction plate	Empty
E	Grid 10, Site 3	RNA plate	MicroAmp® EnduraPlate™ reaction plate	8 µL/well (1.4 ng/µL)

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.
[2] Combine two tubes from SuperScript® VILO™ cDNA Synthesis Kit.

2. Add the following reagents according to the transfer mode selected:

Transfer Mode	Reagent Setup
Standard	1. Place the tube containing 10X SuperScript® III Enzyme Mix in position 1 of the chilled metal cooling block at Grid 2, Site 1 (**A**). 2. Place the tube containing 5X VILO™ RT Reaction Mix in position 2 of the chilled metal cooling block at Grid 2, Site 1 (**B**).
Fast	1. Aliquot 10X SuperScript® III Enzyme Mix into all wells of column 2 and 5X VILO™ RT Reaction Mix into all wells of column 4 in a MicroAmp® EnduraPlate™. 2. Place the plate on the incubator at Grid 10, Site 1 (**C**).

3. Place a new MicroAmp® EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**D**).
4. Place MicroAmp® EnduraPlate™ reaction plate containing RNA (1.4 ng/µL) on the incubator at Grid 10, Site 3 (**E**).
5. Replace the tip boxes as needed.

Run the script

1. Open EVOware.
2. Click **Run an existing script**.
3. Select the script "**RNA_RT**."
4. Enter the number of reactions to be prepared.
5. If you are using Fast Mode, enter "**1**" when prompted "**Enable Fast Transfer?**." Otherwise leave the value as "**0**."
6. Click **RUN**.
   The run time is ~45 minutes (Standard) or 20 minutes (Fast) for 96 reactions.
7. When the script is complete, seal the plate with a MicroAmp® Adhesive Film. Vortex the plate 3 times for three seconds each at setting 7–10, then centrifuge the plate briefly.
8. Place a MicroAmp® Compression Pad on the plate, load it in the thermal cycler, and run the following program:

Temperature	Time
42°C	30 min
85°C	5 min
10°C	Hold

STOPPING POINT Samples may be stored at 10°C overnight (12–16 hours) in the thermal cycler. For longer periods, store at –20°C.

Amplify cDNA targets

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below:

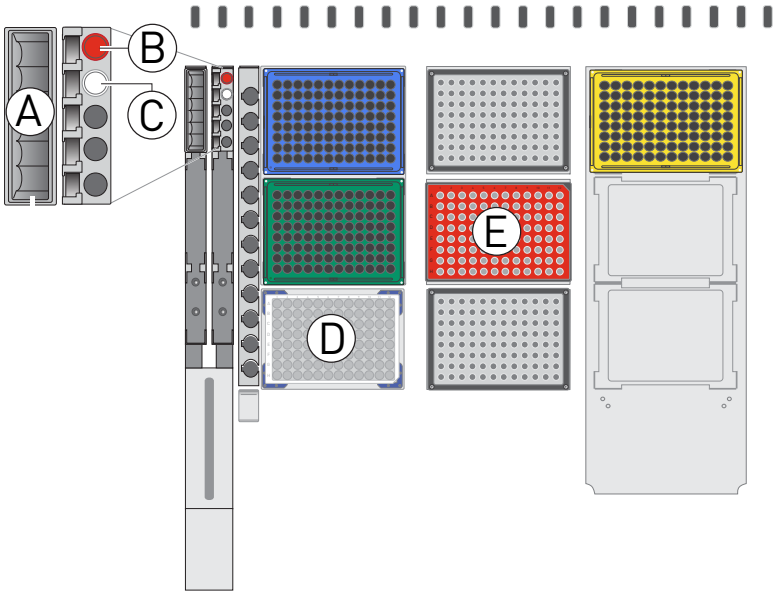


Table 2 Reagent setup summary: Amplify cDNA

No.	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 1	Water	25-mL reservoir	1 mL
B	Grid 2, Site 1, Well 1	5X HiFi Mix (red cap)	0.5-mL tube	500 µL[2]
C	Grid 2, Site 1, Well 2	5X RNA Primer Panel	1.5-mL tube	500 µL
D	Grid 4, Site 3	Deepwell plate	Eppendorf® Deepwell plate 96/500	Empty
E	Grid 10, Site 2	cDNA plate	EnduraPlate™ reaction plate	From previous reaction

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.

[2] To ensure sufficient volume for 96 reactions, add 50 µL of 5X HiFi Mix to a full tube from a 96-LV kit to accommodate dead volumes. This tube may be refilled from another tube later to minimize reagent loss.

2. Fill a 25-mL trough with Nuclease-Free Water and place it inside a 100-mL trough at Grid 1, Site 1 (**A**).

3. Place the tube containing 5X Ion AmpliSeq™ HiFi Master Mix (red cap) in position 1 of the chilled metal cooling block at Grid 2, Site 1 (**B**).

4. Place the tube containing 5X Ion AmpliSeq™ RNA panel in position 2 of the chilled metal cooling block at Grid 2, Site 1 (**C**).

5. Place a 500-µL plate onto the shaking incubator at Grid 4, Site 3 (**D**).

6. Place the MicroAmp® EnduraPlate™ reaction plate containing the cDNA from the previous reaction on the incubator at Grid 10, Site 2 (**E**).

7. Replace the tip boxes as needed.

Run the script

1. Open EVOware.

2. Click **Run an existing script**.

3. Select the script "**RNA_Amp**."

4. Enter the number of reactions to be prepared.

5. Click **RUN**.
   The run time is ~10 minutes for 96 reactions.

6. When the script is complete, seal the plate with MicroAmp® Adhesive Film and spin down to collect the droplets.

7. Proceed immediately to “Thermal cycling conditions“ on page 15.

Amplify DNA targets

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below:

