

# Advanta RNA-Seq XT NGS Library Prep Kit and Kit II with UDIs and 96-Well Plates

**IMPORTANT** Before using this Quick Reference, read and understand the detailed instructions and safety guidelines in the protocol Advanta RNA-Seq XT NGS Library Prep Kit (101-9950) and Kit II (101-9959) for Use with Unique Dual Indexes (UDIs), FLDM-00146.

The Advanta™ RNA-Seq XT NGS Library Prep Kit (101-9950) contains 4 A plates of UDIs in PCR Master Mix, and the Advanta RNA-Seq XT NGS Library Prep Kit II (101-9959) contains 2 A plates and 2 B plates of UDIs in PCR Master Mix. Each kit uses unique dual indexes (UDIs) with the 48.Atlas™ IFC and produces up to 192 cDNA libraries.

**NOTE** Before you begin, print the full-size pipetting map of the 48.Atlas IFC (integrated fluidic circuit) at the end of this document.

## Workflow Overview

### Workflow Step

#### Pre-PCR room







- 1 Prepare reagent mixes, unique dual indexes (UDIs), samples, and IFC:
  - a Prepare the reverse transcription (RT) and fragmentation reagent mixes.
  - b Prepare the UDI-PCR Mix plate by thawing on ice.
  - c Prepare the RNA samples.
  - d Load reagents, UDI-PCR mixes, samples, and mRNA Seq Beads in 48.Atlas IFC.
- 2 Run 48.Atlas RNA-Seq LP script on Juno™.

#### Post-PCR room





- 3 Off the IFC, collect the sample libraries for pooling.
- 4 Quantify and normalize the sample libraries.
- 5 Generate 6 pooled libraries.
- 6 Clean up and enrich the sample libraries.
  - a Purify the pooled libraries using Agencourt® AMPure XP® beads.
  - b Enrich the pooled libraries by PCR.
  - c Further purify the libraries using the Agencourt AMPure XP beads.
- 7 Quantify the final libraries by qPCR.
- 8 Pool libraries prior to sequencing.
- 9 Perform sequencing on an Illumina® instrument.

## Prepare the RT and Fragmentation Reagents

### RT Master Mix (4 on Loading map)

Component	Volume (μL)
Nuclease-Free Water (101-9100) 	4.1
RT Buffer (101-9093) 	2.4
TSO Mix (101-9091) 	9.2
Loading Reagent Type 1 (101-9301) 	1.1
RNase Inhibitor (101-9095) 	1.1
RT Enzyme (101-9094) 	4.1
<b>Total (Vortex 5 sec, centrifuge 10 sec, keep tube on ice.)</b>	<b>22.0</b>

### Fragmentation Master Mix (3 on Loading map)

Component	Volume (μL)
Nuclease-Free Water (101-9100) 	11.0
RT Buffer (101-9093) 	7.3
Loading Reagent Type 1 (101-9301) 	1.1
N6 (101-9092) 	2.6
<b>Total (Vortex 5 sec, centrifuge 10 sec, keep tube on ice.)</b>	<b>22.0</b>

## Thaw the UDIs On Ice

Example of UDI-PCR Mix **Plate A** index mixes **1–48**—10 μL per well (101-9954):

