Advanta CFTR NGS Library Preparation on the LP 48.48 IFC with Juno

IMPORTANT Before using this quick reference, read and understand the detailed instructions and safety guidelines in the Advanta™ CFTR NGS Library Preparation on the LP 48.48 IFC with Juno™ Protocol (PN 101-6270).

Workflow Overview

	Workflow Step
1	Determine the estimated read depth.
2	Preamplify gDNA, if the concentration is <30 ng/µL. For more information about preamplification, see the Advanta CFTR NGS Preamp Reagent Kit quick reference (PN 101-6273).
3	Prepare the CFTR assay, non-assay mix, and sample mixes.
4	Prime the LP 48.48 IFC.
5	Load and run LP 48.48 IFC on Juno and harvest samples.*
6	Pool the harvested samples.*
7	Clean up the pooled samples (3x).*
8	Thermal-cycle the samples to add P5 sequencing adapters to the library.
9	Clean up the final sequencing library.*
10	Quantify the sequencing library.*

*Potential stopping point.

Determine the Estimated Read Depth

Determine the estimated read depth based on the estimated total of paired-end reads and the number of amplicons and samples.

Prepare CFTR Assay Mixes and Sample Mixes

IMPORTANT Pipet reagents slowly and carefully to transfer entire volumes and to minimize bubbles. Reagents tend to cling to tip surfaces and can form bubbles easily.

Prepare the Assay Pre-Mix

☐ **1** Vortex reagents for 20 sec, and then briefly centrifuge them before use.

☐ **2** In a DNA-free hood, combine the components shown in Table 1 in a new 1.5 mL microcentrifuge tube.

Table 1. Assay pre-mix

Component	Vol. for 48 Assay Inlets (µL)*
PCR Water (PN 100-5941)	165
TSP Assay Loading Reagent (PN 101-0409)	15
Total	**180**

*Includes overage

☐ **3** Gently vortex the assay pre-mix for 5 sec at medium speed, and then use a microcentrifuge for ≥3 sec to bring down all components and remove bubbles.

Prepare the CFTR Assay Mixes

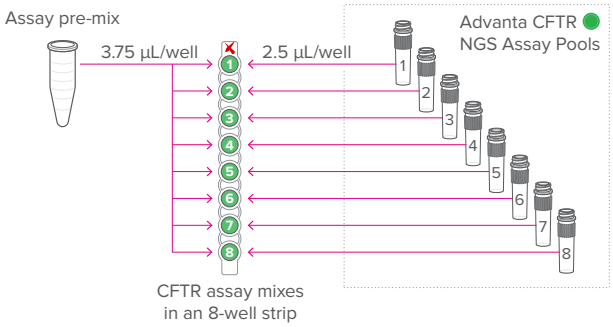


Figure 1. Preparation of CFTR assay mixes

☐ **1** Immediately before use, ensure that each tube of Advanta CFTR NGS Assay Pools is securely sealed, and then vortex at medium-high speed for 10–20 sec to mix. Centrifuge the CFTR NGS Assay Pools tubes at 3,000 × *g* for 5 min.

☐ **2** In a DNA-free hood, prepare a CFTR assay mix for each of the eight tubes of Advanta CFTR NGS Assay Pools in a new 8-well strip by adding each component as shown in Table 2 and Figure 1. Pipet gently up and down to mix, being careful to avoid creating bubbles.

Table 2. CFTR assay mixes

Component	Vol. per CFTR Assay Mix (µL)*
Assay pre-mix (see Table 1)	3.75
Advanta CFTR NGS Assay Pools (PN 101-6155, 8 tubes)	2.50
Total	**6.25**

*Includes overage

☐ **3** Cap the 8-well strip, and then centrifuge for 3 sec to bring down contents. Centrifuge longer if any bubbles are present. Label the strip "CFAM" and set aside until you are ready to load the IFC.