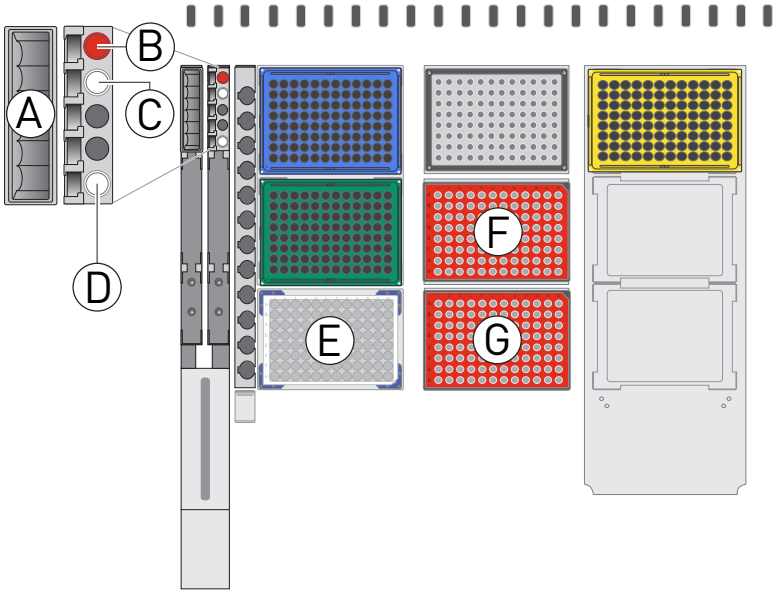


Table 3 Reagent setup summary: Amplify DNA

No.	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 1	Water	25-mL reservoir	1 mL
B	Grid 2, Site 1, Well 1	5X HiFi Mix (red cap)	0.5-mL tube	500 µL[2]
C	Grid 2, Site 1, Well 2	Primer Mix (diluted to 2X)[3]	1.5-mL tube	1.2 mL
D	Grid 2, Site 1, Well 5	*(Optional)* 20X Sample ID Panel	0.5-mL tube	120 µL
E	Grid 4, Site 3	Deepwell plate	Eppendorf® Deepwell plate 96/500	Empty
F	Grid 10, Site 2	Amplification plate (output)	EnduraPlate™ reaction plate	Empty
G	Grid 10, Site 3	gDNA	EnduraPlate™ reaction plate	8 µL/well, 2 ng/µL

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.

[2] To ensure sufficient volume for 96 reactions, add 50 µL of 5X HiFi Mix to a full tube from a 96-LV kit to accommodate dead volumes. This tube may be refilled from another tube later to minimize reagent loss.

[3] Single primer pool only.

IMPORTANT! The script uses primers at 2X concentration only. If you are using a 5X primer pool, dilute to 2X with Nuclease-Free Water in a 1.5-mL tube prior to use.

2. Fill a 25-mL trough with Nuclease-Free Water and place it inside a 100-mL trough at Grid 1, Site 1 (**A**).
3. Place the tube containing 5X Ion AmpliSeq™ HiFi Master Mix (red cap) in position 1 of the chilled metal cooling block at Grid 2, Site 1 (**B**).
4. Place the tube containing 2X Ion AmpliSeq™ Primer Pool in position 2 of the chilled metal cooling block at Grid 2, Site 1 (**C**).
5. Optional: Place the tube containing 20X Ion AmpliSeq™ Sample ID Panel in position 5 of the chilled metal cooling block at Grid 2, Site 1 (**D**).

   Note: If you are using the Sample ID Panel with a Custom or Ready-to-use Panel containing multiple primer pools, you only need to add the Sample ID Panel to one of the target amplification reactions.
6. Place a 500-µL plate onto the shaking incubator at Grid 4, Site 3 (**E**).
7. Place a new MicroAmp® EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**F**).
8. Place the MicroAmp® EnduraPlate™ reaction plate containing the prepared gDNA (2 ng/µL) on the incubator at Grid 10, Site 3 (**G**).
9. Replace the tip boxes as needed.

Run the script

1. Open EVOware.
2. Click **Run an existing script**.
3. Select the script "**Target_Amp**."
4. Enter the number of reactions to be prepared.
5. Click **RUN**.
   The run time is ~20 min for 96 reactions.
6. When the script is complete, seal the plate with MicroAmp® Adhesive Film and spin down to collect the droplets.
7. Proceed immediately to “Thermal cycling conditions“ on page 15.

Thermal cycling conditions

Place a MicroAmp® Compression Pad on the plate, load the plate in the thermal cycler, and run the following program to amplify target DNA regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 min
Cycle; set number according to the following table	Denature	99°C	15 sec
	Anneal and extend	60°C	4 min/8 min/ 16 min[1]
Hold	—	10°C	Hold[2]

[1] 4 minutes for ≤1536 primer pairs per pool; 8 minutes for 1,537–6,144; 16 minutes for 6,145–24,576.

[2] Samples may be held at 4°C overnight.

Primer pairs per pool (see notes below)	Recommended number of amplification cycles	
	Normal DNA/RNA	FFPE DNA/RNA
gene fusion	27	30
12–24	21	24
25–48	20	23
49–96	19	22
97–192	18	21
193–384	17	20
385–768	16	19
769–1,536	15	18
1,537–3,072	14	17
3,073–6,144	13	16
6,145–12,288	12	15
12,289–24,576	11	14

Note: *Ready-to-use panels:* The Ion AmpliSeq™ Cancer Hotspot Panel v2 is 207 primer pairs, the Comprehensive Cancer Panel is ~4,000 primer pairs/pool, and the Inherited Disease Panel is ~3,500 primer pairs/pool.

Note: Cycle numbers can be increased when input material quality or quantity is questionable. The cycle number does not need to be adjusted when using the Ion AmpliSeq™ Sample ID panel.

STOPPING POINT PCR products may be stored at 10°C overnight. For longer periods, store at –20°C.

Partially digest primer sequences

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below: :

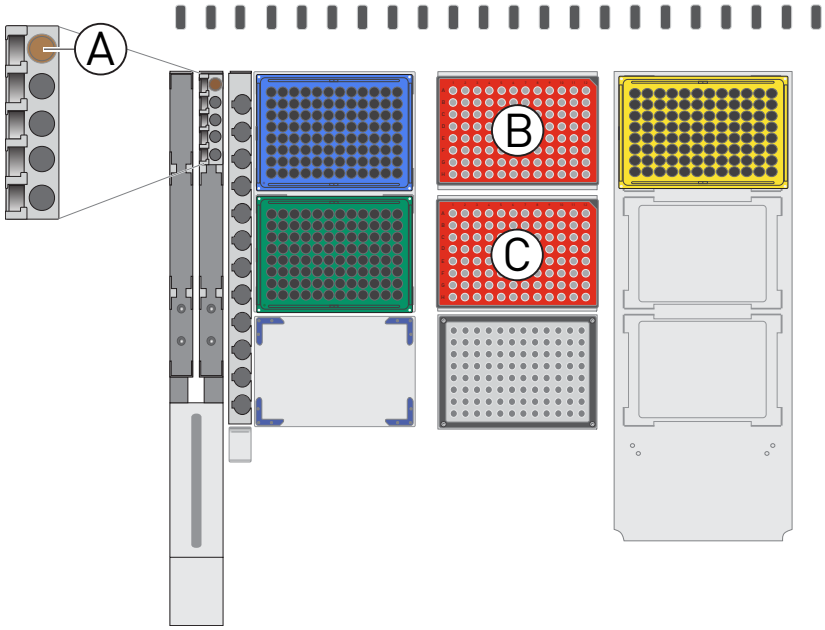


Table 4 Reagent setup summary: Partially digest primers

No.	Position	Description	Labware	Fill volume[1]
A	Grid 2, Site 1, Well 1 ***(Standard Transfer Only)***	FuPa Reagent (brown cap)	0.5-mL tube	240 µL[2]
B	Grid 10, Site 1 ***(Fast Transfer Only)***	FuPa Reagent	EnduraPlate™ reaction plate	40 µL/well in column 1
C	Grid 10, Site 2	Amplified DNA	EnduraPlate™ reaction plate	From previous reaction

