Advanta 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™ NGS Library Preparation with Juno™ Protocol (PN 101-7878).

**Workflow Overview**

<table>
<thead>
<tr>
<th>Workflow Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-PCR room</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Combine and dilute the stock assays (ASY-MPX or ASY-MPX-P).*</td>
</tr>
<tr>
<td>2</td>
<td>Prepare the 20X assay pools and sample mixes.</td>
</tr>
<tr>
<td>3</td>
<td>Prime the LP 48.48 IFC (integrated fluidic circuit) on Juno.</td>
</tr>
<tr>
<td>4</td>
<td>Load and run the LP 48.48 IFC on Juno and harvest samples.†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Post-PCR room</strong></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Pool the harvested samples.†</td>
</tr>
<tr>
<td>6</td>
<td>Clean up the pooled samples (3x).†</td>
</tr>
<tr>
<td>7</td>
<td>Thermal-cycle the samples to add P5 sequencing adapters to the library.</td>
</tr>
<tr>
<td>8</td>
<td>Clean up the final sequencing library.†</td>
</tr>
<tr>
<td>9</td>
<td>Quantify the sequencing library.</td>
</tr>
</tbody>
</table>

*Skip this step if using the ASY-MPX-P diluted assay plate (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** Thaw reagents at room temperature. Mix and centrifuge reagents as directed. 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.

**Combine and Dilute the Stock Assays**

**IMPORTANT** If you are using the ASY-MPX-P diluted assay 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, combine and dilute one pool of stock assays at a time with DNA Suspension Buffer as calculated in Table 1, where:
  - \( X \) = unique stock assay pool
  - \( N \) = number of assays in pool \( X \)
  - \( y \) = μL volume to use per assay well (recommended: 2 μL)

  Following Figure 1 as an example:
  - a Using an 8-channel pipette with fresh pipette tips for each well, transfer the appropriate assay volume 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.

**Workflow Step**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for Dilution (μL)</th>
<th>Example Dilution from Figure 1 (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeted DNA Seq Library Assay, from pool ( X ) (Fluidigm PN ASY-MPX or ASY-MPX-P)</td>
<td>( N \times y )</td>
<td>96 × 2</td>
</tr>
<tr>
<td>DNA Suspension Buffer (Teknova PN T0221)</td>
<td>((180 − N) \times y)</td>
<td>((180 − 96) \times 2)</td>
</tr>
<tr>
<td>Total</td>
<td>((N \times y) + [(180 − N) \times y])</td>
<td>((96 \times 2) + [(180 − 96) \times 2])</td>
</tr>
</tbody>
</table>

**NOTE** The concentration of forward primers for each unique assay is 450 μM (before pooling) and 2.5 μM (after pooling). The concentration of reverse primers is 150 μM (before pooling) and 833 nM (after pooling).

Figure 1. Example dilution of a 96-assay pool

☐ 3 Repeat Step 2 for each unique stock assay pool \( X \).

☐ 4 Reseal each stock assay plate and store at ~20 °C.
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 2.0 mL microcentrifuge tube.

Table 2. Assay pre-mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol. for 48 Assay Inlets (μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSP Assay Loading Reagent (Fluidigm PN 101-0409)</td>
<td>150</td>
</tr>
<tr>
<td>PCR Water (Fluidigm PN 100-5941)</td>
<td>1,650</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1,800</strong></td>
</tr>
</tbody>
</table>

*Includes overage. Volume is sufficient for 10 IFCs. Scale down appropriately for fewer 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 20X 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. 20X assay mixes

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Assay Pool (μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay pre-mix (see Table 2) or PCR Water†</td>
<td>30</td>
</tr>
<tr>
<td>Diluted assay pools (see Table 1) or PCR Water†</td>
<td>20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

*Includes overage.†For unused assay inlets, replace the diluted assay pools with 20.0 μL of PCR Water (Fluidigm PN 100-5941).

