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

USER BULLETIN

Publication Number MAN0010822

Revision E.0

ThermoFisher SCIENTIFIC

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Revision history: Pub. No. MAN0010822

Revision	Date	Description of Change
E.0	27 August 2020	• Added Ion Torrent™ NGS Reverse Transcription Kit to “Required materials not supplied” on page 10. • Updated the script file name in “Import the script file” on page 13. • Added a note in “Reverse transcribe RNA” on page 15. • Updated instructions to accommodate the use of Ion Torrent™ NGS Reverse Transcription Kit in “Set up the worktable” on page 15. • Added thermal cycler conditions specific to the Ion Torrent™ NGS Reverse Transcription Kit in “Reverse transcribe RNA” on page 17. • Updated instructions and figures to accommodate the 2-pool protocol in “Set up the worktable” on page 18, page 21, and page 25. • Updated the script selections in “Run the script” on page 19, page 23, and page 26. • Updated thermal cycler settings in “Ligate adapters” on page 32.
D.0	07 April 2020	• Updated “Ion AmpliSeq™ Library Kits” on page 7 and “Required materials not supplied” on page 10. • Added references to Ion AmpliSeq™ Library Kit Plus and *Ion AmpliSeq™ Library Kit Plus User Guide*. • Removed references to Ion Community. • Added reference to Support for obtaining script and plate files (see “Import the script file” on page 13 and “Barcode transfer” on page 30). • Removed the informational note about the number of primer pairs per pool for specific *ready-to-use* panels in “Amplify the targets” on page 24. The number of primer pairs per pool for specific *ready-to-use* panels are listed in the respective user guides. • Added “Recommended reagent substitutions for calibration” on page 14. • Re-organized the procedural guidelines and methods for ease of use.
C.0	02 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: • “Amplify the library” on page 33 • “Add Equalizer™ beads, wash, and elute the equalized library” on page 36 • “Amplify the library” on page 41
A.0	29 August 2014	• First release

Contents

Methods

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Protocol information

Description

This user bulletin describes how to prepare the Ion AmpliSeq™ libraries using Tecan™ Freedom EVO™ NGS Workstation. The workflow for library preparation described in this bulletin is similar to the Ion AmpliSeq™ Library Kit 2.0 or Ion AmpliSeq™ Library Kit Plus workflow. Additional steps to set up the Tecan™ Freedom EVO™ NGS Workstation and import and run the scripts are described. For more information, see the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) or *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

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 barcode adapters 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.

Kit	Requirements
Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4480441 (96 reactions), or Cat. No. 4480442 (384 reactions)) *or* Ion AmpliSeq™ Library Kit Plus[1] (Cat. No. A35907 (96 reactions), or Cat. No. A38875 (384 reactions))	Each kit contains reagents for the rapid preparation of either 96 or 384 libraries containing 12–24,576 amplicons per reaction. Due to higher dead volume requirements for library preparation using the automated platform, you will need more than one kit to set up 96 or 384 reactions. Each kit uses a 96-well plate-based protocol for easy sample handling and tracking, and for compatibility with automation and high-throughput laboratories.

(continued)

Kit	Requirements
Ion Library Equalizer™ Kit (Cat. No. 4482298)	This kit provides an optional streamlined method for normalizing library concentration without the need for quantification.
Ion Xpress™ Barcode Adapters Kit (Cat. No. 4474517)[2]	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).

[1] Cat. No. 4488990 is also supported for preparing <24 samples.

[2] Other kit configurations are available (see "Ion Xpress™ Barcode Adapters Kits" on page 9).

Kits used in this protocol

Ion AmpliSeq™ Library Kits

Use Ion AmpliSeq™ Library Kit 2.0 or Ion AmpliSeq™ Library Kit Plus for library preparation. If preparing the maximum number of libraries per kit, multiple kits may be required to accommodate for higher dead volume requirements that are associated with the automated platform.

Ion AmpliSeq™ Library Kit 2.0 (96 or 384 reactions)

The Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4480441) provides reagents for preparing 96 libraries for 1- or 2-pool panels (64 libraries for 3-pool panels, and 48 libraries for 4-pool panels).

The Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4480442) provides reagents for preparing 384 libraries for 1- or 2-pool panels (256 libraries for 3-pool panels, and 192 libraries for 4-pool panels).

Contents	Amount		Storage
	Cat. No. 4480441 (96 reactions)	Cat. No. 4480442 (384 reactions)	
5X Ion AmpliSeq™ HiFi Mix (red cap)	480 µL	4 × 480 µL	–30°C to –10°C
FuPa Reagent (brown cap)	192 µL	4 × 192 µL	
Switch Solution (yellow cap)	384 µL	4 × 384 µL	
DNA Ligase (blue cap)	192 µL	4 × 192 µL	
Ion AmpliSeq™ Adapters (green cap)	192 µL	4 × 192 µL	
Platinum™ PCR SuperMix High Fidelity (black cap)	3 × 1.6 mL	12 × 1.6 mL	
Library Amplification Primer Mix (white cap)	192 µL	4 × 192 µL	
Low TE	2 × 6 mL	8 × 6 mL	15°C to 30°C[1]

[1] Can be stored at –30°C to –10°C for convenience.

Ion AmpliSeq™ Library Kit Plus (96 or 384 reactions)

The Ion AmpliSeq™ Library Kit Plus (Cat. No. A35907) provides reagents for manually preparing 96 libraries for 1- or 2-pool panels (64 libraries for 3-pool panels or 48 libraries for 4-pool panels).

The Ion AmpliSeq™ Library Kit Plus (Cat. No. A38875) provides reagents for manually preparing 384 libraries for 1- or 2-pool panels (256 libraries for 3-pool panels, and 192 libraries for 4-pool panels).

Note: 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.

Component	Amount		Storage
	Cat. No. A35907 (96 reactions)	Cat. No. A38875 (384 reactions)	
5X Ion AmpliSeq™ HiFi Mix (red cap)	480 µL	4 × 480 µL	–30ºC to –10ºC
FuPa Reagent (brown cap)	192 µL	4 × 192 µL	
Switch Solution (yellow cap)	384 µL	4 × 384 µL	
DNA Ligase (blue cap)	192 µL	4 × 192 µL	
25X Library Amp Primers (pink cap)	192 µL	4 × 192 µL	
1X Library Amp Mix (black cap)	4 × 1.2 mL	16 × 1.2 mL	
Low TE	2 × 6 mL	8 × 6 mL	15°C to 30°C[1]

[1] Can be stored at –30ºC to –10ºC.

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit (Cat. No. 4482298) provides an optional, streamlined method for normalizing library concentration without quantification. This kit should be used when library yields are consistently above the minimum expected concentration.

The Ion Library Equalizer™ Kit contains reagents sufficient for 96 libraries.

Component	Amount	Storage
Equalizer™ Primers (pink cap)	200 µL	2ºC to 8ºC
Equalizer™ Capture (purple cap)	1 mL	
Equalizer™ Elution Buffer (clear cap)	10 mL	
Equalizer™ Beads (orange cap)	300 µL	
Equalizer™ Wash Buffer (clear cap)	35 mL	15–30°C[1]

[1] Can be stored at 2–8°C.

Ion Xpress™ Barcode Adapters Kits

Each kit provides 16 different barcode adapters, sufficient for ~640 Ion AmpliSeq™ libraries. Ion Xpress™ Barcode Adapters, IonCode™ Barcode Adapters, or Ion Torrent™ Dual Barcode Adapters are required to run multiple libraries per sequencing chip, and are ordered separately.