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.
[2] Use a full tube(s) from a 96-LV kit to accommodate dead volumes.

2. Add the following reagents according to the transfer mode selected:

Transfer Mode	Reagent Setup
Standard	1. Place the tube containing FuPa Reagent (brown cap) in position 1 of the chilled metal cooling block at Grid 2, Site 1 (**A**).
Fast	1. Aliquot FuPa Reagent (brown cap) into all wells of column 1 in a MicroAmp® EnduraPlate™ reaction plate and place iton the incubator at Grid 10, Site 1 (**B**). This plate can be stored at -20°C for later use.

3. Carefully remove the plate seal from the amplified DNA plate and place it on the incubator at Grid 10, site 2 (**C**).

4. Replace the tip boxes as needed.

Run the script

1. Open EVOware.

2. Click **Run an existing script**.

3. Select the script "**Fupa**."

4. Enter the number of reactions to be prepared.

5. If you are using Fast Mode, enter "**1**" when prompted "**Enable Fast Transfer?**." Otherwise leave value as "**0**."

6. Click **RUN**.
   The run time is ~20 minutes (Standard) or 7 minutes (Fast) for 96 reactions.

7. When the script is complete, seal the plate with MicroAmp® Adhesive Film. Vortex the plate three times for 3 seconds each at setting 7–10, then centrifuge the plate briefly.

8. Place a MicroAmp® Compression Pad on the plate, load the plate in the thermal cycler, and run the following program:

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

Ligate adapters to the amplicons and purify

Barcoded libraries only: Combine and dilute adapters

If you are running multiple sample libraries on a single chip, you can assign a unique barcode to each library. For each 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 in a MicroAmp® EnduraPlate™ 96-well plate.

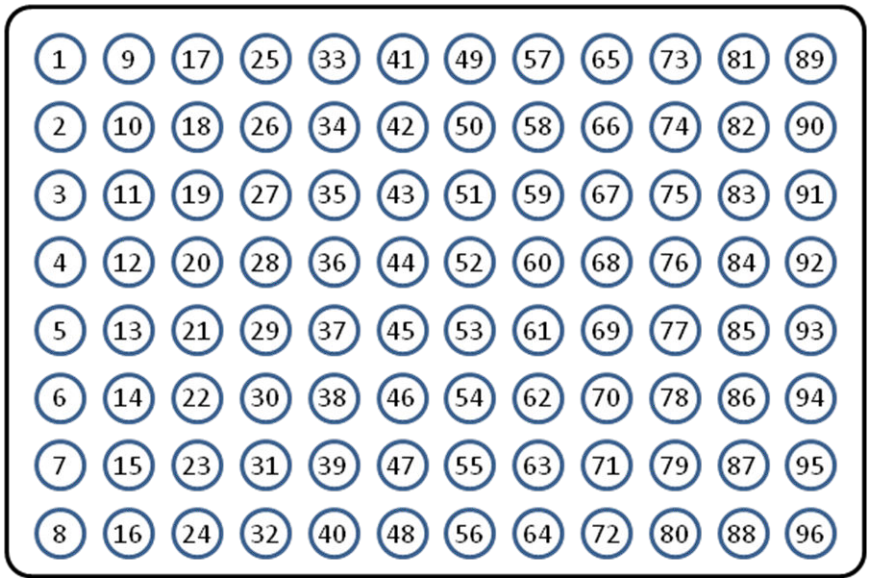
IMPORTANT! When handling barcoded adapters, be careful to avoid cross-contamination by changing gloves frequently and opening one tube at a time.

Table 5 Example barcode adapter mix for up to 40 reactions

Component	Volume
Ion P1 Adapter	20 µL
Ion Xpress™ Barcode X[1]	20 µL
Nuclease-Free Water	40 µL
Total	**80 µL**

[1] X = Barcode chosen

IMPORTANT! Barcodes *must* be arrayed in the following pattern (i.e., barcode number must match position number on the plate):



Ligate adapters

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below: :