Prepare the Non-Assay Mix

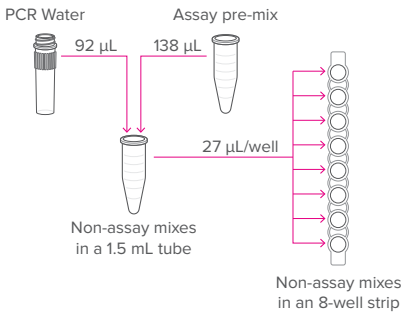


Figure 2. Preparation of non-assay mixes

☐ **1** Vortex reagents for 20 sec, and then briefly centrifuge them before use.

☐ **2** In a DNA-free hood, prepare the non-assay mix in a new 1.5 mL microcentrifuge tube as shown in Table 3:

Table 3. Non-assay mix

Component	Vol. for 40 Non-Assay Inlets (µL)*
PCR Water (PN 100-5941)	92
Assay pre-mix (see Table 1 on page 1)	138
Total	**230**

*Includes overage

☐ **3** Gently vortex the non-assay mix for 5 sec at medium speed, and then use a microcentrifuge for ≥3 sec to bring down all components and remove bubbles.

☐ **4** Aliquot 27 µL of the non-assay mix into each well of an 8-well strip. Label the strip "NAM" and set it aside until you are ready to load the IFC.

Prepare the Sample Pre-Mix

☐ **1** Manually flick the bottom of TSP DNA Polymerase tube with your forefinger to mix the enzyme (**do not vortex**). Vortex all other reagents for 20 sec, and then briefly centrifuge all reagents before use.

☐ **2** In a DNA-free hood, prepare the sample pre-mix in a new 1.5 mL microcentrifuge tube as shown in Table 4.

IMPORTANT

- Add the 4X TSP Master Mix to the PCR Water to dilute it before adding the remaining reagents.
- While pipetting, do not go past the first stop on the pipette.
- 4X TSP Master Mix is viscous. Pipet slowly.

Table 4. Sample pre-mix. Combine and mix in order shown.

	Component	Vol. per IFC (µL)*
1	PCR Water (PN 100-5941)	40
2	4X TSP Master Mix (PN 101-3055)	100
3	TSP Sample Loading Reagent v2 (PN 101-7634)	20
4	TSP DNA Polymerase (PN 101-0995)	16
Total		**176**

*Includes overage

☐ **3** Vortex the sample pre-mix for 10–20 sec at a medium speed, and then use a microcentrifuge for 10 sec to bring down all components and remove bubbles.

☐ **4** Pipet 19 µL of the sample pre-mix into each well of a new 8-well strip (see Figure 3).

IMPORTANT To prevent introducing bubbles, pipet only to the first stop during this transfer process. To help ensure that all liquid can be retrieved during the next step, we recommend using a microcentrifuge at maximum speed for 3 sec.

Prepare the Sample Mixes

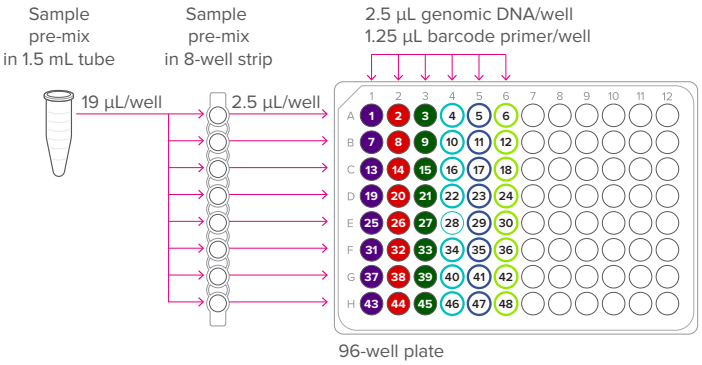


Figure 3. Preparation of sample mixes

☐ **1** Centrifuge the Targeted DNA Seq Barcode Plate at 3,000 × *g* for 3 min before using.

☐ **2** In a DNA sample hood, prepare the sample mixes by pipetting the components shown in Table 5 into each well of a new 96-well plate. Pipet reagents and samples according to the diagram shown in Figure 3. Use an 8-channel pipette to transfer the sample pre-mix from the 8-well strip.

