Targeted DNA Sequencing Library Preparation with the LP 48.48 IFC on Juno

For safety information and detailed procedures, see the Targeted DNA Sequencing Library Preparation with Juno™ Getting Started Guide (PN 101-0414) and the Juno System User Guide (PN 100-7070).

IMPORTANT

- This document is for preparing targeted DNA sequencing libraries with Targeted DNA Seq Library Reagent kits only. To prepare sequencing libraries with Advanta™ NGS Library Prep Reagent Kits, see the Advanta NGS Library Preparation with Juno Protocol (PN 101-7878).
- Be certain that all reagents are thawed completely to room temperature and mixed thoroughly prior to use.

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).

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 the stock assay plate is securely sealed, and then vortex for 10–20 seconds to mix. Centrifuge the assay plate at 3,000 × *g* for 5 minutes.

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

Component	Starting Concentration (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.

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.

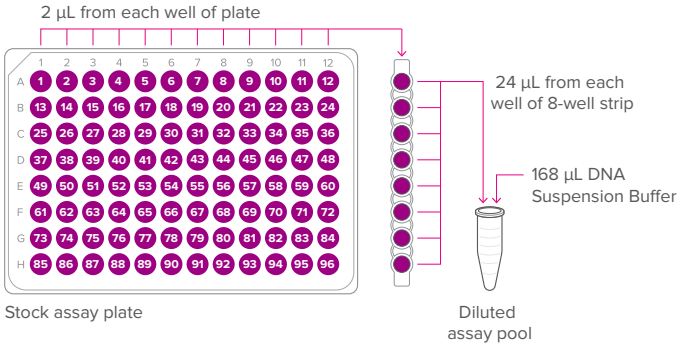


Figure 1. Example dilution of a 96-assay pool

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 In a DNA-free hood, combine the following components in a new 2 mL microcentrifuge tube:

Component	Volume per Assay Pool (µL)	Volume for 48 Assay Pools (µL)*
TSP Assay Loading Reagent (PN 101-0409)	2.5	150
PCR Water (PN 100-5941)	27.5	1,650
Total	**30.0**	**1,800**

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

2 Vortex the assay pre-mix for ≥20 seconds, and then briefly centrifuge it to bring down all components.

Prepare the 20X Assay Pools

1 Vortex the diluted assay pools for 10–20 seconds to mix, and then centrifuge them at 3,000 x *g* for 5 minutes.

2 In a DNA-free hood, combine the following components in wells of a new PCR plate or in 8-well strips according to the layout shown (see Figure 2):

Component	Volume per Assay Pool (µL)*
Assay pre-mix (See Prepare the Assay Pre-Mix on page 1.)	30.0
Diluted assay pools (see Dilute the Stock Assays on page 1) or PCR Water†	20.0
Total	**50.0**

*Includes overage.
†For unused assay inlets, replace the diluted assay pools with 20.0 µL of PCR Water.

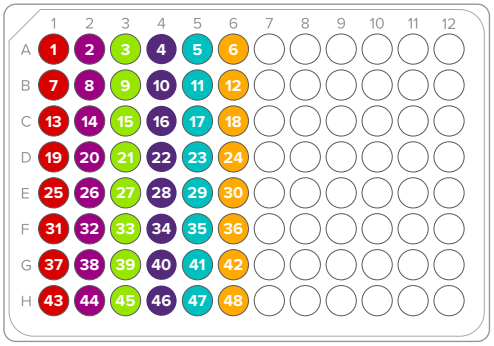


Figure 2. Layout of 20X assay pools and sample mixes

3 Seal the assay plate with clear adhesive film or cap the 8-well strips.

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, combine the following components in a new 1.5 mL microcentrifuge tube:

IMPORTANT Components must be combined in the order below. You add 4X TSP Master Mix to the PCR Water to dilute it before adding the remaining reagents.

