Advanta NGS Library Preparation on the LP 192.24 IFC with Juno

IMPORTANT Before using this quick reference, read and understand the detailed instructions and safety guidelines in the Advanta™ NGS Library Preparation with Juno™ Protocol (PN 101-7878).

Workflow Overview

	Workflow Step
Pre-PCR room	
1	Dilute the stock assay pools (ASY-MPX or ASY-MPX-P).*
2	Prepare the 10X assay pools and sample mixes.
3	Load and run the LP 192.24 IFC on Juno and harvest samples.†
Post-PCR room	
4	Pool the harvested samples.†
5	Clean up the pooled samples (3x).†
6	Thermal-cycle the samples to add P5 sequencing adapters to the library.
7	Clean up the final sequencing library.†
8	Quantify the sequencing library.

*Skip this step if using the diluted ASY-MPX-P assay pools (see Obtain Target-Specific Assays).

† Potential stopping point.

Obtain Target-Specific Assays

Design and order the target-specific assays ASY-MPX or ASY-MPX-P from d3.fluidigm.com. For detailed instructions about designing and ordering assays, see the D3™ Assay Design User Guide (PN 100-6812).

Prepare Assay Mixes and Sample Mixes

Prepare the assay and sample mixes in a **pre-PCR** room.

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.

Dilute the Stock Assays

IMPORTANT If you are using the ASY-MPX-P diluted assay pools plate, skip this dilution step and proceed to Prepare the Assay Pre-Mix.

☐ **1** Immediately before use, ensure that each stock assay plate is securely sealed, and then vortex for 10–20 sec to mix. Centrifuge each assay plate at 3,000 × *g* for 5 min.

☐ **2** In a DNA-free hood, dilute each pool of stock assays with DNA Suspension Buffer as calculated in Table 1. An example using the recommended volume of 2 µL for each assay from a single 96-assay pool is shown in Figure 1.

- **a** Using an 8-channel pipette with fresh pipette tips for each well, transfer the appropriate assay volume (y) from each column in the stock assay plate to a new 8-well strip.
- **b** Pipet the appropriate volume of DNA Suspension Buffer into a new 1.5 mL microcentrifuge tube.
- **c** Transfer the entire volume from each well of the 8-well strip to the tube.

Table 1. Diluted stock assays

Component	Starting Conc. (µM)	Number of Assays in Pool	Volume for 20X Assay Mix (µL)
Targeted DNA Seq Library Assay, one pool (Fluidigm PN ASY-MPX or ASY-MPX-P)	450/150	*N*	y
DNA Suspension Buffer (Teknova PN T0221)	—	—	(180 − *N*) × y
Total	**—**	***N***	**y + [(180 − *N*) × y]**

NOTE The final concentration for forward primers in each unique pool is 2.5 µM, and the final concentration for reverse primers is 833 nM.

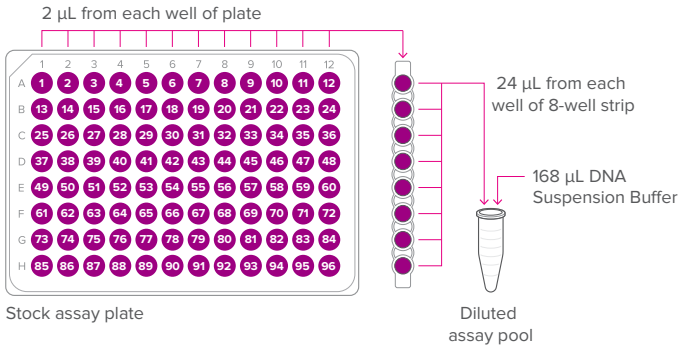


Figure 1. Example dilution of a 96-assay pool

☐ **3** Repeat Step 2 for all other stock assay pools.

☐ **4** Keep diluted assay pools at room temperature until use.

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 2 in a new 1.5 mL microcentrifuge tube.

Table 2. Assay pre-mix

Component	Volume for 24 Assay Inlets (µL)*
TSP Assay Loading Reagent (Fluidigm PN 101-0409)	75
PCR Water (Fluidigm PN 100-5941)	1,125
Total	**1,200**

*Includes overage. Volume is sufficient for 1 IFC. Scale up appropriately for multiple IFCs.