Table 5. Sample mixes

Component	Vol. per Reaction (µL)*
Sample pre-mix (see page 1)	2.5
Genomic DNA sample (30–60 ng/µL)	2.5
Barcode primer from Targeted DNA Seq Barcode Plate (PN 101-0744)	1.25
Total	**6.25**

*Includes overage

☐ **3** Reseal the Targeted DNA Seq Barcode Plate. If using the barcode plate again within 2 days, store at 4 °C. Otherwise, store at −20 °C.

☐ **4** Tightly seal the 96-well plate with clear adhesive film, vortex thoroughly for 20 sec, and then centrifuge the plates at 2,500–3,000 × *g* for 5 min.

☐ **5** If you observe bubbles in the wells following centrifugation, manually flick or gently snap the bottom of the affected wells with your forefinger, and then centrifuge the plates again at 2,500–3,000 × *g* for 5 min.

☐ **6** Continue to Prime the LP 48.48 IFC on Juno.

Prime the LP 48.48 IFC on Juno

For detailed instructions about injecting Control Line Fluid, see the Control Line Fluid Loading Procedure (PN 68000132). For detailed instructions about using Juno, see the Juno System User Guide (PN 100-7070).

IMPORTANT Use the following best practices when injecting Control Line Fluid into the IFC.

- To ensure correct accumulator volume, use only syringes containing LP 48.48 Control Line Fluid.
- Be careful when removing the syringe cap to prevent drips.
- Avoid getting Control Line Fluid on the exterior of the IFC or in the inlets because this makes the IFC unusable. If this occurs, use a new IFC.

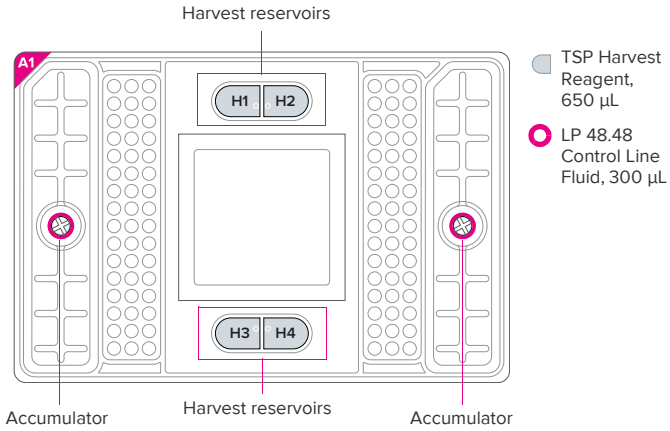


Figure 4. Priming map for the LP 48.48 IFC

☐ **1** Ensure that the MX Interface Plate is installed in Juno.

☐ **2** Pull the protective tape down and away from bottom of IFC.

☐ **3** Inject LP 48.48 Control Line Fluid into each accumulator on the IFC (see Figure 4).

☐ **4** Place the IFC on a flat surface and pipet 650 µL of TSP Harvest Reagent into each of the harvest reservoirs H1, H2, H3, and H4 (see Figure 4).

☐ **5** Place the IFC on the tray of the Juno instrument, and then tap **LOAD**.

☐ **6** On the Juno Scripts screen, tap **Prime LP—48.48**. Priming the IFC takes approximately 5 min.

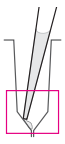
☐ **7** After the script is finished, tap **EJECT** to eject the IFC.

IMPORTANT Load the IFC within 60 min of completing the prime script.

Load and Run the LP 48.48 IFC on Juno

IMPORTANT Use the following best practices to load assay and sample mixes on the IFC.

- Before pipetting reagents, maintain traceability by noting the orientation of the A1 corner, assay inlets, and sample inlets, as shown in Figure 5.
- When pipetting reagents:
  - Dispense the reagents while making contact with the side of the inlet near the bottom of the inlet, as shown.
  - Pipet reagents slowly and carefully to transfer entire volumes and to minimize bubbles. Reagents tend to cling to tip surfaces and can form bubbles easily.
  - Do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets, which can result in sample or amplicon dropout due to load failure.



Pipetting near the bottom of the inlet

☐ **1** Pipet the CFTR assay mixes and sample mixes into the LP 48.48 IFC as shown in Figure 5.

a Carefully pipet 4.0 µL of each CFTR assay mix from the 8-well strip labeled CFAM into the designated assay inlets on the IFC.

b Carefully pipet 4.0 µL of non-assay mix from the 8-well strip labeled NAM into the remaining assay inlets (N).

c Carefully pipet 4.0 µL of each sample mix into the designated sample inlet of the IFC based on the predefined sample map.