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 tube per barcode)	20 µL each	

The following Ion Xpress™ Barcode Adapters Kits are available:

- Ion Xpress™ Barcode Adapters 1–16 Kit (Cat. No. 4471250)
- Ion Xpress™ Barcode Adapters 17–32 Kit (Cat. No. 4474009)
- Ion Xpress™ Barcode Adapters 33–48 Kit (Cat. No. 4474518)
- Ion Xpress™ Barcode Adapters 49–64 Kit (Cat. No. 4474519)
- Ion Xpress™ Barcode Adapters 65–80 Kit (Cat. No. 4474520)
- Ion Xpress™ Barcode Adapters 81–96 Kit (Cat. No. 4474521)
- *(Complete set)* Ion Xpress™ Barcode Adapters 1–96 Kit (Cat. No. 4474517)

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
Instruments and Equipment	
Tecan™ Freedom EVO™ NGS Workstation	**www.tecan.com/NGS**
One of the following thermal cyclers: • GeneAmp™ PCR System 9700[1] or GeneAmp™ PCR System 9700 96-Well[1] • 2720 Thermal Cycler[1] • Veriti™ 96-Well Thermal Cycler • ProFlex™ 96-well PCR System	See web product pages
MicroAmp™ Splash-Free 96-Well Base	4312063
MicroAmp™ Optical Film Compression Pad	4312639
DynaMag™–96 Side Magnet, or other plate magnet	12331D
96-well plate centrifuge	MLS
Reagents and consumables	
Tecan™ Pure LiHa Disposable Tips, Filtered, 50-, 200-, and 1000-µL	30057813, 30057815, 30057817 (Tecan™)
Disposable Reagent Troughs, 25-mL and 100-mL	10613102, 10613048 (Tecan™)
Eppendorf™ Deepwell Plates, 96-well, 500 µL	951031801 (Eppendorf™)
MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode	4483354, 4483352
MicroAmp™ Clear Adhesive Film	4306311
Agencourt™ AMPure™ XP Reagent	A63880, A63881, or A63882 (Beckman Coulter™)
Nuclease-free Water	AM9932
Absolute ethanol	MLS
One or more of the following kits for nucleic acid isolation and quantification	
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
MagMAX™ FFPE DNA/RNA Ultra Kit	A31881
PureLink™ Genomic DNA Mini Kit	K1820-00

(continued)

Item	Source
(RNA only) SuperScript™ VILO™ cDNA Synthesis Kit *or* Ion Torrent™ NGS Reverse Transcription Kit	11754250 A45003
(Recommended for DNA quantification) TaqMan™ RNase P Detection Reagents Kit	4316831
(Recommended for RNA quantification) Qubit™ RNA HS Assay Kit	Q32852 or Q32855
One or more of the following kits for library preparation and quantification	
Ion AmpliSeq™ Library Kit 2.0	4480441 (96 reactions) or 4480442 (384 reactions)
Ion AmpliSeq™ Library Kit Plus[2]	A35907 (96 reactions) or A38875 (384 reactions)
Ion Xpress™ Barcode Adapters Kit	Various[3]
Ion Library TaqMan™ Quantitation Kit	4468802
Ion Library Equalizer™ Kit	4482298
If you are not using the Ion Library Equalizer™ Kit for library normalization, select one of the following kits:	
Qubit™ Fluorometer[4] and Qubit™ dsDNA HS Assay Kit	Q33226, Q32851 or Q32854
Agilent™ 2100 Bioanalyzer™ and Agilent™ High Sensitivity DNA Kit	G2939BA, 5067-4626 (Agilent™)

[1] Supported but no longer available for purchase.
[2] Cat. No. 4488990 is also supported for preparing <24 samples.
[3] For Cat. Nos., see "Ion Xpress™ Barcode Adapters Kits" on page 9.
[4] Qubit™ 2.0 Fluorometer or later

Procedural guidelines

Guidelines for RNA isolation, quantification, and input

- For recommended kits for isolating RNA, see “Required materials not supplied” on page 10.
- Each reverse transcription reaction requires 1–100 ng of DNase-treated RNA (≥0.14 ng/µL), prepared from normal or formaldehyde- or paraformaldehyde-fixed paraffin-embedded (FFPE) tissue.
- For quantifying RNA, we recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32852 or Q32855).
- In general, the library yield from high-quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.
- Having more RNA starting material generally results in higher quality libraries. However, if RNA is not degraded, high-quality libraries can be generated from as little as 1 ng starting material.

Guidelines for DNA isolation, quantification, and input

- For recommended kits for isolating DNA, see “Required materials not supplied” on page 10.
- We recommend the TaqMan™ RNase P Detection Reagents Kit (Cat. No. 4316831) or Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) for quantification of amplifiable human genomic DNA (gDNA).
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- For DNA isolated from formaldehyde- or paraformaldehyde-fixed paraffin-embedded (FFPE) tissue samples, use an FFPE-compatible Ion AmpliSeq™ panel. Standard designs with longer amplicons can perform poorly with FFPE DNA.
- Each target amplification reaction requires 10 ng of 2 ng/µL gDNA or FFPE DNA. The instrument requires at least 8 µL for each reaction due to dead volume. If sample volumes are low, dilute samples to bring up the volume and increase the recommended cycle number by 1 when you amplify the targets (see “Amplify the targets” on page 24).

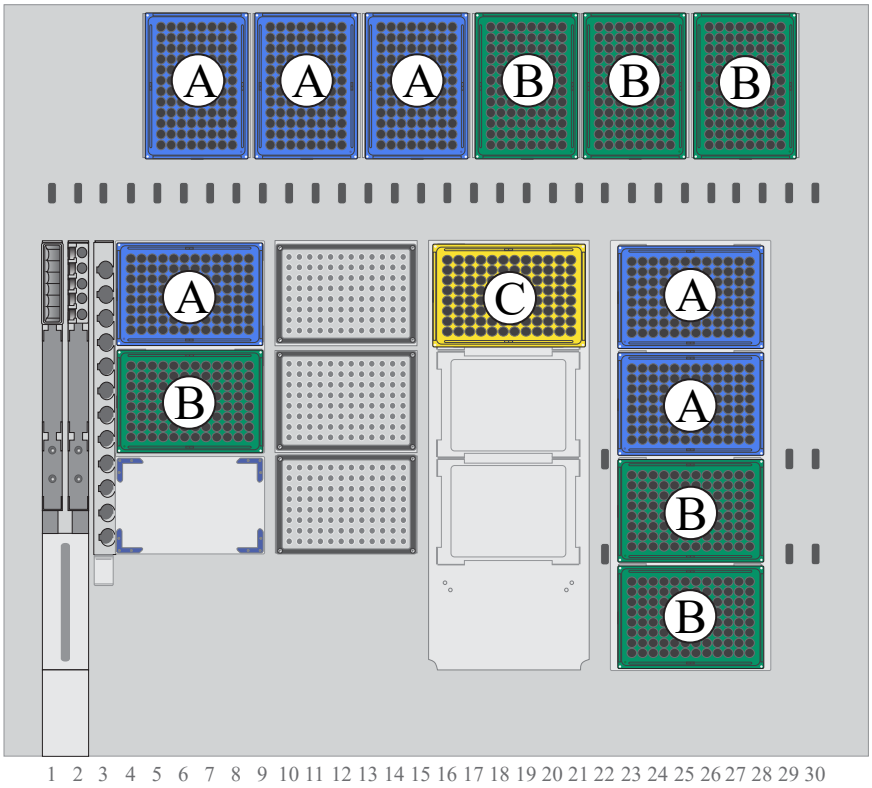
Using the Tecan™ Freedom EVO™ NGS Workstation

Import the script file

1. Contact Technical Support or your support representative to obtain the `AmpliSeq_Tecan_1or2pool.exd` script file (see “Customer and technical support” on page 48).
2. Open the **Tecan™ Export Import** tool.
3. Click **File** ▸ **Load** ▸ **Import file**.
4. Select **AmpliSeq_Tecan_1or2pool.exd**, then click **Open**.
5. Click **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 are only required to replace tips when all six boxes are empty. Alternatively, at the start of a run you can replace all tip boxes, then enter a value of ***1*** when prompted with **New tips boxes?** dialog. This action resets the tip count. The 1000-µL tips are handled separately and replaced as needed.