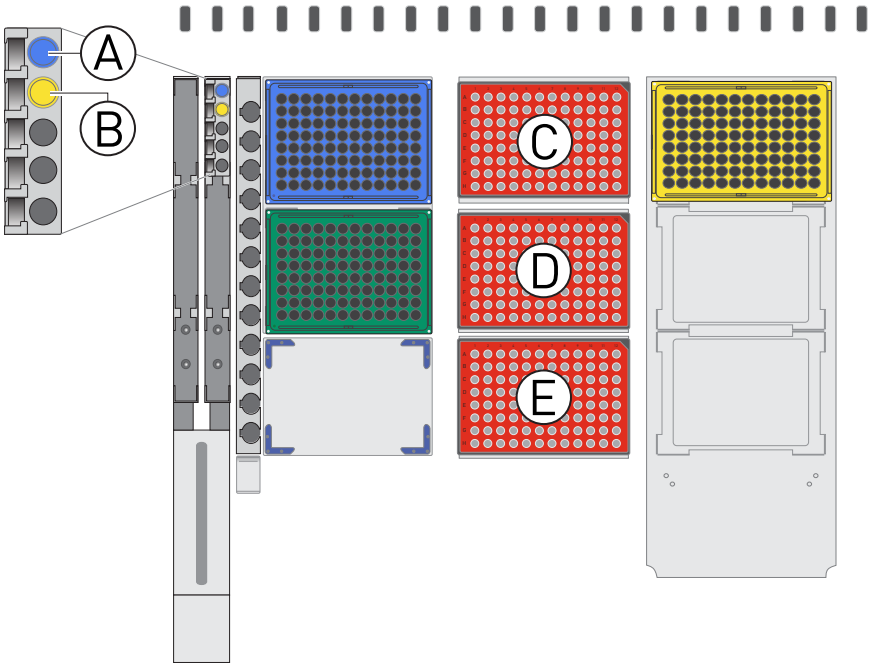


Table 6 Reagent setup summary: Ligate adapters

No.	Position	Description	Labware	Fill volume[1]
A	Grid 2, Site 1, Well 1 ***(Standard Transfer Only)***	Ligase (blue cap)	0.5-mL tube	240 µL[2]
B	Grid 2, Site 1, Well 2 ***(Standard Transfer Only)***	Switch solution (yellow cap)	0.5-mL tube	480 µL[2]
C	Grid 10, Site 1 ***(Fast Transfer Only)***	Ligase and Switch Solution	EnduraPlate™ reaction plate	Switch Solution: 80 µL/well in column 3
				Ligase: 40 µL/well in column 5
D	Grid 10, Site 2	Digested DNA	EnduraPlate™ reaction plate	From previous reaction
E	Grid 10, Site 3	Barcode Plate (5 µM each)	EnduraPlate™ reaction plate	8 µL/well

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.
[2] Use a full tube(s) from a 96-LV kit to accommodate dead volumes.

2. Add the following reagents according to the transfer mode selected:

Transfer Mode	Reagent Setup
Standard	1. Place the tube containing Switch Solution (yellow cap) in position 2 of the chilled metal cooling block at Grid 2, Site 1 (**A**). 2. Place the tube containing Ligase in position 1 of the chilled metal cooling block at Grid 2, Site 1 (**B**).
Fast	1. Aliquot Switch Solution (yellow cap) into all wells of column 3 and Ligase into all wells of column 5 in a MicroAmp® EnduraPlate™ reaction plate and place it on the incubator at Grid 10, Site 1 (**C**). This plate can be stored at -20°C for later use.

3. Carefully remove the plate seal from the amplified and partially digested DNA plate (from "Partially digest primer sequences" on page 16) and place it on the incubator at Grid 10, Site 2 (**D**).

4. Place the Barcode Plate with the diluted barcode adapter mix in the appropriate locations on the incubator at Grid 10, Site 3 (**E**).

5. Replace the tip boxes as needed.

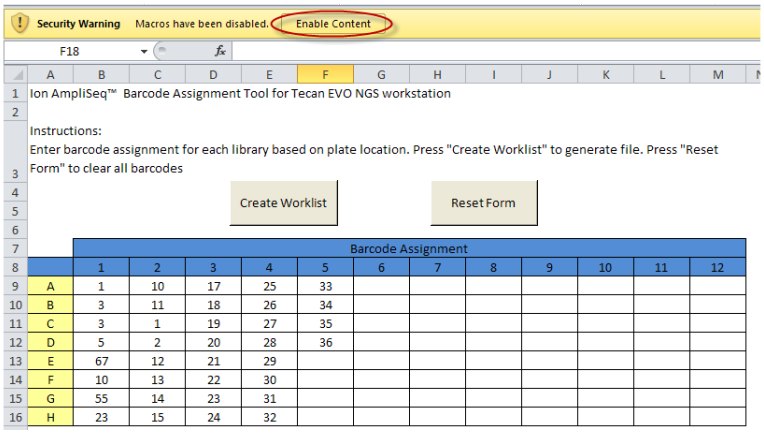
Barcode transfer

1. Download the file "Ion AmpliSeq Adapter Worklist PLATE.xlsm" from the Ion Community (**http://ioncommunity.lifetechnologies.com/docs/DOC-9140**).

2. Open the file and enable the macros.

3. For each reaction position, assign a barcode by typing a number (1–96) into the cell.

   Note: The instrument will always prepare libraries from top to bottom and left to right. The position on the plate map refers to the library position, and the number entered into each cell refers to the well number on the diluted barcode source plate. The example below assigns various barcodes for the preparation of 40 libraries:



	1	2	3	4	5	6	7	8	9	10	11	12
A	1	10	17	25	33							
B	3	11	18	26	34							
C	3	1	19	27	35							
D	5	2	20	28	36							
E	67	12	21	29								
F	10	13	22	30								
G	55	14	23	31								
H	23	15	24	32								

4. When completed, press the "Create Worklist" button and save the file to the desktop.

Run the script

1. Open EVOware.
2. Click **Run an existing script**.
3. Select the script "**Ligation**."
4. Enter the number of reactions to be prepared.
5. If you are using Fast Mode, enter "**1**" when prompted "**Enable Fast Transfer?**." Otherwise leave value as "**0**."
6. Click **RUN**.
   The run time is ~45 minutes (Standard) or 20 minutes (Fast) for 96 reactions.
7. When the script is complete, seal the plate with MicroAmp® Adhesive Film. Vortex the plate three times for 3 seconds each at setting 7–10, then centrifuge the plate briefly.
8. Place a MicroAmp® Compression Pad on the plate, load the plate in the thermal cycler, and run the following program.

Temperature	Time
22°C	30 min[1]
72°C	10 min
10°C	Hold (for up to 1 hour)

[1] For libraries from FFPE samples, 60 min may increase yield.

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

Option 1: Equalize the library

The Ion Library Equalizer™ Kit (Cat. no. 4482298) provides a method for normalizing library concentration at ~100 pM without the need for quantitation. First amplify the unamplified Ion AmpliSeq™ library, then capture the library on Equalizer™ Beads. After elution of the equalized library, proceed directly to combining libraries and/or template preparation.

Purify, elute, and amplify the library

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below:

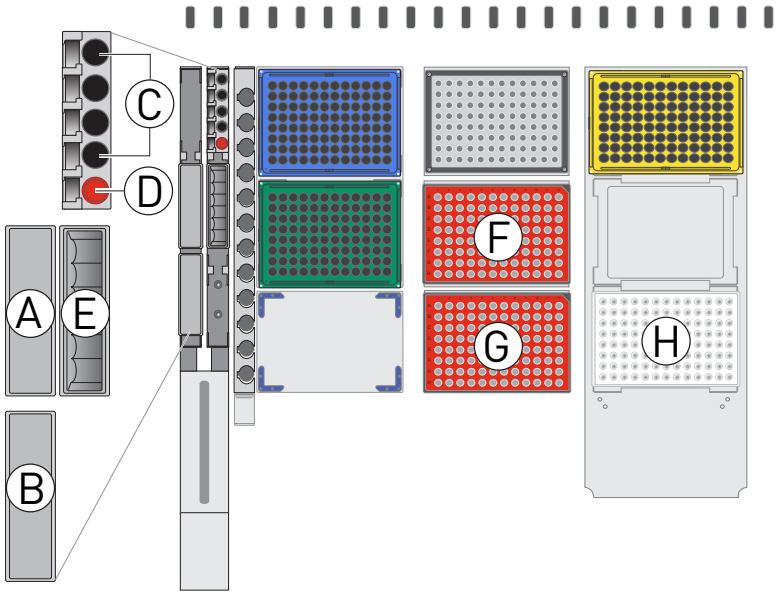


Table 7 Reagent setup summary: Purify, elute, and amplify the library

No.	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 2	70% Ethanol	100-mL reservoir	35 mL
B	Grid 1, Site 3	Waste	100-mL reservoir	Empty
C	Grid 2, Site 1, Wells 1–4	Platinum® PCR SuperMix HiFi (black cap)	2.0-mL tube	1.3 mL each[2]
D	Grid 2, Site 1, Well 5	Amplification Primers[3]	0.5-mL tube	240 µL [2]
E	Grid 2, Site 2	AMPure® XP Beads	25-mL reservoir	3.5 mL
F	Grid 10, Site 2	Amplification plate (output)	EnduraPlate™ reaction plate	Empty
G	Grid 10, Site 3	Ligated DNA plate (input)	EnduraPlate™ reaction plate	From previous reaction
H	Grid 16, Site 3	Plate magnet	DynaMag™ Side-96	n/a

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.

[2] Use a full tube(s) from a 96-LV kit to accommodate dead volumes.

[3] See the *Ion AmpliSeq™ DNA and RNA Library Preparation User Guide* (Pub. no. MAN0006735) for proper primer selection.

2. Fill a 100-mL trough with freshly prepared 70% ethanol and place it at Grid 1, Site 2 (**A**).

3. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).

4. Place the tubes containing Platinum® PCR SuperMix High Fidelity (black cap) in positions 1–4 of the chilled metal cooling block at Grid 2, Site 1 (**C**).

   Note: All four tubes are required.

5. Place the tube containing the appropriate Amplification Primers in position 5 of the chilled metal cooling block at Grid 2, Site 1 (**D**).

Primer tube label	Protocol compatibility
Equalizer™ Primers	Equalizer™ or Agilent® 2100 Bioanalyzer®/ Qubit® 2.0 Fluorometer
25X Library Amplification Primers	Equalizer™ or Agilent® 2100 Bioanalyzer®/ Qubit® 2.0 Fluorometer
Library Amplification Primer Mix	Agilent® 2100 Bioanalyzer®/ Qubit® 2.0 Fluorometer

6. Fill a 25-mL trough with AMPure® XP Beads and place it inside a 100-mL trough at Grid 2, Site 2 (**E**).

7. Place a new MicroAmp® EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**F**).

8. Place a MicroAmp® EnduraPlate™ reaction plate containing ligated DNA on the incubator at Grid 10, Site 3 (**G**).

9. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**H**).

10. Replace the tip boxes as needed.

Run the script

1. Open EVOware.

2. Click **Run an existing script**.

3. Select the script "**Library_Amp**."

4. Enter the number of reactions to be prepared.

5. Click **RUN**.
   The run time is ~85 minutes for 96 reactions.

6. When the script is complete, seal the plate with a MicroAmp® Adhesive Film, place a MicroAmp® Compression Pad on the plate, load the plate in the thermal cycler, and run the following program:

   Note: Wash the Equalizer™ Beads (described in "Wash the Equalizer™ beads" on page 24) while cycling, if necessary.

Stage	Temperature	Time
Hold	98°C	2 min
7 cycles	98°C	15 sec
	64°C	1 min
Hold	10°C	Hold (for up to 1 hour)

Before starting

Warm all the reagents in the Colon and Lung Kit Box 2 (4°C) to room temperature. Vortex and spin down all reagents before use.

Wash the Equalizer™ beads

If not previously performed:

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

   Note: Beads for multiple reactions may be prepared in bulk, and can be stored in Equalizer™ Wash Buffer at 4°C for up to 6 months until use. After 6 months, beads should be re-washed with an equal volume of Equalizer™ Wash Buffer.

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

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

4. Carefully remove and discard the supernatant without disturbing the pellet.

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

Add Equalizer™ beads and wash

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below:

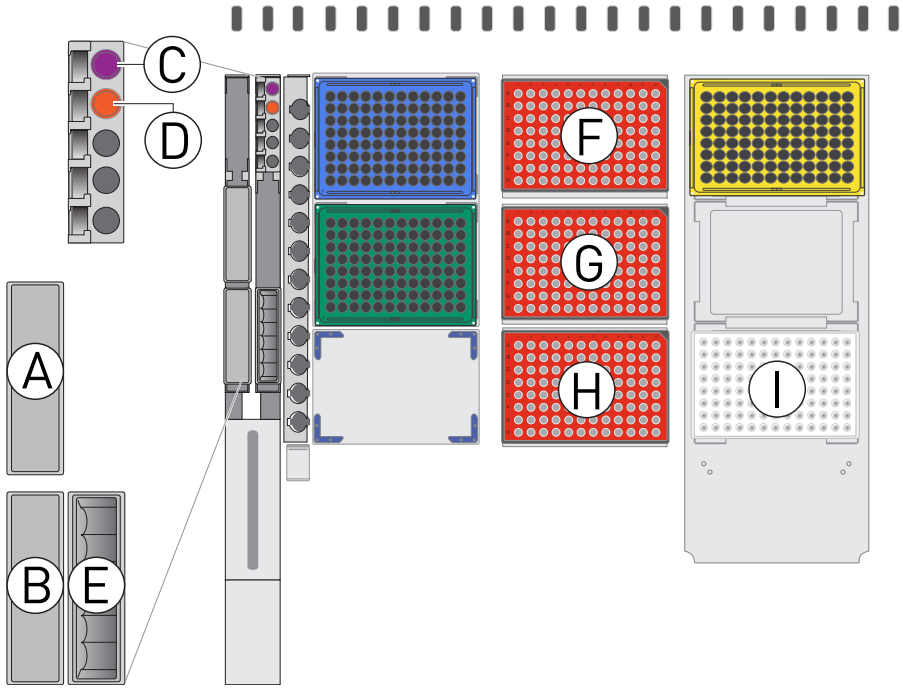


Table 8 Reagent setup summary: Add Equalizer™ Beads and wash

No.	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 2	Equalizer™ Wash Buffer	100-mL reservoir	35 mL[2]
B	Grid 1, Site 3	Waste	100-mL reservoir	Empty
C	Grid 2, Site 1, Well 1 ***(Standard Transfer Only)***	Equalizer™ Capture	2.0-mL tube	1.2 mL[2]
D	Grid 2, Site 1, Well 2 ***(Standard Transfer Only)***	Washed Equalizer™ Beads	1.5-mL tube	750 µL
E	Grid 2, Site 3	Equalizer™ Elution Buffer	25-mL reservoir	12 mL[2]
F	Grid 10, Site 1 ***(Fast Transfer Only)***	Equalizer™ Capture and Beads	EnduraPlate™ reaction plate	Capture: 150 µL/well in column 6 Washed Beads: 92 µL/well in column 8
G	Grid 10, Site 2	Equalizer™ Library (Output)	EnduraPlate™ reaction plate	Empty
H	Grid 10, Site 3	Amplified Library Plate (input)	EnduraPlate™ reaction plate	From previous reaction
I	Grid 16, Site 3	Plate Magnet	DynaMag™ Side-96	n/a

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.
[2] Use a full tube(s) from an Ion Library Equalizer™ Kit to accommodate dead volumes.

2. Add the following reagents according to the transfer mode selected:

Transfer Mode	Reagent Setup
Standard	1. Place the tube containing Equalizer™ Capture in position 1 of the chilled metal cooling block at Grid 2, Site 1 (**C**). 2. Place the tube containing washed Equalizer™ Beads in position 2 of the chilled metal cooling block at Grid 2, Site 1 (**D**).
Fast	1. Aliquot Equalizer™ Capture into all wells of column 6 and washed Equalizer™ Beads into all wells of column 8 in a MicroAmp® EnduraPlate™ reaction plate and place it on the incubator at Grid 10, Site 1 (**F**). This plate can be stored at 4°C for later use.

3. Fill a 100-mL trough with Equalizer™ Wash Buffer and place it at Grid 1, Site 2 (**A**).

4. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).

5. Fill 25-mL trough with Equalizer™ Elution Buffer and place it inside a 100-mL trough at Grid 2, Site 3 (**E**).

6. Place a new MicroAmp® EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**G**).

7. Place a MicroAmp® EnduraPlate™ reaction plate containing amplified library (from "Purify, elute, and amplify the library" on page 22) on the incubator at Grid 10, Site 3 (**H**).

8. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**I**).

9. Replace the tip boxes as needed.

Run the script

1. Open EVOware.

2. Click **Run an existing script**.

3. Select the script "**Equalizer**."

4. Enter the number of reactions to be prepared.

5. Click **RUN**.
   The run time is ~100 minutes (Standard) or 85 minutes (Fast) for 96 reactions.

6. When the script is complete, the plate at Grid 10, site 3 contains the Equalized library. Proceed immediately to template preparation, or combine and/or store the library as described in the *Ion AmpliSeq™ DNA and RNA Library Preparation User Guide* (Pub. no. MAN0006735).

   Note: The final concentration of each Equalized library is ~100 pM.

Store libraries

Libraries may be stored at 4–8°C for up to 1 month. For longer term storage, store at -20°C.

Option 2: Purify and quantify the unamplified library by qPCR

Purify and elute the unamplified Ion AmpliSeq™ library, then determine the concentration by qPCR with the Ion Library Quantitation Kit (Cat. no. 4468802). After quantitation, determine the dilution factor that results in a concentration of ~100 pM, which is suitable for template preparation using an Ion template kit.

Note: The Ion Library Quantitation Kit may also be used to quantify libraries that have been amplified using the procedure described in "Elute and amplify the library" on page 24.

Purify the unamplified library

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below:

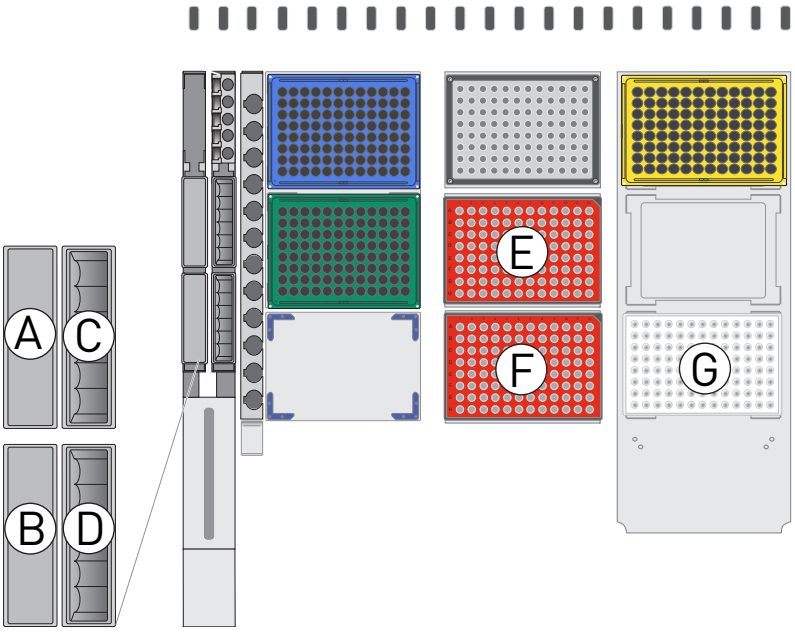


Table 9 Reagent setup summary: Purify the unamplified library

No.	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 2	70% Ethanol	100-mL reservoir	35 mL
B	Grid 1, Site 3	Waste	100-mL reservoir	Empty
C	Grid 2, Site 2	AMPure® XP Beads	25-mL reservoir	3.5 mL
D	Grid 2, Site 3	Low TE	25-mL reservoir	6 mL
E	Grid 10, Site 2	Finished library (output)	EnduraPlate™ reaction plate	Empty
F	Grid 10, Site 3	Ligated DNA plate (input)	EnduraPlate™ reaction plate	From previous reaction
G	Grid 16, Site 3	Plate Magnet	DynaMag™ Side-96	n/a

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.

2. Fill a 100-mL trough with freshly prepared 70% ethanol and place it at Grid 1, Site 2 (**A**).
3. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).
4. Fill a 25-mL trough with AMPure® XP Beads and place it at Grid 2, Site 2 (**C**).
5. Fill a 25-mL trough with Low TE and place it at Grid 2, Site 3 (**D**).
6. Place a new MicroAmp® EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**E**).
7. Place a MicroAmp® EnduraPlate™ reaction plate containing ligated DNA (from "Ligate adapters" on page 19) on the incubator at Grid 10, Site 3 (**F**).
8. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**G**).
9. Replace the tip boxes as needed.