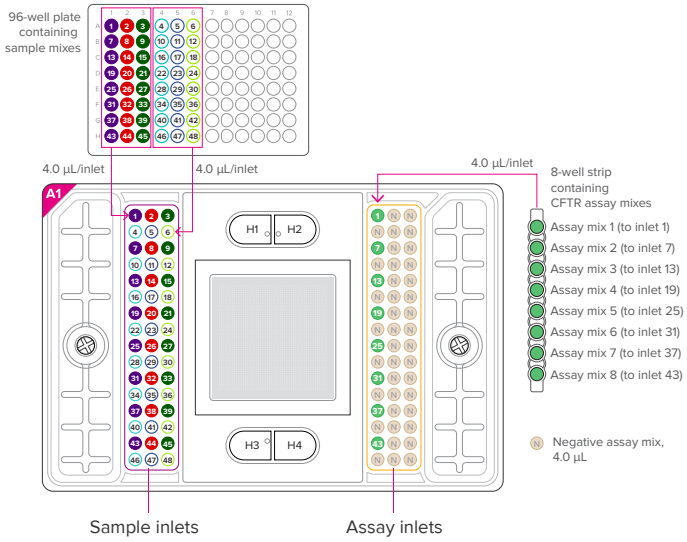


Figure 5. Loading map

☐ **2** Cover the sample and assay inlets with LP 48.48 Barrier Tape.

☐ **3** On Juno, tap **OPEN** and place the IFC on the tray of the Juno instrument, and then tap **LOAD**.

☐ **4** On the Juno Scripts screen, tap **One Step LP—48.48**.

☐ **5** Select when the script should finish (if necessary, adjust the harvest time), and then tap **RUN**. The run takes approximately 4 hr to complete loading, thermal cycling, and harvesting. You can delay the harvest for up to 16 hr (total of 20 hr, including run time).

☐ **6** After the run is finished, tap **EJECT** to eject the IFC.

IMPORTANT Eject the IFC ≤60 min after run is complete.

☐ **7** After ejecting the IFC, immediately proceed to the next section.

Pool the Harvested Samples from the Calculated Number of Samples

Pool the harvested samples in a post-PCR room.

IMPORTANT If you ran preamplified and non-preamplified samples on the same IFC, harvest the preamplified, barcoded amplicons to a different tube than the non-preamplified, barcoded amplicons

Each processed sample is harvested from the same sample inlet that was used to dispense sample mix into the IFC. Pool the harvested samples by first transferring them from the IFC to an 8-well strip, and then transferring them to a 1.5 mL tube.

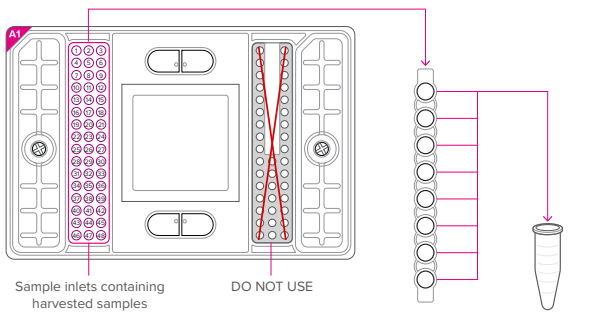


Figure 6. Process for pooling harvested samples

☐ **1** Carefully remove the LP 48.48 Barrier Tape from the sample chamber of the IFC (Figure 6).

☐ **2** Set an 8-channel pipette to 12.0 µL to transfer the entire harvest volumes from the sample inlets of the LP 48.48 IFC. Each harvest volume should be >5 µL.

IMPORTANT Be sure to transfer the entire volume from each sample inlet for best barcode uniformity of mapped reads.

☐ **3** Combine entire harvest volumes from the samples directly into an 8-well strip.

☐ **4** Combine volumes from the 8-well strip into a single new 1.5 mL microcentrifuge tube.