Label	Tip type	Location
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

Recommended reagent substitutions for calibration

Use the following reagent substitutions during initial setup of the Tecan™ Freedom EVO™ NGS Workstation for calibration. You can also use the recommended reagent substitutions for troubleshooting of any performance problems and inconsistencies.

Reagent	Recommended substitution
DNA	Water
Primers	
5X Ion AmpliSeq™ HiFi Mix	40% glycerol solution in water
FuPa Reagent	50% glycerol solution in water
DNA Ligase	
Switch Solution	20% Polyethylene Glycol 8000 (PEG-8000) solution in water
Agencourt™ AMPure™ XP Reagent	

Set up cDNA target amplification reactions

Reverse transcribe RNA

IMPORTANT! SuperScript™ VILO™ cDNA Synthesis Kit and NGS Reverse Transcription Kit are not interchangeable, buffer and enzyme mixes must be from a single kit type.

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

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

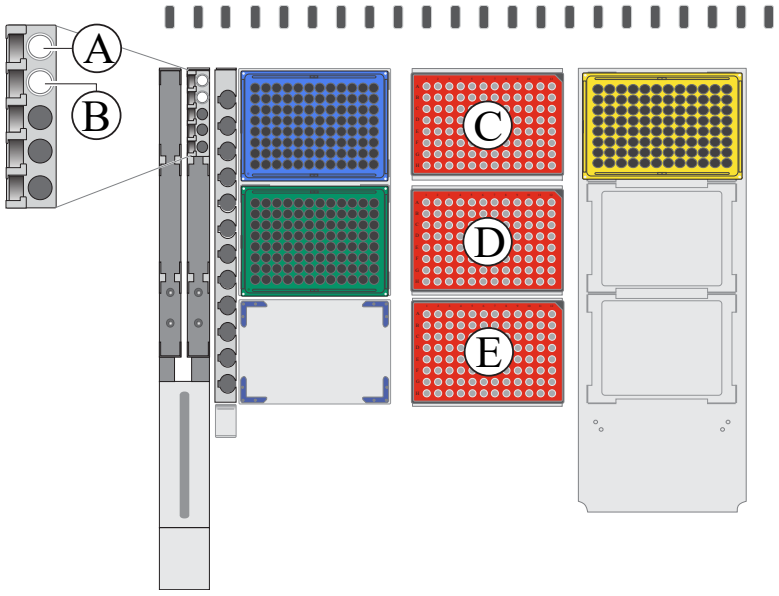


Table 2 Reagent setup summary: Reverse transcribe RNA

Label	Position	Description	Labware	Fill volume[1]
A	Grid 2, Site 1, Well 1 ***(Standard Transfer Only)***	10X SuperScript™ Enzyme Mix *or* 10X RT Enzyme Mix	1.5-mL tube[2]	120 µL[1]
B	Grid 2, Site 1, Well 2 ***(Standard Transfer Only)***	5X VILO™ Reaction Mix *or* 5X Reaction Buffer	1.5-mL tube[2]	240 µL[1]
C	Grid 10, Site 1 ***(Fast Transfer Only)***	10X SuperScript™ Enzyme Mix *or* 10X RT Enzyme Mix	MicroAmp™ EnduraPlate™ reaction plate	10X SuperScript™ Enzyme Mix *or* 10X RT Enzyme Mix: 20 µL/well in column 2

Table 2 Reagent setup summary: Reverse transcribe RNA *(continued)*

Label	Position	Description	Labware	Fill volume[1]
C	Grid 10, Site 1 ***(Fast Transfer Only)***	5X VILO™ Reaction Mix *or* 5X Reaction Buffer	MicroAmp™ EnduraPlate™ reaction plate	5X VILO™ Reaction Mix *or* 5X Reaction Buffer: 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 multiple tubes in a 1.5-mL tube to obtain the full volume.

1. Add the following reagents according to the transfer mode that you selected.

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

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

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **RNA_RT** script.
4. Enter the number of reactions to be prepared.

5. If you are using **Fast Mode**, enter ***1*** when prompted to **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™ Clear Adhesive Film. Vortex the plate 3 times for 3 seconds each time at setting 7–10, then centrifuge the plate briefly.

Reverse transcribe RNA

Place a MicroAmp™ Optical Film Compression Pad on the plate, load it in the thermal cycler, then run one the following programs depending on the kit that was used to set up the reverse transcription reactions.

Table 3 SuperScript™ VILO™ cDNA Synthesis Kit conditions

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

Table 4 NGS Reverse Transcription Kit conditions

Temperature	Time
25°C	10 min
50°C	10 min
85°C	5 min
10°C	Hold

STOPPING POINT Samples can be stored at 10°C for up to 16 hours in the thermal cycler. For longer term, store at –20°C.

Set up cDNA target amplification reactions

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

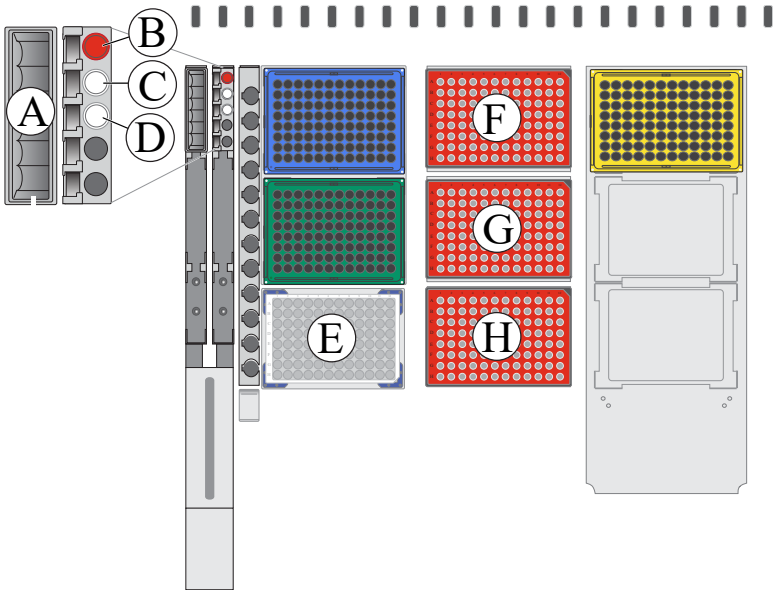


Table 5 Reagent setup summary: Amplify cDNA

Label	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)	1.5-mL tube[2]	500 µL[3]
C	Grid 2, Site 1, Well 2	5X RNA Primer Panel 1	1.5-mL tube	1-pool: 500 µL 2-pool: 300 µL
D	Grid 2, Site 1, Well 3	5X RNA Primer Panel 2[4]	1.5-mL tube	300 µL
E	Grid 4, Site 3	Deepwell plate	Eppendorf™ Deepwell plate 96/500	Empty
F	Grid 10, Site 1	cDNA plate	EnduraPlate™ reaction plate	From previous reaction

Table 5 Reagent setup summary: Amplify cDNA *(continued)*

Label	Position	Description	Labware	Fill volume[1]
G	Grid 10, Site 2	Amplification plate (Panel 1)	EnduraPlate™ reaction plate	Empty
H	Grid 10, Site 3	Amplification plate (Panel 2)[4]	EnduraPlate™ reaction plate	Empty

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

[2] Transfer reagent to a 1.5-mL tube if provided in a 0.5-mL tube.