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 <sup>15-17,1</sup>	A2 <sup>15-9,7,9</sup>	A3 <sup>15-17,17</sup>	A4 <sup>15-25,7,25</sup>	A5 <sup>15-33,33</sup>	A6 <sup>15-41,41</sup>						
B	B1 <sup>15-2,7,2</sup>	B2 <sup>15-10,7,10</sup>	B3 <sup>15-18,7,18</sup>	B4 <sup>15-26,7,26</sup>	B5 <sup>15-34,34</sup>	B6 <sup>15-42,42</sup>						
C	C1 <sup>15-3,7,3</sup>	C2 <sup>15-11,7,11</sup>	C3 <sup>15-19,7,19</sup>	C4 <sup>15-27,7,27</sup>	C5 <sup>15-35,35</sup>	C6 <sup>15-43,43</sup>						
D	D1 <sup>15-4,7,4</sup>	D2 <sup>15-12,7,12</sup>	D3 <sup>15-20,7,20</sup>	D4 <sup>15-28,7,28</sup>	D5 <sup>15-36,36</sup>	D6 <sup>15-44,44</sup>						
E	E1 <sup>15-5,7,5</sup>	E2 <sup>15-13,7,13</sup>	E3 <sup>15-21,7,21</sup>	E4 <sup>15-29,7,29</sup>	E5 <sup>15-37,37</sup>	E6 <sup>15-45,45</sup>						
F	F1 <sup>15-6,7,6</sup>	F2 <sup>15-14,7,14</sup>	F3 <sup>15-22,7,22</sup>	F4 <sup>15-30,7,30</sup>	F5 <sup>15-38,38</sup>	F6 <sup>15-46,46</sup>						
G	G1 <sup>15-7,7,7</sup>	G2 <sup>15-15,7,15</sup>	G3 <sup>15-23,7,23</sup>	G4 <sup>15-31,7,31</sup>	G5 <sup>15-39,39</sup>	G6 <sup>15-47,47</sup>						
H	H1 <sup>15-8,7,8</sup>	H2 <sup>15-16,7,16</sup>	H3 <sup>15-24,7,24</sup>	H4 <sup>15-32,7,32</sup>	H5 <sup>15-40,7,40</sup>	H6 <sup>15-48,48</sup>						

Example of UDI-PCR Mix **Plate B** index mixes **49–96**—10 μL per well (101-9955):

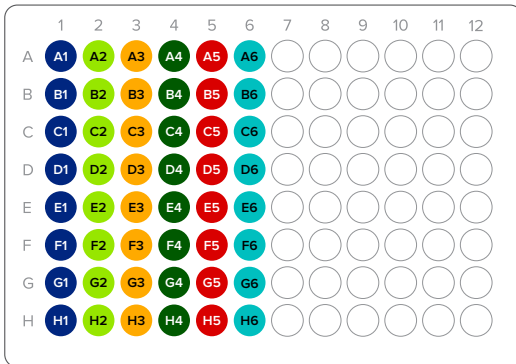
	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 <sup>15-49,7,49</sup>	A2 <sup>15-57,7,57</sup>	A3 <sup>15-65,7,65</sup>	A4 <sup>15-73,7,73</sup>	A5 <sup>15-81,7,81</sup>	A6 <sup>15-89,7,89</sup>						
B	B1 <sup>15-50,7,50</sup>	B2 <sup>15-58,7,58</sup>	B3 <sup>15-66,7,66</sup>	B4 <sup>15-74,7,74</sup>	B5 <sup>15-82,7,82</sup>	B6 <sup>15-90,7,90</sup>						
C	C1 <sup>15-51,7,51</sup>	C2 <sup>15-59,7,59</sup>	C3 <sup>15-67,7,67</sup>	C4 <sup>15-75,7,75</sup>	C5 <sup>15-83,7,83</sup>	C6 <sup>15-91,7,91</sup>						
D	D1 <sup>15-52,7,52</sup>	D2 <sup>15-60,7,60</sup>	D3 <sup>15-68,7,68</sup>	D4 <sup>15-76,7,76</sup>	D5 <sup>15-84,7,84</sup>	D6 <sup>15-92,7,92</sup>						
E	E1 <sup>15-53,7,53</sup>	E2 <sup>15-61,7,61</sup>	E3 <sup>15-69,7,69</sup>	E4 <sup>15-77,7,77</sup>	E5 <sup>15-85,7,85</sup>	E6 <sup>15-93,7,93</sup>						
F	F1 <sup>15-54,7,54</sup>	F2 <sup>15-62,7,62</sup>	F3 <sup>15-70,7,70</sup>	F4 <sup>15-78,7,78</sup>	F5 <sup>15-86,7,86</sup>	F6 <sup>15-94,7,94</sup>						
G	G1 <sup>15-55,7,55</sup>	G2 <sup>15-63,7,63</sup>	G3 <sup>15-71,7,71</sup>	G4 <sup>15-79,7,79</sup>	G5 <sup>15-87,7,87</sup>	G6 <sup>15-95,7,95</sup>						
H	H1 <sup>15-56,7,56</sup>	H2 <sup>15-64,7,64</sup>	H3 <sup>15-72,7,72</sup>	H4 <sup>15-80,7,80</sup>	H5 <sup>15-88,7,88</sup>	H6 <sup>15-96,7,96</sup>						

## Prepare the Samples

- 1 Vortex, centrifuge, and pipet 20 µL mRNA Binding Buffer (101-9079) into each 0.2 mL tube of a new 8-tube strip.
- 2 Pipet 2.4 µL per well mRNA Binding Buffer from the 8-tube strip into the first 6 columns of a new 96-well plate.
- 3 Mix total RNA and add 3.6 µL per well into the 48 wells of the 96-well plate that contain the mRNA Binding Buffer.