NOTE If you are processing more than one sample pool at the same time for cleanup, label a new 1.5 mL tube for each sample pool to be processed.

STOPPING POINT Store the 1.5 mL tube of pooled samples at 4 °C for up to one week or at –20 °C for longer storage.

☐ **5** If continuing immediately to cleaning up the pooled samples, retrieve the Agencourt AMPure XP magnetic beads from storage now and warm them to room temperature for 30 min.

Clean Up the Pooled Samples

Clean up the pooled samples in a post-PCR room.

Process the harvested samples pooled from a single IFC independently for the cleanup and PCR steps.

IMPORTANT

- To minimize sample-cross-contamination, it is critical to remove all excess primers from the pooled samples before adapter addition. Due to the high concentration of primers remaining in the harvest product, three sequential solid-phase reversible immobilization (SPRI) bead cleanup steps are required. Pipet carefully to ensure proper SPRI (bead:DNA) ratios.
- Fully dispense magnetic bead suspension from pipette tip.

Prepare the Reagents for Cleanup

A 5 mL preparation of 80% ethanol is sufficient for three cleanups of a single pool of harvested samples and the final cleanup of the sequencing library. Scale the preparation of 80% ethanol as necessary to process all IFC harvested sample pools that might be prepared in parallel.

IMPORTANT Ethanol is hygroscopic. Prepare fresh 80% ethanol before library cleanup. Cap the tube of 80% ethanol when not in use. A batch of ethanol can be kept for 24 hr.

☐ **1** Remove the Agencourt AMPure XP magnetic beads from 4 °C, and then warm the beads to room temperature for 30 min before use.

☐ **2** Vortex the Agencourt AMPure XP magnetic beads vigorously to ensure that they are fully suspended.

☐ **3** Using a new graduated tube, prepare 5 mL of fresh 80% ethanol for each pool of harvested samples:

- **a** Pipet 1 mL of DNase-free water into the tube.
- **b** Add 4 mL of absolute alcohol to bring the volume to 5 mL.
- **c** Cap the tube, and then invert to mix.

First Cleanup (0.4X/0.9X Double-Size SPRI)

IMPORTANT In step 5 of this section, do not discard the supernatant.

☐ **1** Suspend magnetic beads in pooled samples:

Component	Vol. for First Cleanup (µL)
Pooled samples	150
Agencourt AMPure XP magnetic beads (Beckman Coulter PN A63880)	60
Total	**210**

- **a** Pipet pooled samples into a new 1.5 mL microcentrifuge tube. If the volume of pooled samples is <150 µL, add DNA Dilution Reagent or PCR Water to bring the volume to 150 µL. Label and store the remaining pooled samples for possible contingencies.
- **b** Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 min. The beads should appear homogeneous and uniform in color.
- **c** Pipet Agencourt AMPure XP magnetic beads into each tube that contains pooled samples. Expel any beads left in the pipette tip by pipetting the suspension up and down 10 times.

☐ **2** Vortex the suspension at high speed for 20 sec.

☐ **3** Incubate the suspension at room temperature for 10 min.

☐ **4** Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 min.

☐ **5** Without disturbing the beads, and keeping the tube on the magnetic stand, carefully pipet the **supernatant** to a new tube.

IMPORTANT Retain all of the supernatant.

☐ **6** Use a P10 pipette to transfer any residual volume to ensure that all supernatant has been transferred. Dispose of the tube containing the remaining beads.

☐ **7** Vortex the bottle of AMPure XP magnetic beads at high speed for 20 sec, and then pipet 75 µL of the beads into the supernatant. Vortex the suspension at high speed for 20 sec.

☐ **8** Incubate the suspension at room temperature for 10 min.

☐ **9** Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 min.

☐ **10** Without disturbing the beads, and keeping the tube on the magnetic stand, remove and discard the supernatant.

☐ **11** Use a P10 pipette to remove any residual supernatant from the tube.

☐ **12** Wash the beads three times with 80% ethanol:

- **a** Keeping the tube on the magnetic stand, pipet 400 µL of 80% ethanol to wash the beads.
- **b** Incubate the tube at room temperature for 30–60 sec.
- **c** Without disturbing the beads, and keeping the tube on the magnetic stand, remove and discard the ethanol.
- **d** Repeat Steps 12a–12c two more times. Completely remove and discard all of the 80% ethanol.