Run the script

1. Open EVOware.
2. Click **Run an existing script**.
3. Select the script "**qPCR_Purification**."
4. Enter the number of reactions to be prepared.
5. Click **RUN**.
   The run time is ~85 minutes for 96 reactions.
6. When the script is complete, the plate at Grid 10, Site 2 contains the purified library. Prepare a 100-fold dilution of the library by removing 2 µL of supernatant and combining with 198 µL of Nuclease-Free Water for quantitation.
7. Proceed to "Quantify library by qPCR and calculate dilution factor," in the *Ion AmpliSeq™ DNA and RNA Library Preparation Guide* (Pub. no. MAN0006735).

Option 3: Quantify the amplified library with Qubit® 2.0 Fluorometer or Agilent® 2100 Bioanalyzer®

Ion AmpliSeq™ libraries must be amplified prior to quantitation with the Qubit® 2.0 Fluorometer or Agilent® 2100 Bioanalyzer®.

Note: Library amplification is required for this method, to enrich amplifiable material and obtain sufficient material for accurate quantification using these instruments. The Ion Library Quantitation Kit may also be used to quantify amplified libraries.

Amplify the library

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by-step instructions provided below:

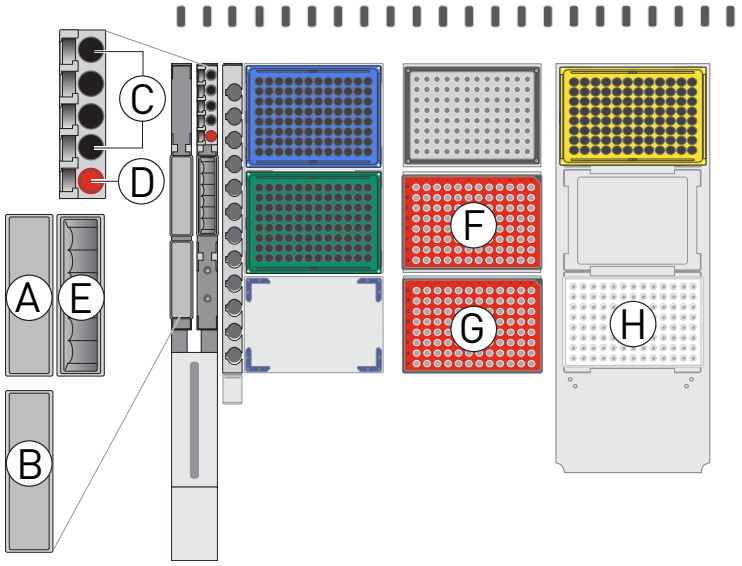


Table 10 Reagent setup summary: Amplify the library

No.	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 2	70% Ethanol	100-mL reservoir	35 mL
B	Grid 1, Site 3	Waste	100-mL reservoir	Empty
C	Grid 2, Site 1, Wells 1–4	Platinum® PCR SuperMix HiFi (black cap)	2.0-mL tube	8 mL
D	Grid 2, Site 1, Well 5	Amplification Primers[2]	0.5-mL tube	6 mL
E	Grid 2, Site 2	AMPure® XP Beads	25-mL reservoir	Empty
F	Grid 10, Site 2	Amplification plate (output)	EnduraPlate™ reaction plate	From previous reaction
G	Grid 10, Site 3	Ligated DNA plate (input)	EnduraPlate™ reaction plate on MicroAmp® Splash-free 96-well Base	Empty
H	Grid 16, Site 3	Plate magnet	DynaMag™ Side-96	n/a

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.
[2] See the *Ion AmpliSeq™ DNA and RNA Library Preparation User Guide* (Pub. no. MAN0006735) for proper primer selection.

2. Fill a 100-mL trough with freshly prepared 70% ethanol and place it at Grid 1, Site 2 (**A**).

3. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).

4. Place the tubes containing Platinum® PCR SuperMix HiFi in positions 1–4 of the chilled metal cooling block at Grid 2, Site 1 (**C**).

   Note: All four tubes are required.

5. Place the tube containing the appropriate library Amplification Primers in position 5 of the chilled metal cooling block at Grid 2, Site 1 (**D**).

Primer tube label	Protocol compatibility
Equalizer™ Primers	Equalizer™ or Agilent® 2100 Bioanalyzer®/ Qubit® 2.0 Fluorometer
25X Library Amplification Primers	Equalizer™ or Agilent® 2100 Bioanalyzer®/ Qubit® 2.0 Fluorometer
Library Amplification Primer Mix	Agilent® 2100 Bioanalyzer®/ Qubit® 2.0 Fluorometer

6. Fill a 25-mL trough with AMPure® XP Beads and place it inside a 100-mL trough at Grid 2, Site 2 (**E**).

7. Place a new MicroAmp® EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**F**).

8. Place a MicroAmp® EnduraPlate™ reaction plate containing ligated DNA on the incubator at Grid 10, Site 3 (**G**).

9. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**H**).

10. Replace the tips boxes as needed.

Run the script

1. Open EVOware.

2. Click **Run an existing script**.

3. Select the script "**Library_Amp**."

4. Enter the number of reactions to be prepared.

5. Click **RUN**.
   The run time is ~85 minutes for 96 reactions.

6. When the script is complete, seal the plate with a MicroAmp® Adhesive Film.

7. Place a MicroAmp® Compression Pad on the plate, load the plate in the thermal cycler and run the following program:

Stage	Temperature	Time
Hold	98°C	2 min
5 cycles	98°C	15 sec
	64°C	1 min
Hold	10°C	Hold