[3] 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.

[4] Optional for 2-panel designs.

1. Fill a 25-mL trough with Nuclease-Free Water, then place the 25-mL trough inside a 100-mL trough at Grid 1, Site 1 (**A**).
2. 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**).
3. Place the tube containing 5X Ion AmpliSeq™ RNA panel 1 in position 2 of the chilled metal cooling block at Grid 2, Site 1 (**C**).
4. *(Optional)* Place the tube containing 5X Ion AmpliSeq™ RNA panel 2 in position 3 of the chilled metal cooling block at Grid 2, Site 1 (**D**).
5. Place a 500-µL plate onto the shaking incubator at Grid 4, Site 3 (**E**).
6. Place the MicroAmp™ EnduraPlate™ reaction plate that contains the cDNA from the previous reaction on the incubator at Grid 10, Site 1 (**F**).
7. Place an empty MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**G**)
8. *(Optional)* Place an empty MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 3 (**H**).
9. Replace the tip boxes as needed.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Target_Amp_1or2Pool** script.
4. Enter the number of reactions to be prepared.
5. Enter ***0*** for RNA panel.
6. Enter the number of panels for the library being prepared.

7. Click **RUN**.
   The run time is ~10 minutes for 1-pool panels or ~20 minutes for 2-pool panels.

8. When the script is complete, seal the plate with MicroAmp™ Clear Adhesive Film, then centrifuge to collect the droplets.

9. Proceed immediately to “Amplify the targets” on page 24.

Set up DNA target amplification reactions

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

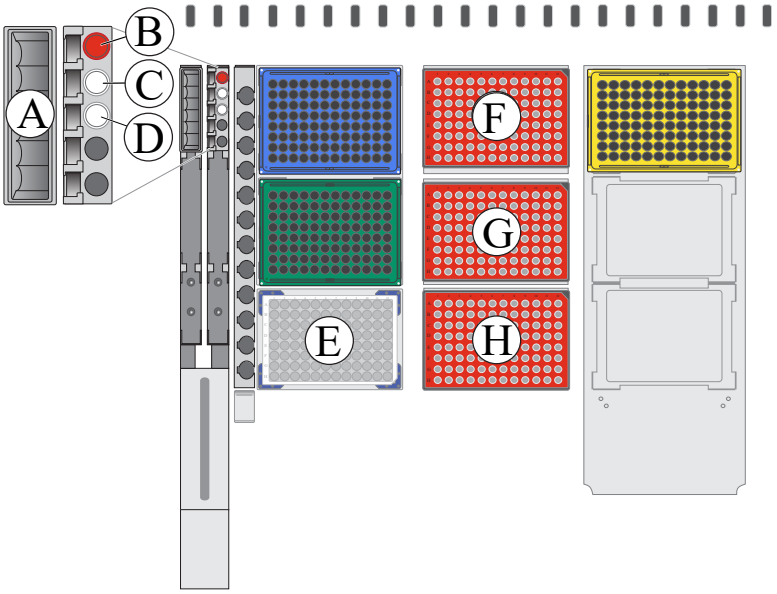


Table 6 Reagent setup summary: Amplify DNA

Label	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 Ion AmpliSeq™ HiFi Mix (red cap)	1.5-mL tube[2]	500 µL[3]
C	Grid 2, Site 1, Well 2	Primer Pool 1 (diluted to 2X)	1.5-mL tube	1-pool: 1.2 mL 2-pool: 600 µL
D	Grid 2, Site 1, Well 3	Primer Pool 2 (diluted to 2X)	1.5-mL tube	600 µL
E	Grid 4, Site 3	Deepwell plate	Eppendorf™ Deepwell plate 96/500	Empty
F	Grid 10, Site 1	gDNA	EnduraPlate™ reaction plate	2 ng/µL, 8 µL

Table 6 Reagent setup summary: Amplify DNA *(continued)*

Label	Position	Description	Labware	Fill volume[1]
G	Grid 10, Site 2	Amplification plate Panel 1 (output)	EnduraPlate™ reaction plate	Empty
H	Grid 10, Site 3	Amplification plate Panel 2[4] (output)	EnduraPlate™ reaction plate	Empty

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

[2] Transfer reagent to a 1.5-mL tube if provided in a 0.5-mL tube

[3] To ensure sufficient volume for 96 reactions, add 50 µL of 5X Ion AmpliSeq™ 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.

[4] Optional for 2-pool panel designs.

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 before use.

1. Fill a 25-mL trough with Nuclease-Free Water, then place the filled 25-mL trough inside a 100-mL trough at Grid 1, Site 1 (**A**).
2. Place the tube containing 5X Ion AmpliSeq™ HiFi Mix (red cap) in position 1 of the chilled metal cooling block at Grid 2, Site 1 (**B**).
3. Place the tube containing 2X Ion AmpliSeq™ Primer Pool 1 in position 2 of the chilled metal cooling block at Grid 2, Site 1 (**C**).
4. *(Optional)* Place the tube containing 2X Ion AmpliSeq™ Primer Pool 2 in position 3 of the chilled metal cooling block at Grid 2, Site 1 (**D**).
5. Place a 500-µL plate onto the shaking incubator at Grid 4, Site 3 (**E**).
6. Place the MicroAmp™ EnduraPlate™ reaction plate containing the prepared gDNA (2 ng/µL) on the incubator at Grid 10, Site 3 (**F**).
7. Place an empty MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**G**).
8. *(Optional)* Place an empty MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 3 (**H**).
9. Replace the tip boxes as needed.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Target_Amp_1or2Pool** script.
4. Enter the number of reactions to be prepared.
5. Enter ***1*** for the DNA panel.
6. Enter the number of panels for the library being prepared.
7. Click **RUN**.
   The run time is ~20 minutes for 1-pool panels and ~30 minutes for 2-pool panels for 96 reactions.
8. When the script is complete, seal the plate with MicroAmp™ Clear Adhesive Film, then centrifuge to collect the droplets.
9. Proceed immediately to “Amplify the targets” on page 24.

Amplify the targets

Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program to amplify target regions.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 min
Cycle (see Table 7 for recommended number of amplification cycles)	Denature	99°C	15 sec
	Anneal and extend	60°C	4 min/8 min/ 16 min[1]
Hold	—	10°C	Hold

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

Table 7 Recommended number of amplification cycles

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.

Primer pairs per pool	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

STOPPING POINT PCR products can be stored at 10°C for up to 16 hours in the thermal cycler. For longer term, store at –20°C.