☐ Wash 1 ☐ Wash 2 ☐ Wash 3

☐ **13** Transfer the tube to a rack and open the tube. Remove any remaining ethanol by drying the beads at 37 °C for 1 min or air-drying the beads at room temperature for 10–15 min.

☐ **14** Prepare the eluate:

- **a** To the dried beads, pipet 30 µL of DNA Dilution Reagent. Vortex the suspension at high speed for 20 sec.
- **b** Incubate the suspension at room temperature for 2 min.
- **c** Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 min.
- **d** Keeping the tube on the magnetic stand, pipet 30 µL of the eluate to a new tube.

STOPPING POINT You can store the eluate at 4 °C for up to one week or at –20 °C for longer storage.

Second and Third Cleanup (0.8X SPRI)

☐ **1** Suspend magnetic beads in eluate:

Component	Vol. for Second and Third Cleanup (µL)
Eluate	30
Agencourt AMPure XP magnetic beads (Beckman Coulter PN A63880)	24
Total	**54**

- **a** Vortex the Agencourt AMPure XP magnetic beads at high speed for 20 sec. The beads should appear homogeneous and uniform in color.
- **b** Pipet Agencourt AMPure XP magnetic beads into the same tube with the eluate from the previous cleanup. Expel any beads left in the pipette tip by pipetting the suspension up and down 10 times.

☐ **2** Vortex the suspension at high speed for 20 sec.

☐ **3** Incubate the suspension at room temperature for 10 min.

☐ **4** Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 min.

☐ **5** Without disturbing the beads, and keeping the tube on the magnetic stand, remove and discard the supernatant.

☐ **6** Use a P10 pipette to remove any residual supernatant from the tube.

☐ **7** Wash the beads three times with 80% ethanol:

- **a** Keeping the tube on the magnetic stand, pipet 190 µL of 80% ethanol to wash the beads.
- **b** Incubate the tube at room temperature for 30–60 sec.
- **c** Without disturbing the beads, and keeping the tube on the magnetic stand, remove and discard the ethanol.
- **d** Repeat Steps 7a–7c two more times. Completely remove and discard all of the 80% ethanol.

☐ Wash 1 ☐ Wash 2 ☐ Wash 3

☐ **8** Transfer the tube to a rack and open the tube. Remove any remaining ethanol by drying the beads at 37 °C for 1 min or air-drying the beads at room temperature for 10–15 min.

☐ **9** Prepare the eluate:

- **a** To the dried beads, pipet 30 µL of DNA Dilution Reagent. Vortex the suspension at high speed for 20 sec.
- **b** Incubate the suspension at room temperature for 2 min.
- **c** Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 min.
- **d** Keeping the tube on the magnetic stand, pipet 30 µL of the eluate to a new tube.

STOPPING POINT You can store the eluate from the second cleanup at 4 °C for up to one week or at –20 °C for longer storage.

☐ **10** Perform the third cleanup (0.8X SPRI) by repeating Steps 1–9 with 30 µL of the eluate from the second cleanup. The eluate from the third cleanup is the purified library (before sequencing adapter is added). Label this tube "LIB w/o SA."

STOPPING POINT You can store the eluate from the third cleanup at 4 °C for up to one week or at –20 °C for longer storage.

After the harvest pools have undergone the cleanup and PCR steps, they can be combined into a single IFC library for sequencing. We recommend that you normalize and combine the single IFC libraries volumetrically for an even representation of each single IFC library in the sequencing run. This recommendation might need to be adjusted if the single IFC libraries have an uneven number of samples in each one of them.

Add Sequencing Adapter to Purified Library

Add the sequencing adapter in a post-PCR room.

☐ **1** Vortex reagents for 20 sec, and then briefly centrifuge them before use.

☐ **2** Combine the components in Table 6 in a new PCR tube to prepare the PCR mix.