☐ **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 10X Assay Mixes

☐ **1** Vortex the diluted assay pools for 10–20 sec to mix, and then centrifuge them at 3,000 × *g* for 5 min.

☐ **2** In a DNA-free hood, combine the components shown in Table 3 in the wells of a new PCR plate according to the layout shown in Figure 2.

Table 3. 10X assay mixes

Component	Volume per Assay Pool (µL)*
Assay pre-mix (see Table 2)	40
Diluted assay pools (see Table 1) or PCR Water†	10
Total	**50**

*Includes overage

†For unused assay inlets, replace the diluted assay pools with 10.0 µL of PCR Water (Fluidigm PN 100-5941).

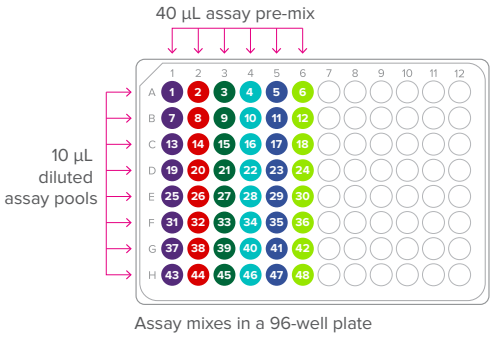


Figure 2. Layout of 10X assay mixes (per-well transfer volumes)

☐ **3** Seal the assay plate with clear adhesive film.

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 using volumes shown in Table 4.

IMPORTANT

- Components must be combined in the order shown in Table 4. 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. Pipette slowly.

Table 4. Sample pre-mix

	Component	Volume per IFC (µL)*
1	PCR Water (Fluidigm PN 100-5941)	72
2	4X TSP Master Mix (Fluidigm PN 101-5786)	300
3	TSP Sample Loading Reagent v2 (Fluidigm PN 101-7633)	60
4	TSP DNA Polymerase (Fluidigm PN 101-0995)	48
Total		**480**

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

IMPORTANT To prevent introducing bubbles, pipette 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.

☐ **5** Keep the sample pre-mix on ice until use.

Prepare the Sample Mixes

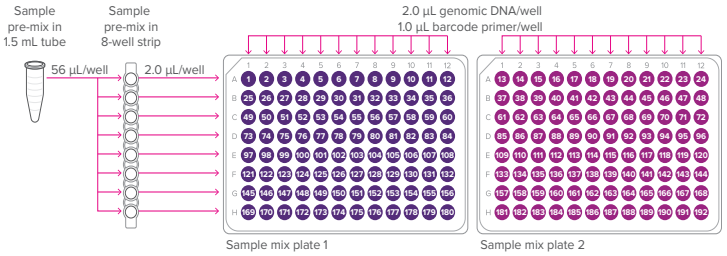


Figure 3. Preparation of sample mixes (per-well transfer volumes)

☐ **1** Centrifuge the TSP 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 two new 96-well plates. 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	Volume per Reaction (µL)*
Sample pre-mix (see Table 4)	2.0
Genomic DNA sample (100–200 ng/µL)	2.0
Barcode primer from TSP Barcode Plate (Fluidigm PN 101-0744)	1.0
Total	**5.0**

*Includes overage

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

☐ **4** Tightly seal each 96-well sample-mix 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** Keep sample mixes on ice until use.

Load and Run the LP 192.24 IFC

Load the Control Line Fluid and Harvest Reagent

For detailed instructions about injecting Juno Control Line Fluid 192.24, 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

- To ensure correct accumulator volume, use only syringes containing Juno Control Line Fluid 192.24.
- 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.

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

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

☐ **3** Inject the entire contents of one 150 µL Juno Control Line Fluid 192.24 syringe into each accumulator on the IFC (see Figure 4), **avoiding spills**.

IMPORTANT Before removing the syringe from the accumulator, ensure that all of the Control Line Fluid and air are purged from the syringe to avoid dripping fluid on the surface of the IFC.

☐ **4** Place the IFC on a flat surface and pipet 27 µL of TSP Harvest Reagent into each of the inlets P1, P2, P3, and P4 (see Figure 4).

☐ **5** Pipet 220 µL of TSP Harvest Reagent into each of the harvest reservoirs H1, H2, H3, and H4 (see Figure 4).