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

Purify the amplified library

Setup the worktable

1. Setup the worktable as shown. For more details, see the step-by step instructions provided below:

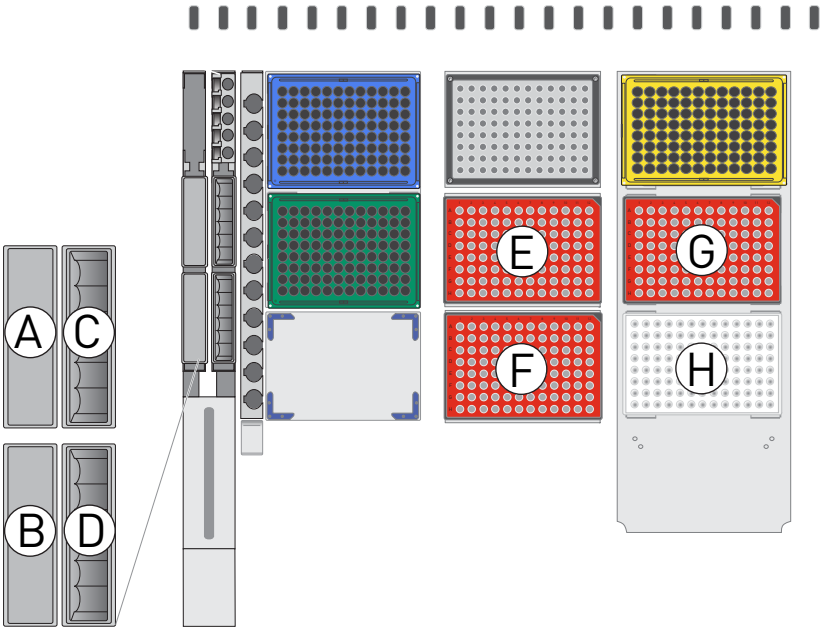


Table 11 Reagent setup summary: Purify the amplified library

No.	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 2	70% Ethanol	100-mL reservoir	35 mL
B	Grid 1, Site 3	Waste	100-mL reservoir	Empty
C	Grid 2, Site 2	AMPure® XP Beads	25-mL reservoir	8 mL
D	Grid 2, Site 3	Low TE	25-mL reservoir	6 mL
E	Grid 10, Site 2	Finished library (output)	EnduraPlate™ reaction plate	Empty
F	Grid 10, Site 3	Amplified library (input)	EnduraPlate™ reaction plate	From previous reaction
G	Grid16, Site 2	Clean-up plate	EnduraPlate™ reaction plate on MicroAmp® Splash-Free 96-Well Base	Empty
H	Grid 16, Site 3	Plate magnet	DynaMag™ Side-96	n/a

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.

2. Fill a 100-mL trough with freshly prepared 70% ethanol and place it at Grid 1, Site 2 (**A**).
3. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).
4. Fill a 25-mL trough with AMPure® XP Beads and place it inside a 100-mL trough at Grid 2, Site 2 (**C**).

5. Fill at 25-mL trough with Low TE and place it inside a 100-mL trough at Grid 2, Site 3 (**D**).
6. Place a new MicroAmp® EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**E**).
7. Place a MicroAmp® EnduraPlate™ reaction plate containing amplified DNA (from "Amplify the library" on page 30) on the incubator at Grid 10, Site 3 (**F**).
8. Place a new MicroAmp® EnduraPlate™ on a MicroAmp® Splash-Free 96-Well Base at Grid 16, Site 2 (**G**).
9. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**H**).
10. Replace the tip boxes as needed.

Run the script

1. Open EVOware.
2. Click **Run an existing script**.
3. Select the script "**Library_Amp_Purification**."
4. Enter the number of reactions to be prepared.
5. Click **RUN**.
   The run time is ~85 minutes for 96 reactions.
6. Once the script is complete, the plate at Grid 10, Site 2 contains the purified library.

Quantify the library using the Qubit® 2.0 Fluorometer

Analyze 10 µL of each amplified library using the Qubit® 2.0 Fluorometer (Cat. no. Q32866) and the Qubit® dsDNA HS Assay Kit. Amplified libraries typically have concentrations of 300–1500 ng/mL. For more information, see the *Qubit® dsDNA HS Assay Kits User Guide.* (Pub. no. MAN0002326)

1. Determine the amplified library concentration:
   a. Make a 1:200 working dilution of Qubit® dsDNA HS reagent using the Qubit® dsDNA HS Buffer.
   b. Combine 10 µL of the amplified Ion AmpliSeq™ library with 190 µL of dye reagent, mix well, and incubate for at least 2 minutes.
   c. Prepare each Qubit® standard as directed in the user guide.
   d. Measure the concentration on the Qubit® 2.0 Fluorometer.

e. Calculate the concentration of the undiluted library by multiplying by 20. This can be calculated automatically using the "Calculate Stock Concentration" button and inputting 10 µL as the sample volume.

2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM (15 ng/ml for amplicons up to 225 bp, 22 ng/ml for amplicons up to 275 bp)
   For example, with a FFPE-compatible 125–175 bp design:
   - The library concentration is 450 ng/mL.
   - The dilution factor is 450 ng/mL divided by 15 ng/mL = 30.
   - Therefore, 10 µL of library mixed with 290 µL of Low TE (1:30 dilution) yields approximately 15 ng/mL (~100 pM).

3. Dilute library to ~100 pM as described and proceed to combining libraries or template preparation.

Quantify the library using Agilent® 2100 Bioanalyzer® Instrument

Analyze 1 µL of amplified library on the Agilent® 2100 Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit (Cat. no. 5067-4626). Amplicon libraries should have multiple peaks in the 125–300 bp size range. Amplified libraries typically have concentrations of 1,000–5,000 pM. If the library concentration is over 20,000 pM, dilute the library 1:10 and repeat the quantification to obtain a more accurate measurement.

1. Determine the molar concentration of the amplified library using the Bioanalyzer® software. Ensure that the upper and lower marker peaks are identified and assigned correctly. Follow the manufacturer's instructions to perform a region analysis (smear analysis). Briefly:
   a. Select the **Data** icon in the Contexts panel, and view the electropherogram of the sample to be quantified.

   b. Select the **Region Table** tab below, and create a region spanning the desired amplicon peaks. Correct the baseline if needed.

   c. The molarity is automatically calculated and displayed in the table in pmol/L (pM).

2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.
   For example:
   - The library concentration is 3000 pM.
   - The dilution factor is 3000 pM/100 pM = 30.
   - Therefore, 10 µL of library mixed with 290 µL of Low TE (1:30 dilution) yields approximately 100 pM.

3. Dilute library to ~100 pM as described and proceed to combining libraries or template preparation.

(Optional) Combine amplicon libraries

There are multiple strategies for combining Ion AmpliSeq™ libraries, as described in *Ion AmpliSeq™ DNA and RNA Library Preparation User Guide* (Pub. no. MAN0006735).

Store libraries

Libraries may be stored at 4–8°C for up to 1 month. For longer term storage, store at -20°C.

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  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Ion contact information

Web site: lifetechnologies.com/iontorrent

Ion community: ioncommunity.lifetechnologies.com

Support email: ionsupport@lifetech.com

Phone numbers
In North America: 1-87-SEQUENCE (1-877-378-3623)
Outside of North America: +1-203-458-8552

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30 March 2015

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