**NOTE** If there are less than 48 samples, use Nuclease-Free Water with the mRNA Binding Buffer.

The 48 samples are arranged in the 96-well plate as 8 rows (A–H) by 6 columns (1–6):



- 4 Tightly seal the 96-well plate, vortex thoroughly for 20 sec at a high speed, and centrifuge at 2,200–3,000 x g for 1–2 min.
- 5 Preheat the heat block and lid of the thermal cycler to 75 °C for at least 5 min prior to incubation for denaturation of total RNA.
- 6 Incubate the sample plate (just prior to loading the IFC) using the following thermal cycle program for denaturation of total RNA:

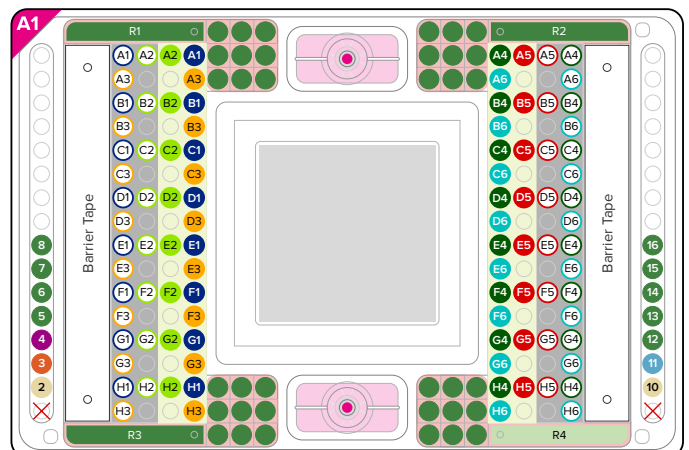
Temperature	Time
75 °C (Preheat block and lid before use.)	
75 °C	5 min
4 °C	5 min
4 °C (Samples can be kept on ice for up to 30 min prior to loading them into the IFC.)	Hold

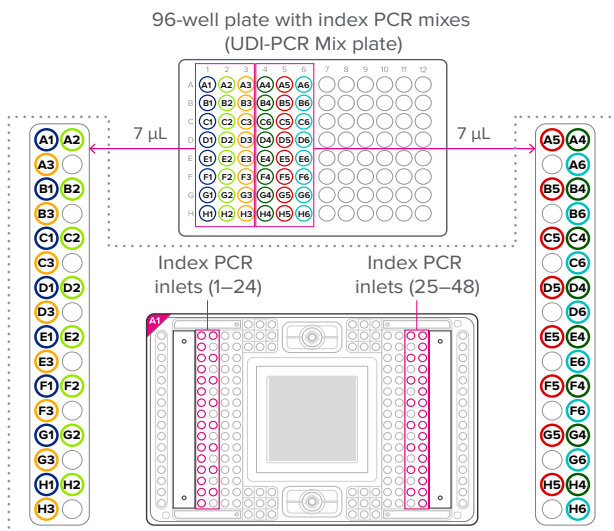
## Load the 48.Atlas IFC

### IMPORTANT

- Ensure that the Juno is on, the TX Interface Plate is loaded, and that the software version is 3.13.1 or later.
- Before pipetting reagents, maintain traceability by noting the orientation of the A1 corner or placing the barcode on the left side.
- To prevent introducing bubbles, pipet only to the first stop during this transfer process.
- Prior to loading the IFC, mix Harvest Reagent, **Pre-Capture Buffer**, and **Post-Capture Buffer** by vortexing for 5 sec at medium speed and then centrifuging for 10 sec to collect the contents and remove bubbles.
- Run the IFC on Juno as soon as possible after the mRNA Seq Beads are loaded.
- 1 **Control Line Fluid (101-9307) in accumulators.** Inject the entire contents of one Control Line Fluid syringe into each of the 2 accumulators on the IFC, avoiding spills and bubbles.