Partially digest amplicons

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

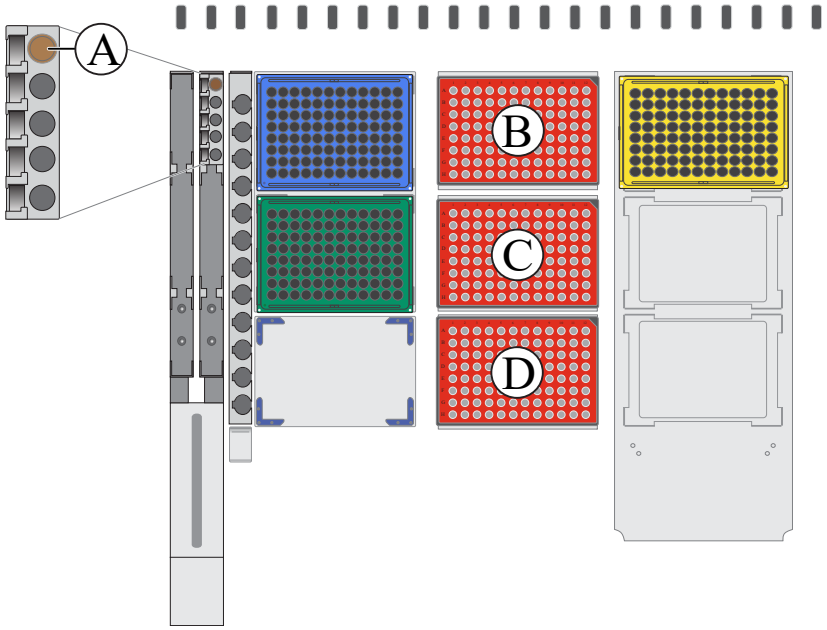


Table 8 Reagent setup summary: Partially digest primers

Label	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	30 µL/well in column 1
C	Grid 10, Site 2	Amplified DNA panel 1	EnduraPlate™ reaction plate	From previous reaction
D	Grid 10, Site 3	Amplified DNA panel 2[3]	EnduraPlate™ reaction plate	From previous reaction

[1] For 96 reactions. If running fewer than 96 samples, adjust volumes accordingly.
[2] Use one or more full tubes from a 96-LV kit to accommodate dead volumes.
[3] Optional for 2-pool panel designs

1. 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.

2. Carefully remove the plate seal from the amplified DNA plate 1, then place it on the incubator at Grid 10, site 2 (**C**).
3. *(Optional)* Carefully remove the plate seal from the amplified DNA plate 2, then place it on the incubator at Grid 10, site 3 (**D**).
4. Replace the tip boxes as needed.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Fupa_1or2Pool** script.
4. Enter the number of reactions to be prepared.
5. Enter the number of panels for the library being prepared.
6. If you are using **Fast Mode**, enter ***1*** when prompted to **Enable Fast Transfer?**. Otherwise, leave the value as ***0***.
7. Click **RUN**.
   The run time is ~20 minutes (Standard) or 7 minutes (Fast) for 96 reactions.
8. When the script is complete, seal the plate at Grid 10, Site 2 with MicroAmp™ Clear Adhesive Film. Vortex the plate 3 times for 3 seconds each time at setting 7–10, then centrifuge the plate briefly.

Partially digest amplicons

Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then 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

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library. DNA and RNA libraries from the same sample also require different barcodes.

Ion Torrent™ Dual Barcode Adapters are provided at the appropriate concentration. No further handling is necessary. If using Ion Torrent™ Dual Barcode Adapters, proceed to “Set up the worktable” on page 29.

IonCode™ Barcode Adapters are provided at the appropriate concentration and include forward and reverse adapters in a single well. No further handling is necessary. If using IonCode™ Barcode Adapters, proceed to “Set up the worktable” on page 29.

Ion Xpress™ Barcode Adapters require handling and dilution as described in “(Ion Xpress™ Barcode Adapters only) Combine and dilute adapters”.

(Ion Xpress™ Barcode Adapters only) Combine and dilute adapters

For each barcode X selected, 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.

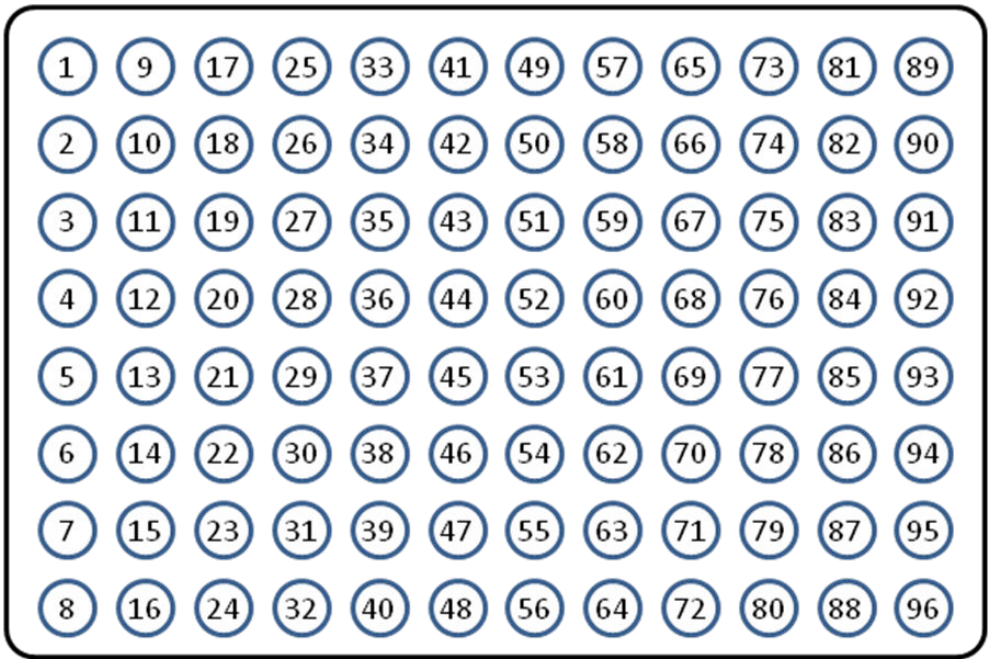
Table 9 Example barcode adapter mix for up to 40 reactions

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!

- When handling barcoded adapters, be careful to avoid cross-contamination by changing gloves frequently and opening one tube at a time.
- Barcodes *must* be arrayed in the following pattern, with barcode number matching position number on the plate:



Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

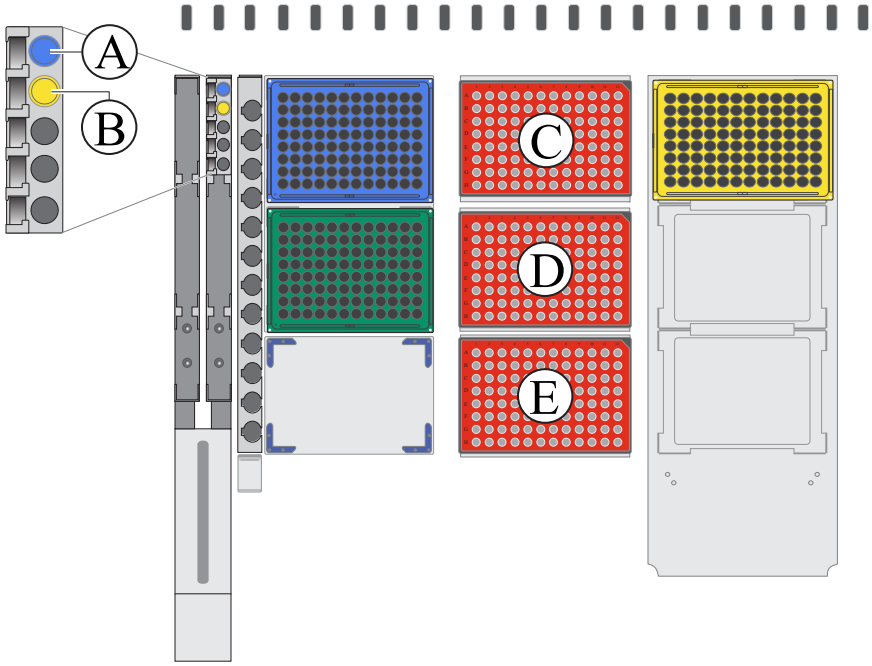


Table 10 Reagent setup summary: Ligate adapters

Label	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: 60 µL/well in column 3
				Ligase: 30 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	≥5 µL/well

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

[2] Use a full tube from a 96 reaction kit to accommodate dead volumes.