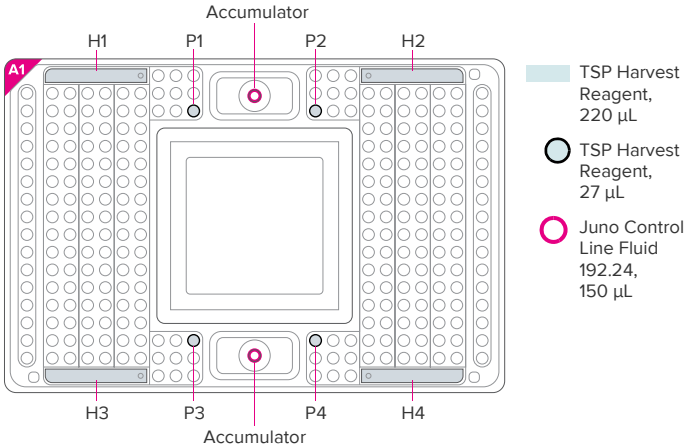
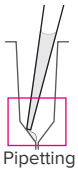


Figure 4. Priming map for the LP 192.24 IFC

Load the Assay and Sample Mixes

IMPORTANT

- 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:
  - We recommend using an 8-channel pipette.
  - Dispense the reagents while making contact with the side of the inlet near the bottom of the well, 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 bottom of the inlet

☐ **1** Pipet the 10X assay mixes and sample mixes into the LP 192.24 IFC.

a Carefully pipet 3.5 µL of each 10X assay mix from the assay plate into the designated assay inlets on the IFC, as shown in Figure 5.

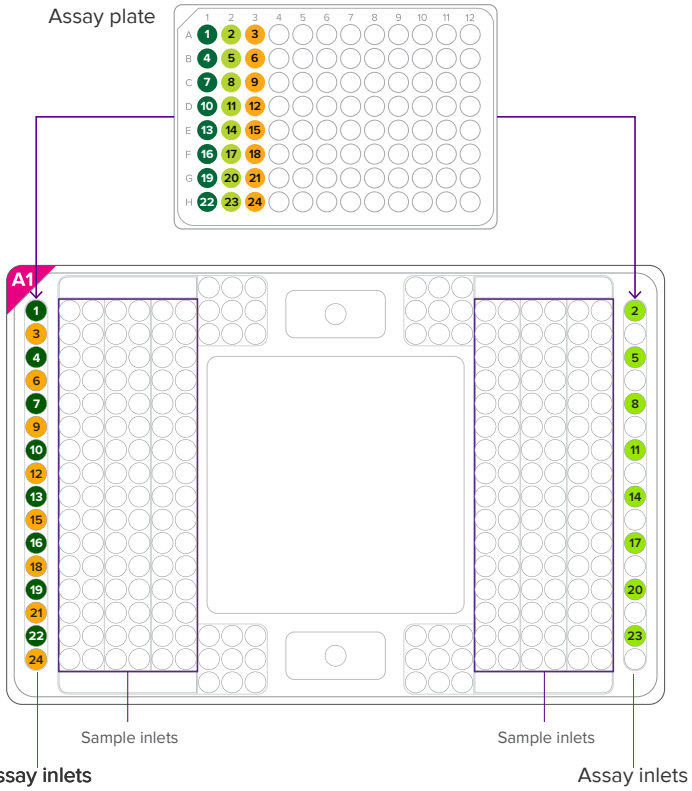


Figure 5. Assay loading map

b Carefully pipet 3.5 µL of each sample mix from the sample mix plates into the designated sample inlet of the IFC based on the predefined sample map, as shown in Figure 6 on page 4.