- 2 **Harvest Reagent Type 1 (HR, 101-9300) in inlets.** Load 20 µL/inlet HR into:
  - The 36 green inlets in 4 groups of 9 inlets on both sides of the top and bottom accumulators.
  - 5 6 7 8 Outer-left inlets 5, 6, 7, and 8.
  - 12 13 14 15 16 Outer-right inlets 12, 13, 14, 15, and 16.
- 3 **Harvest Reagent Type 1 (HR, 101-9300) in 3 reservoirs.** Load 170 µL per reservoir into top-left reservoir R1, top-right reservoir R2, and bottom-left reservoir R3.
- 4 **Post-Capture Buffer (101-9081) in 1 reservoir.** Load 170 µL into bottom-right reservoir R4.
- 5 **Pre-Capture Buffer (101-9080) in right-side inlet.** Load 20 µL into outer-right inlet 11.
- 6 **UDI-PCR Mix in designated inlets.** Ensure that the index plate is securely sealed. Then:
  - a Vortex the index plate thoroughly for 5 sec at a medium speed, and centrifuge at 2,200–3,000 x g for 1–2 min.
  - b Without removing the foil seal from the UDI-PCR Mix plate, pierce the foil seal of each well with a fresh pipette tip and load 7 µL/inlet into Index PCR inlets 1–24 (left side) and 25–48 (right side). See the diagram for 96-well plate with index PCR mixes (UDI-PCR Mix plate) on the next page.
- 7 **RNA samples in designated inlets.** Ensure that the sample plate is securely sealed. Then:
  - a Vortex the sample plate thoroughly for 20 sec at a high speed, and centrifuge at 2,200–3,000 x g for 1–2 min.
  - b Load 5 µL/inlet of sample mix from the sample plate into Sample inlets 1–24 (left side) and 25–48 (right side). See the diagram for 96-well plate with samples on the next page.
- 8 **RT Master Mix in left-side inlet.** Prior to loading the IFC, mix the RT Master Mix by pipetting up and down slowly, and load 18 µL into outer-left inlet 4.
- 9 **Fragmentation Master Mix in left-side inlet.** Prior to loading the IFC, mix Fragmentation Master Mix by pipetting up and down slowly, and load 18 µL into outer-left inlet 3.
- 10 **mRNA Seq Beads (101-9076) in left- and right-side inlets.** Prior to loading, mix the beads in each case by vortexing for 10–20 sec at a high speed, and continue until there is no bead pellet at the bottom of the tube. **Do not centrifuge after vortexing.** Load 15 µL of fully suspended mRNA Seq Beads into:
  - 2 Outer-left inlet 2.
  - 10 Outer-right inlet 10.





- 4 Use an 8-channel pipette to transfer 20 µL per well of the Harvest Reagent Type 1 from the 8-tube strip into the first 6 columns of a new 96-well plate.
  - 5 Set an 8-channel pipette to 6.0 µL to transfer the entire harvest volumes from the sample inlets of the IFC into each of the corresponding wells of the Sample Libraries plate. Each harvest volume should be 3–6 µL.
- IMPORTANT** For best index uniformity of mapped reads, be sure to transfer the entire volume from each sample inlet. The exact volume harvested from each well may vary.
- 6 Tightly seal the 96-well plate, vortex thoroughly for 20 sec at a high speed, and centrifuge at 2,200–3,000 x g for 1–2 min.

## Perform qPCR to Quantify Sample Libraries for Normalization

### Dilute the Sample Libraries 50-Fold for the qPCR Run

- 1 Label a new 96-well plate **50-Fold Dilution**.
- 2 Pour 6 mL of library dilution buffer (Teknova, T1485) into a new reagent reservoir. (Double the volume for 2 IFCs.)
- 3 Use an 8-channel pipette to transfer 98 µL per well of library dilution buffer from the reservoir into the first 6 columns of the qPCR 50-Fold Dilution plate.
- 4 Use an 8-channel pipette to transfer 2 µL of sample libraries into each of the corresponding wells of the 50-Fold Dilution plate.
- 5 Tightly seal the 96-well plate, vortex thoroughly for 20 sec at a high speed, and centrifuge at 2,200–3,000 x g for 1–2 min.