1. 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.

2. Carefully remove the plate seal from the amplified and partially digested DNA plate (from "Partially digest amplicons" on page 25), then place it on the incubator at Grid 10, Site 2 (**D**).

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

4. Replace the tip boxes as needed.

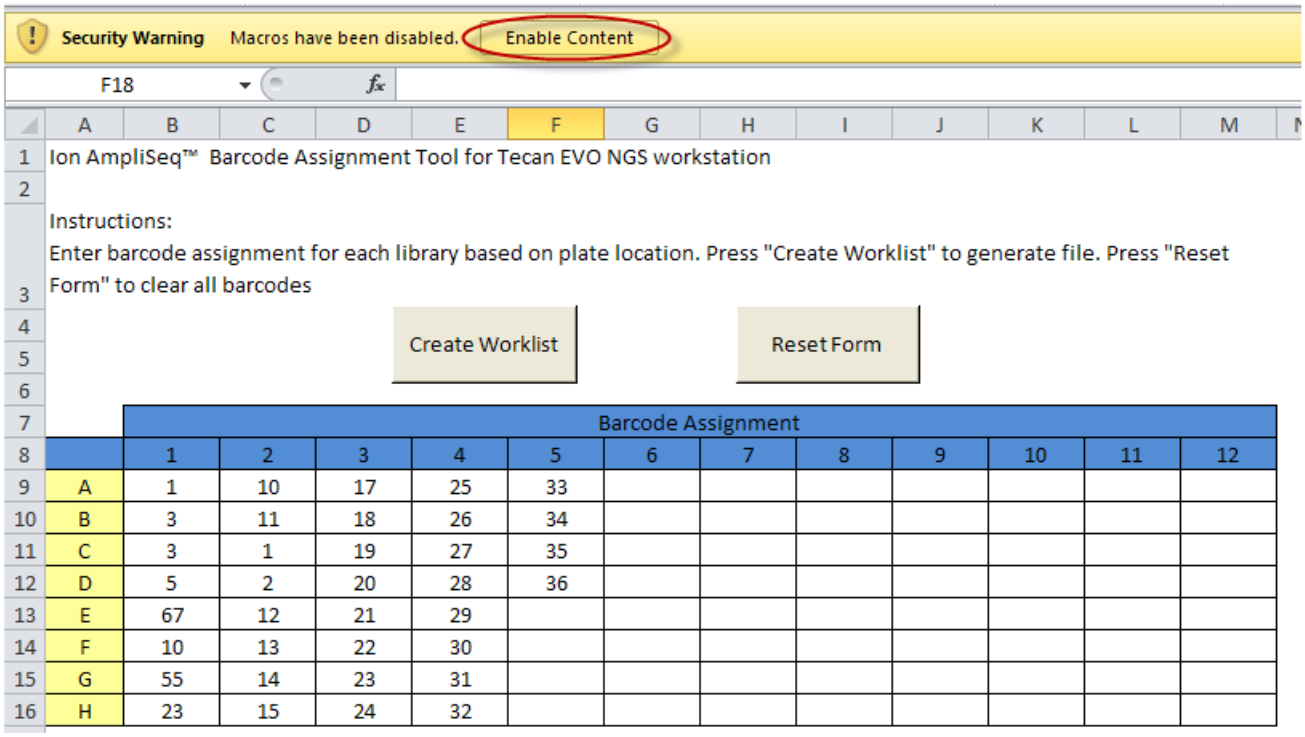
Barcode transfer

1. Contact Technical Support or your support representative to obtain the `Ion AmpliSeq Adapter Worklist PLATE.xlsm` file (see "Customer and technical support" on page 48).

2. Open the file, then enable the macros.

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

   Note: The instrument always prepares libraries from top to bottom and left to right. The position on the plate map refers to the library position. The number that is entered into each cell refers to the well number on the diluted barcode source plate.



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

Figure 1 Example barcode assignment for the preparation of 40 libraries.

4. When completed, click **Create Worklist** , then save the file to the desktop.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Ligation** script.
4. Enter the number of reactions to be prepared.
5. If you are using **Fast Mode**, enter *1* when prompted to **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™ Clear Adhesive Film. Vortex the plate 3 times for 3 seconds each time at setting 7–10, then centrifuge the plate briefly.

Ligate adapters

Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program.

Temperature	Time
22°C	30 min[1]
68°C	5 min
72°C	5 min
10°C	Hold (for up to 1 hour)

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

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

Proceed to one of the following methods.

- “Option 1: Equalize the library” on page 32
- “Option 2: Purify and quantify the unamplified library by qPCR” on page 38
- “Option 3: Quantify the amplified library with Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™” on page 41

If you are not planning to use the prepared libraries immediately, proceed to “Store libraries” on page 47.

Option 1: Equalize the library

IMPORTANT! We recommend using the Ion Library Equalizer™ Kit when library yield is consistently above the minimum concentration outlined in this user bulletin. In cases where sample input is highly variable or unknown (such as RNA libraries), we recommend using the qPCR method (see “Option 2: Purify and quantify the unamplified library by qPCR” on page 38).

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

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

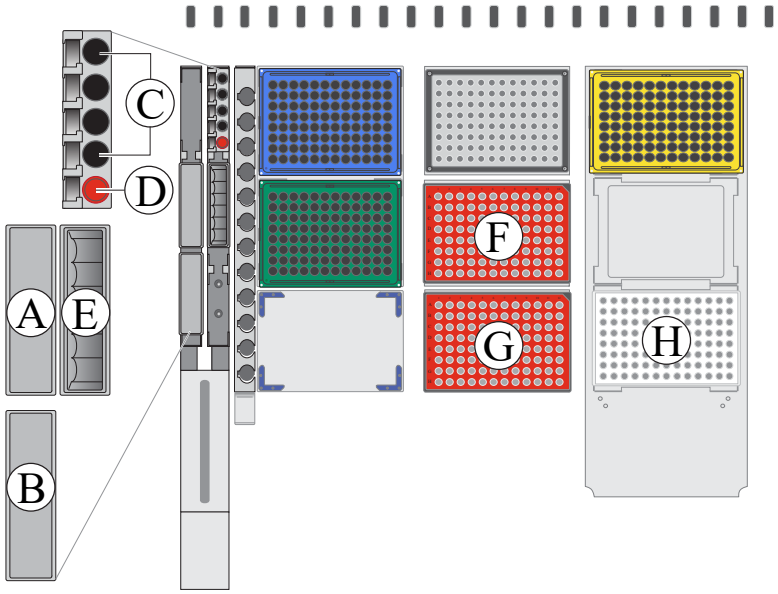


Table 11 Reagent setup summary: Purify, elute, and amplify the library

Label	Position	Description	Labware	Fill volume[1]
A	Grid 1, Site 2	70% Ethanol	100-mL trough	35 mL
B	Grid 1, Site 3	Waste	100-mL trough	Empty
C	Grid 2, Site 1, Wells 1–4	Platinum™ PCR SuperMix HiFi *or* 1X Library Amp Mix (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 from a 96 reaction kit to accommodate dead volumes.

[3] See the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) or *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003) for proper primer selection.

1. Fill a 100-mL trough with freshly prepared 70% ethanol, then place the filled trough at Grid 1, Site 2 (**A**).
2. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).
3. Place the tubes containing Platinum™ PCR SuperMix High Fidelity or 1X Library Amp Mix (black cap) in positions 1–4 of the chilled metal cooling block at Grid 2, Site 1 (**C**).