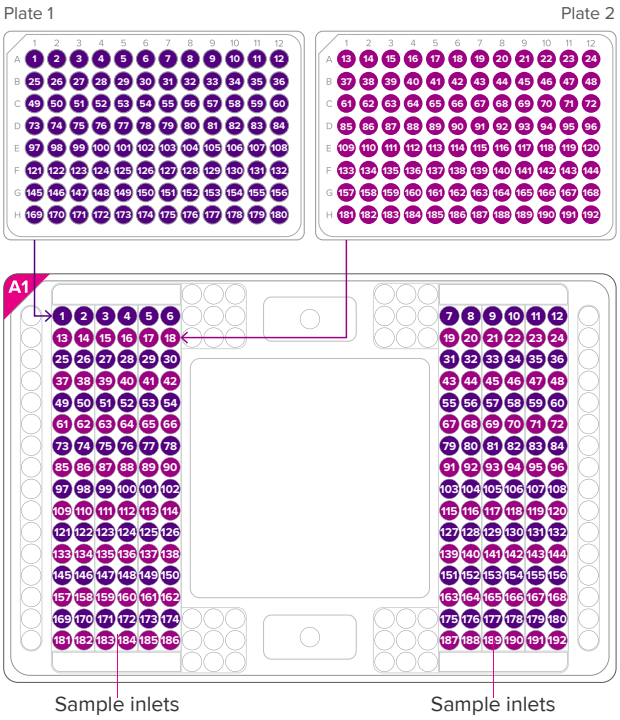


Figure 6. Sample loading map

☐ **2** Cover all sample inlets with LP 192.24 Barrier Tape.

Run the IFC

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

☐ **2** On the Juno Scripts screen, tap **One Step LP—192.24**.

☐ **3** Select when the script should finish (if necessary, adjust the harvest time), and then tap **RUN**. The run takes approximately 5.5 hours to complete the load/mix, thermal cycle, and harvest steps. You can delay the harvest step for up to 16 hours.

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

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

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

Pool the Harvested Samples

Pool the harvested samples in a **post-PCR** room.

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, as shown in Figure 7.

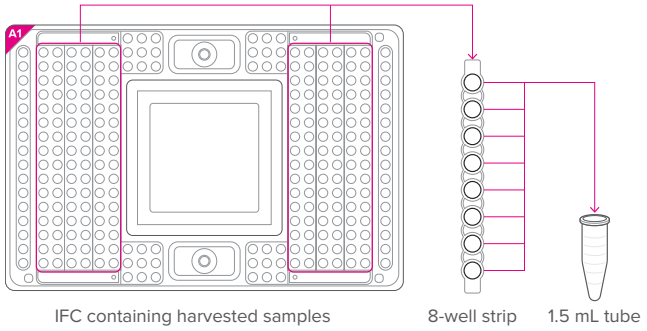


Figure 7. Process for pooling harvested samples

☐ **1** Carefully remove the LP 192.24 Barrier Tape from the IFC by placing the IFC on a flat surface, holding the IFC with one hand, and slowly pulling the tab of the barrier tape until the tape is peeled away from the sample inlets.

☐ **2** Set an 8-channel pipette to 6.0 µL to transfer and combine the entire harvest volumes from the sample inlets of the LP 192.24 IFC directly into an 8-well strip. Each harvest volume should be 3–5 µL.

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

NOTE Because all of the samples are barcoded, it is not necessary to change pipette tips when harvesting and pooling the samples.

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

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.

NOTE 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.

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.
- Before proceeding, see the Agencourt AMPure XP PCR Purification Instructions for Use Guide (Agencourt PN B37419) for further information and troubleshooting tips.
- Fully dispense the magnetic bead suspension from the 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 harvested sample pools.

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 hours.

☐ **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 supernatant.

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

Component	Volume 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 until the solution is clear.

☐ **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 until the solution is clear.

☐ **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 a–c 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 until the solution is clear.
- **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	Volume 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 until the solution is clear.

☐ **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 a–c 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 until the solution is clear.
- **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.

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)
4X TSP Master Mix (Fluidigm PN 101-5786)	7.5
TSP Adapter Mix (Fluidigm PN 101-0408)*	6.0
Purified library	4.5
PCR Water (Fluidigm PN 100-5941)	12.0
Total	**30.0**

*For dual indexing, replace TSP Adapter Mix with a Dual Index Adapter Mix from the Targeted DNA Seq Library 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 hour.

Clean Up the PCR Product (0.8X SPRI)

Clean up the PCR product 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 5).

☐ **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	Volume (µL)
Diluted PCR product	50
Agencourt AMPure XP magnetic beads	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 until the solution is clear.

☐ **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 a–c 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 until the solution is clear.

☐ **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.

For technical support visit fluidigm.com/support.

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