Table 6. Reagents for sequencing adapter PCR

Component	Vol. per Reaction (µL)
PCR Water (PN 100-5941)	12.0
4X TSP Master Mix (PN 101-3055)	7.5
TSP Adapter Mix (PN 101-0408)*	6.0
Purified library	4.5
Total	**30.0**

*For dual indexing, replace TSP Adapter Mix with a Dual Index Adapter Mix from the Advanta NGS Library Prep Adapter Set (PN 101-2412).

☐ **3** To assess library size and purity, store the remaining purified library (before sequencing adapter is added) for performing QC later on an Agilent High Sensitivity DNA chip.

☐ **4** Perform PCR using a stand-alone thermal cycler:

Temperature	Time	Cycles	Description
95 °C	15 min	1	Hot start
95 °C	15 sec	10	PCR
60 °C	90 sec		
68 °C	90 sec		
68 °C	3 min	1	Final extension
4 °C	∞	1	Hold

The run time for the PCR protocol is ~1 hr.

Clean Up the PCR Product (0.8X SPRI)

Clean up the PCR product (see Add Sequencing Adapter to Purified Library on page 5) in a post-PCR room.

IMPORTANT

- The quality of PCR products prepared is critical to the success of amplicon sequencing. Any contamination of primers/tags/adapters or the presence of primer dimers in the PCR products will affect sequencing read quality. Therefore, before sequencing, the sequencing library should be purified and qualified.
- If the 80% ethanol is more than 1 day old, prepare a fresh batch (see Prepare the Reagents for Cleanup on page 4).

☐ **1** In a new 1.5 mL microcentrifuge tube, pipet 25 µL of the PCR product into 25 µL of DNase-free water. Mix to dilute the PCR product, and then briefly centrifuge the tube.

☐ **2** Suspend magnetic beads in diluted PCR product:

Component	Vol. (µL)
Diluted PCR product	50
Agencourt AMPure XP magnetic beads (Beckman Coulter PN A63880)	40
Total	**90**

a Ensure that the Agencourt AMPure XP magnetic beads are at room temperature, and then vortex the beads at high speed for 20 sec.

b Pipet the Agencourt AMPure XP magnetic beads into the same tube with the diluted PCR product (see step 1). Expel any beads left in the pipette tip by pipetting the suspension up and down 10 times.

☐ **3** Vortex the suspension at high speed for 20 sec.

☐ **4** Incubate the suspension at room temperature for 10 min.

☐ **5** Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 min.

☐ **6** Without disturbing the beads, and keeping the tube on the magnetic stand, remove and discard the supernatant.

☐ **7** Use a P10 pipette to remove any residual supernatant from the tube.

☐ **8** Wash the beads three times with 80% ethanol:

a Keeping the tube on the magnetic stand, pipet 190 µL of 80% ethanol to wash the beads.

b Incubate the tube at room temperature for 30–60 sec.

c Without disturbing the beads, and keeping the tube on the magnetic stand, remove and discard the ethanol.

d Repeat Steps 8a–8c two more times. Completely remove and discard all of the 80% ethanol.

☐ Wash 1 ☐ Wash 2 ☐ Wash 3

☐ **9** Transfer the tubes to a rack and open the tube. Remove any remaining ethanol by drying the beads at 37 °C for 1 min or air-drying the beads at room temperature for 10–15 min.

☐ **10** To the dried beads, pipet 45 µL of DNA Dilution Reagent. Vortex the suspension at high speed for 20 sec.

☐ **11** Incubate the suspension at room temperature for 2 min.

☐ **12** Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 min.

☐ **13** Keeping the tube on the magnetic stand, pipet the entire eluate to a new tube labeled "LIB w/ADAP." The eluate contains the final library for sequencing.

STOPPING POINT You can store the sequencing library at 4 °C for up to one week or at –20 °C for longer storage.

Perform Quality Control on Sequencing Library (after sequencing adapter is added)

In a post-PCR room, quantify an aliquot of the library and perform Agilent Bioanalyzer analysis on aliquots of the purified library (before sequencing adapter is added) and sequencing library (after sequencing adapter is added).

Sequence the Library

Sequence the sequencing library (after sequencing adapter is added) on an Illumina sequencer.

IMPORTANT The sequencer must support 300-cycle sequencing chemistry.

For technical support visit fluidigm.com/support.

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