   Note: All four tubes are required.
4. 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™ Fluorometer
25X Library Amplification Primers	Equalizer™ or Agilent™ 2100 Bioanalyzer™/ Qubit™ Fluorometer
Library Amplification Primer Mix	Agilent™ 2100 Bioanalyzer™/ Qubit™ Fluorometer

5. Fill a 25-mL trough with AMPure™ XP beads , then place the filled trough inside a 100-mL trough at Grid 2, Site 2 (**E**).
6. Place a new MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**F**).
7. Place a MicroAmp™ EnduraPlate™ reaction plate containing ligated DNA on the incubator at Grid 10, Site 3 (**G**).
8. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**H**).
9. Replace the tip boxes as needed.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Library_Amp** script.
4. Enter the number of reactions to be prepared.
5. Click **RUN**.
   The run time is ~85 minutes for 96 reactions.

Amplify the library

When the script is complete, seal the plate with a MicroAmp™ Clear Adhesive Film, place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program.

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)

If necessary, wash the Equalizer™ Beads while cycling (see "Wash the Equalizer™ beads").

Wash the Equalizer™ beads

If you have not previously done so, wash the Equalizer™ beads.

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

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

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

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

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

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

Add Equalizer™ beads, wash, and elute the equalized library

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

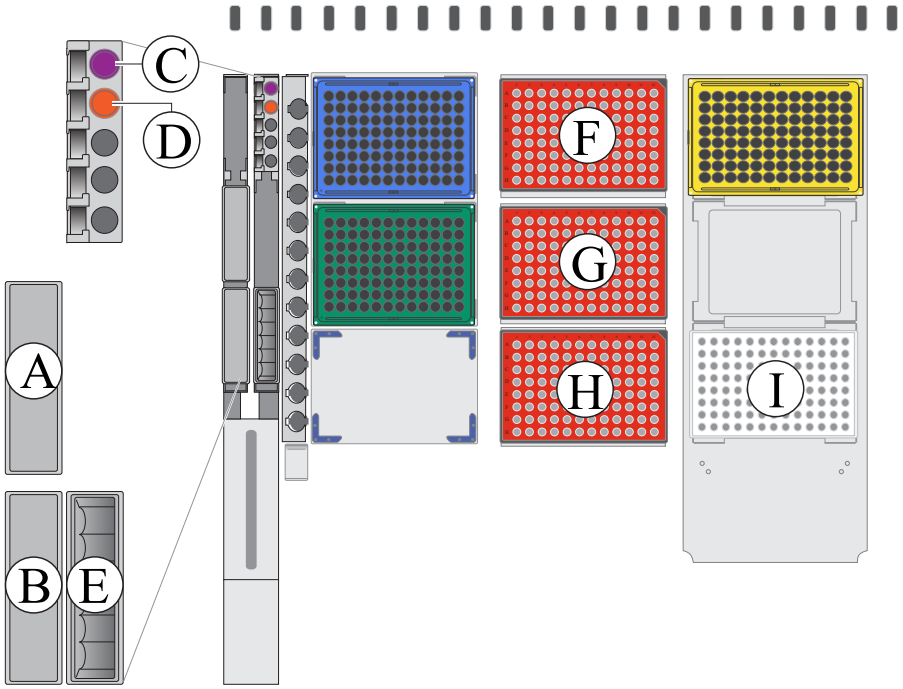


Table 12 Reagent setup summary: Add Equalizer™ Beads and wash

Label	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

Table 12 Reagent setup summary: Add Equalizer Beads and wash *(continued)*

Label	Position	Description	Labware	Fill volume[1]
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	—

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

[2] Use a full tube from an Ion Library Equalizer™ Kit to accommodate dead volumes.

1. 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	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, then place it on the incubator at Grid 10, Site 1 (**F**). This plate can be stored at 4°C for later use.

2. Fill a 100-mL trough with Equalizer™ Wash Buffer, then place the filled trough 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 Equalizer™ Elution Buffer, then place the filled trough inside a 100-mL trough at Grid 2, Site 3 (**E**).
5. Place a new MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**G**).
6. Place a MicroAmp™ EnduraPlate™ reaction plate containing amplified library (from “Amplify the library” on page 33) on the incubator at Grid 10, Site 3 (**H**).
7. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**I**).
8. Replace the tip boxes as needed.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Equalizer** script.
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™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) or *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

The final concentration of each equalized library is ~100 pM.

For more information on library storage, proceed to “Store libraries” on page 47.

Option 2: Purify and quantify the unamplified library by qPCR

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

Note: The Ion Library TaqMan™ Quantitation Kit can also be used to quantify libraries that have been amplified using the procedure that is described in “Option 1: Equalize the library” on page 32.

Purify the unamplified library

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

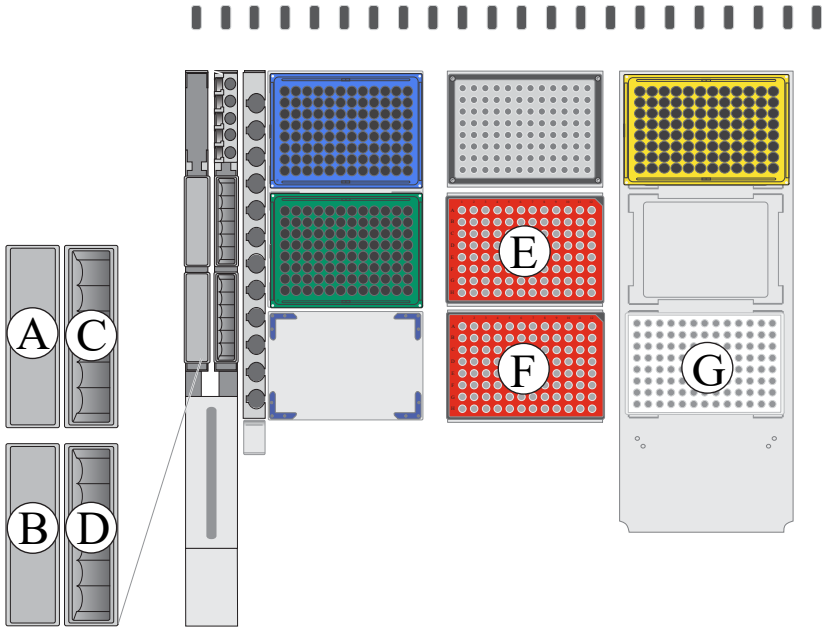


Table 13 Reagent setup summary: Purify the unamplified library

Label	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	Agencourt™ 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™–96 Side Magnet	N/A

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

1. Fill a 100-mL trough with freshly prepared 70% ethanol, then place the filled trough at Grid 1, Site 2 (**A**).
2. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).
3. Fill a 25-mL trough with AMPure™ XP beads, then place the filled trough at Grid 2, Site 2 (**C**).
4. Fill a 25-mL trough with Low TE and place, then place the filled trough at Grid 2, Site 3 (**D**).
5. Place a new MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**E**).
6. Place a MicroAmp™ EnduraPlate™ reaction plate containing ligated DNA (from “Ligate adapters to the amplicons” on page 27) on the incubator at Grid 10, Site 3 (**F**).
7. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**G**).
8. Replace the tip boxes as needed.

Run the script

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

Prepare a 100-fold dilution of the library for quantification by mixing 2 µL of supernatant with 198 µL of Nuclease-Free Water, then proceed to "*Quantify library by qPCR and calculate the dilution factor*" in the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) or *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

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

Before quantification, the Ion AmpliSeq™ libraries must be amplified with the Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™.

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

Amplify the library

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by-step procedure.