Component	Volume per IFC (µL)*
1 PCR Water (PN 100-5941)	40
2 4X TSP Master Mix (PN 101-3050)	100
3 TSP Sample Loading Reagent (PN 101-3049)	20
4 TSP DNA Polymerase (PN 101-0995)	16
Total	**176**

*Includes overage.

3 Vortex the sample pre-mix for 10–20 seconds, and then briefly centrifuge it 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.

IMPORTANT To prevent introducing bubbles during this step, 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 seconds.

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

Prepare Sample Mixes

1 Centrifuge the TSP Barcode Plate at 3,000 × *g* for 3 minutes before using.

2 Obtain one 96-well plate to prepare 48 individual sample mix solutions.

3 In a DNA sample hood, pipet the following components into each well of the new 96-well plate according to the layout shown in Figure 2). Use an 8-channel pipette to transfer the sample pre-mix from the 8-well strip.

Component	Volume per Reaction (µL)*
Sample pre-mix (See Prepare the Sample Pre-Mix.)	2.5
Genomic DNA sample (50–100 ng/µL)	2.5
Barcode primer (Fluidigm PN 101-0744)	1.25
Total	**6.25**

*Includes overage.

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

5 Tightly seal the 96-well sample mix plate with clear adhesive film, vortex thoroughly for 20 seconds, and then centrifuge the plate at 2,500–3,000 × *g* for 5 minutes.

6 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 plate again at 2,500–3,000 × *g* for 5 minutes.

7 Keep the sample mixes on ice until use.

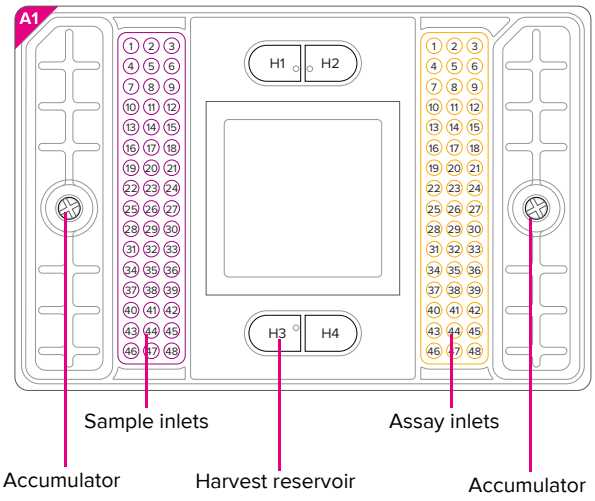
Prime the LP 48.48 IFC on Juno

IMPORTANT

- Control line fluid on the integrated fluidic circuit (IFC) or in the inlets makes the IFC unusable. Use a new IFC.
- Vortex thoroughly and centrifuge all assay and sample mixes before pipetting into IFC inlets. Failure to do so may result in decreased data quality.
- Do not go past the first stop on the pipette.
- Before loading, ensure that there are no bubbles in the inlets.

1 Ensure that the Interface Plate MX is installed in the instrument. [See the Juno System User Guide (PN 100-7070).]

2 Review the LP 48.48 IFC:



3 Pull the protective tape down and away from the bottom of the IFC. Do not invert the IFC.

4 Print the life-size map of the LP 48.48 IFC, place it under the IFC, and align the notched A1 corner at the upper left of the IFC with the pink corner of the IFC map. (For a life-size map, see the Targeted DNA Sequencing Library Preparation with Juno Getting Started Guide.)