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 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

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per IFC (μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PCR Water (Fluidigm PN 100-5941)</td>
<td>40</td>
</tr>
<tr>
<td>2 4X TSP Master Mix (Fluidigm PN 101-3055)</td>
<td>100</td>
</tr>
<tr>
<td>3 TSP Sample Loading Reagent v2 (Fluidigm PN 101-7634)</td>
<td>20</td>
</tr>
<tr>
<td>4 TSP DNA Polymerase (Fluidigm PN 101-0995, do not vortex)</td>
<td>16</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>176</strong></td>
</tr>
</tbody>
</table>

*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, 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.

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.
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 on page 2. Use an 8-channel pipette to transfer the sample pre-mix from the 8-well strip.

Table 5. Sample mixes

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol. per Reaction (μL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample pre-mix (see Table 4)</td>
<td>2.5</td>
</tr>
<tr>
<td>Genomic DNA sample (50–100 ng/μL)</td>
<td>2.5</td>
</tr>
<tr>
<td>Barcode primer from TSP Barcode Plate (Fluidigm PN 101-0744)</td>
<td>1.25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6.25</strong></td>
</tr>
</tbody>
</table>

*Includes overage.

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

Tightly seal the sample mix plate with clear adhesive film.

Prime the LP 48.48 IFC on Juno

For detailed instructions about injecting LP 48.48 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**
- 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.

Ensure that the MX Interface Plate is installed in Juno.

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

Inject the entire contents of one 300 μL LP 48.48 Control Line Fluid 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.

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).

Immediately before use, ensure that the assay and sample mix plates are securely sealed, and then vortex thoroughly for 20 sec to mix. Centrifuge the plates at 2,500–3,000 × g for 5 min.

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.
3. Pipet the 20X assay mixes and sample mixes into the LP 48.48 IFC as shown in Figure 5.
   a. Carefully pipet 4.0 μL of each 20X assay mix from the plate into the designated assay inlets on the IFC.
   b. Carefully pipet 4.0 μL of each sample mix from the plate into the designated sample inlet of the IFC based on the predefined sample map.

4. Cover the sample and assay inlets with LP 48.48 Barrier Tape.

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


7. Select when the script should finish (if necessary, adjust the harvest time), and then tap RUN. The run takes approximately 4 hours to complete the load/mix, thermal cycle, and harvest steps. You can delay the harvest step for up to 16 hours.

8. After the run is finished, tap EJECT to eject the IFC. IMPORTANT Eject the IFC ≤60 min after the run is complete.

9. 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.

1. Carefully remove the LP 48.48 Barrier Tape from the sample chamber of 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 12.0 μL to transfer and combine the entire harvest volumes from the sample inlets of the LP 48.48 IFC directly into an 8-well strip. 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.

   **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 the supernatant.

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol. for First Cleanup (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled samples</td>
<td>150</td>
</tr>
<tr>
<td>Agencourt AMPure XP magnetic beads</td>
<td>60</td>
</tr>
<tr>
<td>(Beckman Coulter PN A63880)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>210</td>
</tr>
</tbody>
</table>
  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.
  - 1 Wash 1  2 Wash 2  3 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol. for Second and Third Cleanup (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate</td>
<td>30</td>
</tr>
<tr>
<td>Agencourt AMPure XP magnetic beads</td>
<td>24</td>
</tr>
<tr>
<td>(Beckman Coulter PN A63880)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>54</td>
</tr>
</tbody>
</table>
  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.
Prepare the eluate:
Transfer the tube to a rack and open the tube. Remove any
residual supernatant from the tube.

Perform the third cleanup (0.8X SPRI) by repeating Steps 1–9
without disturbing the beads, and keeping the tube on
the magnetic stand, remove and discard the 80% ethanol.

To assess library size and purity, store the remaining purified
eluate from the third cleanup is the purified library (before
sequencing adapter is added). Label and save this tube.

Add Sequencing Adapter to Purified Library
Add the sequencing adapter in a post-PCR room.

Vortex reagents for 20 sec, and then briefly centrifuge them
at high speed for 20 sec.

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

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.
☐ 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. The eluate contains the final sequencing library (after sequencing adapter is added). Label and save this tube.

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.