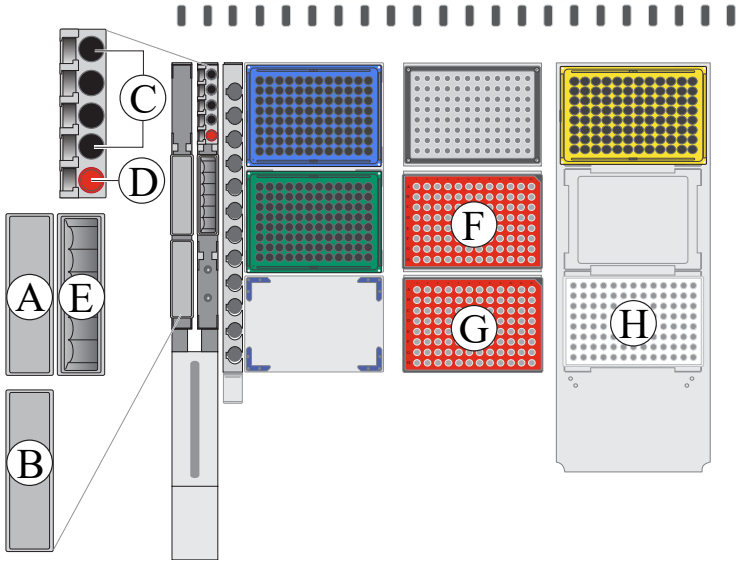


Table 14 Reagent setup summary: Amplify the library

Label	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 *or* 1X Library Amp Mix (black cap)	2.0-mL tube	8 mL
D	Grid 2, Site 1, Well 5	Amplification Primers[2]	0.5-mL tube	6 mL

Table 14 Reagent setup summary: Amplify the library *(continued)*

Label	Position	Description	Labware	Fill volume[1]
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™–96 Side Magnet	N/A

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

[2] For primer selection, see the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) or *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

1. Fill a 100-mL trough with freshly prepared 70% ethanol, then place the filled trough at Grid 1, Site 2 (**A**).
2. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).
3. Place the tubes containing Platinum™ PCR SuperMix HiFi or 1X Library Amp Mix in positions 1–4 of the chilled metal cooling block at Grid 2, Site 1 (**C**).

   Note: All four tubes are required.
4. 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™ Fluorometer
25X Library Amplification Primers	Equalizer™ or Agilent™ 2100 Bioanalyzer™/ Qubit™ Fluorometer
Library Amplification Primer Mix	Agilent™ 2100 Bioanalyzer™/ Qubit™ Fluorometer

5. Fill a 25-mL trough with AMPure™ XP beads, then place the filled trough inside a 100-mL trough at Grid 2, Site 2 (**E**).
6. Place a new MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**F**).
7. Place a MicroAmp™ EnduraPlate™ reaction plate containing ligated DNA on the incubator at Grid 10, Site 3 (**G**).
8. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**H**).
9. Replace the tip boxes if needed.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Library_Amp** script.
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™ Clear Adhesive Film.

Amplify the library

Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then 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 can be stored at –20°C.

Purify the amplified library

Set up the worktable

Set up the worktable as described in the following figure. For detailed instructions, see the step-by step procedure.

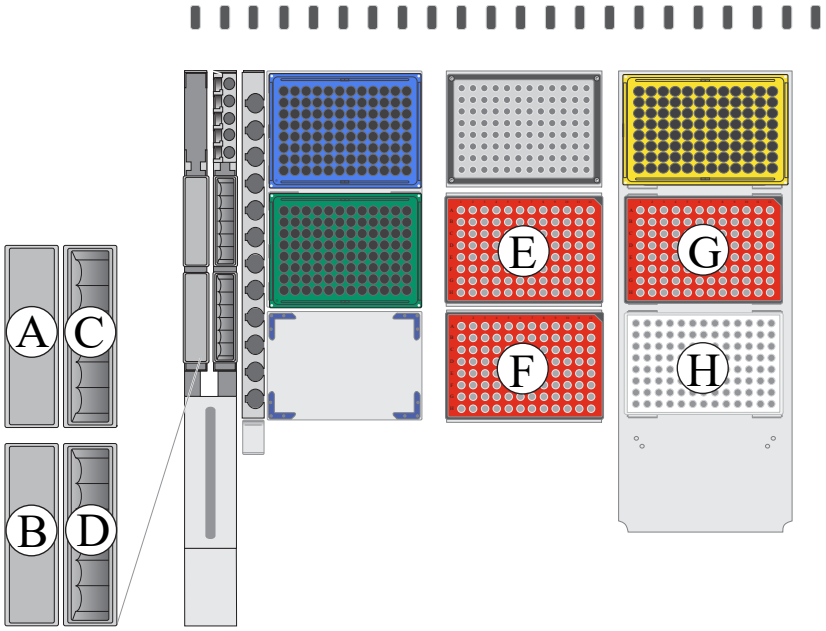


Table 15 Reagent setup summary: Purify the amplified library

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.

1. Fill a 100-mL trough with freshly prepared 70% ethanol, then place the filled trough at Grid 1, Site 2 (**A**).
2. Place an empty 100-mL trough at Grid 1, Site 3 (**B**).
3. Fill a 25-mL trough with AMPure™ XP beads, then place the filled trough inside a 100-mL trough at Grid 2, Site 2 (**C**).
4. Fill at 25-mL trough with Low TE, then place the filled trough inside a 100-mL trough at Grid 2, Site 3 (**D**).
5. Place a new MicroAmp™ EnduraPlate™ reaction plate on the incubator at Grid 10, Site 2 (**E**).
6. Place a MicroAmp™ EnduraPlate™ reaction plate containing amplified DNA (from "Amplify the library" on page 41) on the incubator at Grid 10, Site 3 (**F**).
7. Place a new MicroAmp™ EnduraPlate™ reaction plate on a MicroAmp™ Splash-Free 96-Well Base at Grid 16, Site 2 (**G**).
8. Place the DynaMag™ Side Magnet at Grid 16, Site 3 (**H**).
9. Replace the tip boxes as needed.

Run the script

1. Open Freedom EVOware™ software.
2. Click **Run an existing script**.
3. Select the **Library_Amp_Purification** script.
4. Enter the number of reactions to be prepared.
5. Click **RUN**.
   The run time is ~85 minutes for 96 reactions.

After the script is complete, the plate at Grid 10, Site 2 contains the purified library.

Quantify the library using the Qubit™ Fluorometer

Analyze 10 µL of each amplified library using the Qubit™ Fluorometer 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, then incubate for at least 2 minutes.

c. Prepare each Qubit™ Standard as directed in the user guide.

d. Measure the concentration on the Qubit™ Fluorometer.

e. Calculate the concentration of the undiluted library by multiplying by 20. You can calculate the concentration automatically using the **Calculate Stock Concentration** button and entering 10 µL as the sample volume.

2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.

   Dilute to 15 ng/ml for amplicons up to 225 bp or 22 ng/ml for amplicons up to 275 bp design.

   Example calculation for 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 that is 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, then proceed to combining libraries or template preparation.

Quantify the library using Agilent™ 2100 Bioanalyzer™

Analyze 1 µL of amplified library on the Agilent™ 2100 Bioanalyzer™ instrument using 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, then 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).

   a. Click **Data** in the **Contexts** panel, then view the electropherogram of the sample to be quantified.

   b. Click the **Region Table** tab, then create a region spanning the desired amplicon peaks. Correct the baseline if needed.

   c. The molarity is automatically calculated and displayed in the table as 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, then proceed to combining libraries or template preparation. If you are performing template preparation immediately, store libraries as described in "Store libraries".

   For more information on combining libraries, see "(Optional) Combine amplicon libraries".

(Optional) Combine amplicon libraries

Multiple strategies for combining Ion AmpliSeq™ libraries are available. See the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) and *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

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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