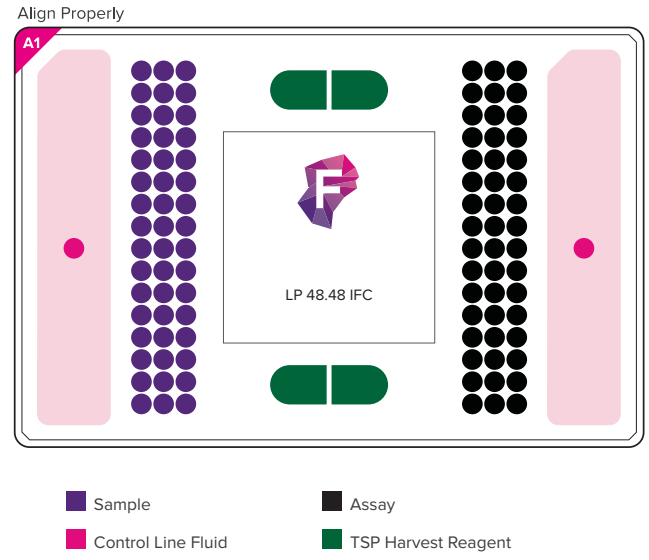


Figure 3. Map of the LP 48.48 IFC

5 Load an entire syringe of LP 48.48 Control Line Fluid into one accumulator and a second syringe into the other accumulator (dark pink circles in Figure 3). Use only LP 48.48 Control Line Fluid.

6 Pipet 650 µL of TSP Harvest Reagent (PN 101-0743) into each of the H1, H2, H3, and H4 harvest reservoirs (green hemispheres in Figure 3).

7 Place the IFC on the tray of the Juno™ instrument, and then tap **LOAD**. [For detailed instructions on Juno operation, see the Juno System User Guide (PN 100-7070).]

8 On the Juno Scripts screen, tap **Prime LP–48.48**. Priming the IFC takes ~5 minutes.

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

Load and Run the LP 48.48 IFC on Juno

1 Place the life-size map of the LP 48.48 IFC under the IFC and align the notched A1 corner at the upper left of the IFC with the pink corner of the IFC map. (For a life-size map, see the Targeted DNA Sequencing Library Preparation with Juno Getting Started Guide.)

2 Immediately before transferring into the IFC, vortex the 20X assays and sample mixes for 10–20 seconds to mix, and then centrifuge both plates at 3,000 x *g* for 5 minutes. If necessary, remove any large bubbles from the wells and centrifuge again at 3,000 x *g* for 5 minutes.

3 Pipet 4.0 µL of each 20X assay pool into an assay inlet on the IFC. [See Figure 3 (black circles) and the IFC pipetting scheme in Figure 4.)

4 Pipet 4.0 µL of each sample mix into a sample inlet on the IFC. [See Figure 3 (dark purple circles) and the IFC pipetting scheme in Figure 4.]

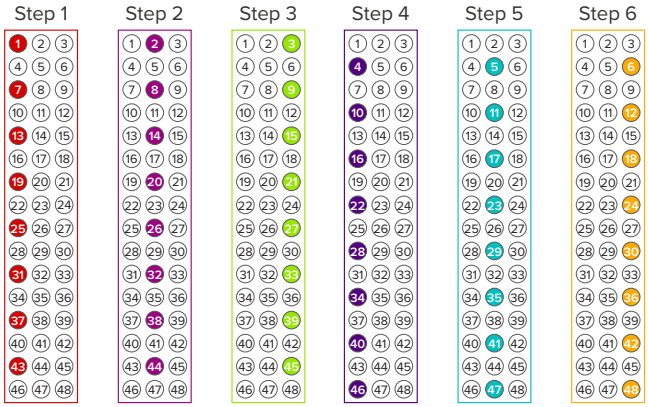


Figure 4. IFC pipetting scheme for samples and assay pools

5 Cover sample and assay inlets with LP 48.48 Barrier Tape. (See the Targeted DNA Sequencing Library Preparation with Juno Getting Started Guide.)

6 On Juno, tap **OPEN** and place the IFC on the tray, tap **LOAD**, and then tap **One Step LP–48.48**. Select when the script should finish (if necessary, adjust the harvest time), and then tap **RUN**.

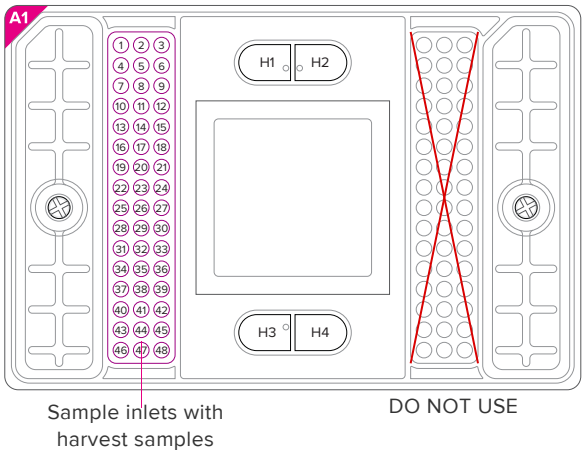
7 After the IFC is finished, tap **EJECT**.

IMPORTANT Eject the IFC ≤60 minutes after the run.

8 After ejecting the IFC, immediately proceed to the next section.

Pool the Harvested Samples

1 Review the map for harvesting samples:



2 Carefully remove the LP 48.48 Barrier Tape from the sample chamber of the IFC by putting 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 chamber

3 Place the life-size map of the LP 48.48 IFC under the IFC and align the notched A1 corner at the upper left of the IFC with the pink corner of the IFC map. (For a life-size map, see the Targeted DNA Sequencing Library Preparation with Juno Getting Started Guide.)

4 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.

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

5 Combine volumes from each 8-well strip into a single new 1.5 mL microcentrifuge tube per sample pool.

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.

First Cleanup (0.4X/0.9X Double-Sided Solid-Phase Reversible Immobilization)

1 Warm Agencourt® AMPure® XP magnetic beads to room temperature.

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

3 Prepare 5 mL of fresh 80% ethanol per library: Pipet 1 mL of DNase-free water into a graduated tube, and then add absolute ethanol to 5 mL. Cap the tube and mix.

4 Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 minute.

5 In a new 1.5 mL microcentrifuge tube, pipet 150 µL of pooled samples. If the volume of pooled samples is <150 µL, add DNA Dilution Reagent or PCR Water to bring the volume to 150 µL.

6 Pipet 60 µL of AMPure XP magnetic beads into the same tube with the 150 µL of pooled samples. Expel any beads left in the pipette tip by pipetting the suspension up and down 10 times.

7 Vortex the suspension at high speed for 20 seconds.

8 Incubate the suspension at room temperature for 10 minutes.

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

10 Keeping the tube on the magnetic stand, pipet the entire **supernatant** to a new tube.

11 Use a P10 pipette to transfer any residual volume to ensure that all supernatant has been transferred.

12 Pipet 75 µL of AMPure XP magnetic beads into the supernatant. Vortex the suspension at high speed for 20 seconds.

13 Incubate the suspension at room temperature for 10 minutes.

14 Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 minutes.

15 Keeping the tube on the magnetic stand, remove and discard the supernatant.

16 Use a P10 pipette to remove any residual supernatant from the tube.

17 Keeping the tube on the magnetic stand, pipet 400 µL of 80% ethanol to wash the beads.

18 Incubate the tube at room temperature for 30–60 seconds.

19 Keeping the tube on the magnetic stand, remove and discard the ethanol.

20 Repeat steps 17–19 two more times. Remove all ethanol.

21 Remove the tube from the magnetic stand and dry the beads at 37 °C for 1 minute, or at room temperature for 10–15 minutes.

IMPORTANT Ensure that both the beads and tube are dry before proceeding, but be careful not to over dry the beads. If over dried, beads look cracked.

22 To the dried beads, pipet 30 µL of DNA Dilution Reagent (PN 100-9167). Vortex for 20 seconds.

23 Incubate the suspension at room temperature for 2 minutes.

24 Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 minutes.

25 Keeping the tube on the magnetic stand, pipet 30 µL of the eluate to a new tube.

STOPPING POINT Store at 4 °C for up to one week or at –20 °C for longer storage.

Second Cleanup (0.8X SPRI)

1 Vortex the AMPure XP magnetic beads at high speed for 20 seconds.

2 Pipet 24 µL of Agencourt AMPure XP magnetic beads into the same tube with the 30 µL of eluate from the first cleanup. 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 seconds.

4 Incubate the suspension at room temperature for 10 minutes.

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

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

9 Incubate the tube at room temperature for 30–60 seconds.

10 Keeping the tube on the magnetic stand, remove and discard the ethanol.

11 Repeat steps 8–10 two more times. Remove all ethanol.

12 Remove the tube from the magnetic stand and dry the beads at 37 °C for 1 minute, or at room temperature for 10–15 minutes.

IMPORTANT Ensure that both the beads and tube are dry before proceeding, but be careful not to over dry the beads. If over dried, beads look cracked.

13 To the dried beads, pipet 30 µL of DNA Dilution Reagent. Vortex 20 seconds.

14 Incubate the suspension at room temperature for 2 minutes.

15 Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 minutes.

16 Keeping the tube on the magnetic stand, pipet 30 µL of the eluate to a new tube.

STOPPING POINT Store the tube at 4 °C for up to 1 week –20 °C for longer storage.

Third Cleanup (0.8X SPRI) and Quality Control

1 Repeat steps 1–16 in Second Cleanup (0.8X SPRI) with 30 µL eluate from the second cleanup. This is the purified library (before sequencing adapter is added). Store the purified library from the third cleanup at 4 °C or perform QC.

2 Perform QC by estimating the purified library concentration by fluorometer and, if necessary, analyzing the purified library by Agilent® Bioanalyzer®.

Add the Sequencing Adapter to the Purified Library

1 Combine in a new PCR tube:

Component	Vol. per Reaction (µL)*
4X TSP Master Mix (PN 101-3050)	7.5
TSP Adapter Mix † (PN 101-0408)	6.0
Purified library (before sequencing adapter is added)	4.5
PCR Water (PN 100-5941)	12.0
Total	**30.0**

*Includes overage.
† For dual barcoding, replace the TSP Adapter Mix with a dual index adapter mix from the Targeted DNA Seq Library Adapter Set (PN 101-2412).

2 Perform PCR using a stand-alone thermal cycler:

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

Clean Up the PCR Product (0.8X SPRI)

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

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

3 Pipet 40 µL of room temperature AMPure XP magnetic beads into the same tube with the 50 µL of diluted PCR product. (See step 1.) Expel any beads left in the pipette tip by pipetting the suspension up and down 10 times.

4 Vortex the suspension at high speed for 20 seconds.

5 Incubate the suspension at room temperature for 10 minutes.

6 Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 minutes.

7 Keeping the tube on the magnetic stand, remove and discard the supernatant.

8 Use a P10 pipette to remove any residual supernatant from the tube.

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

10 Incubate the tube at room temperature for 30–60 seconds.

11 Keeping the tube on the magnetic stand, remove and discard the ethanol.

12 Repeat steps 9–11 two more times. Completely remove and discard all of the 80% ethanol.

13 Remove the tube from the magnetic stand and dry the beads at 37 °C for 1 minute, or at room temperature for 10–15 minutes.

IMPORTANT Ensure that both the beads and tube are dry before proceeding, but be careful not to over dry the beads. If over dried, beads look cracked.

14 To the dried beads, pipet 45 µL of DNA Dilution Reagent. Vortex for 20 seconds.

15 Incubate the suspension at room temperature for 2 minutes.

16 Briefly centrifuge the tube, and then place the tube on a magnetic stand for 1–2 minutes.

17 Keeping the tube on the magnetic stand, pipet the entire eluate to a labeled tube. The eluate is the sequencing library.

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

18 Compare the purified library (before sequencing adapter is added) and the sequencing library (after sequencing adapter is added) using the Agilent Bioanalyzer to ensure that the library with the sequencing adapter passes QC requirements.

19 Sequence the sequencing library (after sequencing adapter is added) on an Illumina® sequencer. Perform data analysis.

For technical support visit fluidigm.com/support.

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