### Prepare qPCR Master Mix for Normalization

To run 2 IFCs from the contents of 2 separate 96-well plates, set up 2 separate 96-well plate runs:

Component	1 Sample Well (µL)	1 IFC with 96-Well Plate (µL)	2 IFCs with 96-Well Plate (µL)
Nuclease-Free Water (not included)	5.8	406.0	812.0
KAPA SYBR® FAST qPCR Master Mix (2X)*, completely thawed	10.0	700.0	1,400.0
qPCR Primer Premix (10X, 101-9755) <sup>†</sup>	0.2	14.0	28.0
<b>Total (Vortex 10 sec, centrifuge 10 sec, keep tube on ice.)</b>	<b>16.0</b>	<b>1,120.0</b>	<b>2,240.0</b>

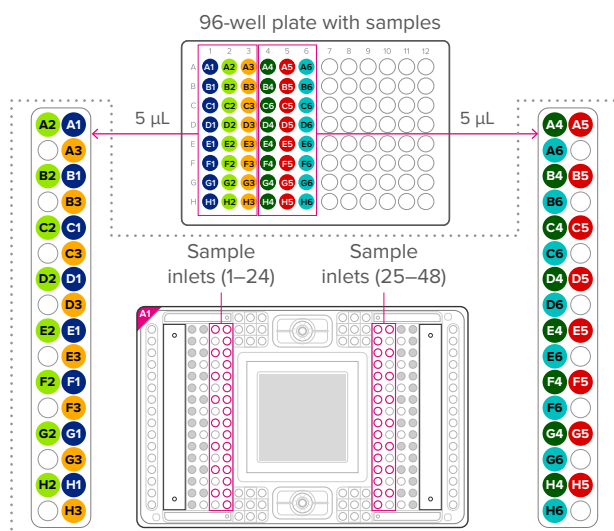
\* The KAPA SYBR FAST qPCR Master Mix must not contain primers.

<sup>†</sup> Do not use the Primer Premix supplied by KAPA. Use only the qPCR Primer Premix (10X, 101-9755) supplied by Fluidigm.

**NOTE** If the qPCR Master Mix does not contain ROX reference dye, reduce the water volume to accommodate the ROX volume.

### Prepare the qPCR Reaction Plate for Normalization

- 1 Label a new optical 96-well reaction plate **qPCR Reaction**.
- 2 Pipet 135 µL of qPCR Master Mix into each 0.2 mL tube of a new 8-tube strip.
- 3 Use an 8-channel pipette to transfer 16 µL per well of qPCR Master Mix from the 8-tube strip into the first 8 columns of the optical qPCR Reaction plate.



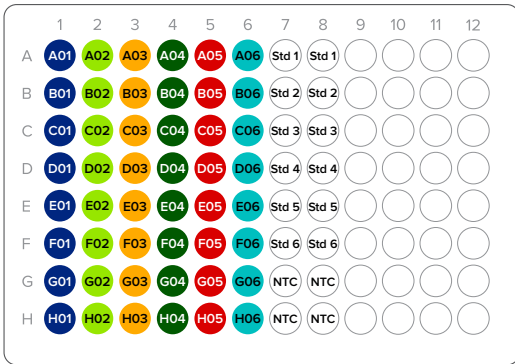
## Run the 48.Atlas IFC on Juno

- 1 Ensure that the TX Interface Plate is installed in Juno.
- 2 Tap **Open**.
- 3 Place the IFC on the tray in Juno and tap **LOAD**.
- 4 On the **Library Prep** tab of the Juno Scripts screen, tap the script **48.Atlas RNA-Seq LP**.
- 5 Select when the script should finish (if necessary, overnight harvest delay) and tap **RUN**. The run takes ~9 hr to complete loading, thermal cycling, and harvesting.
- 6 After the script is finished, tap **EJECT** to eject the IFC.

## Collect the Sample Libraries for Pooling

- 1 Label a new 96-well plate **Sample Libraries**.
- 2 Orient the IFC with the A1 corner at the top-left corner and the barcode on the left.
- 3 Vortex, centrifuge, and pipet 160 µL Harvest Reagent Type 1 (HR, 101-9300) into each 0.2 mL tube of a new 8-tube strip.

- 4 For 1 IFC, use an 8-channel pipette to transfer 4  $\mu$ L of sample library 50-fold dilutions into each of the corresponding wells of columns 1–6 in the optical qPCR Reaction plate, as follows:



- 5 Transfer 4  $\mu$ L of the DNA Standards and NTC (Nuclease-Free Water) into each of the corresponding wells of the qPCR Reaction plate.

**TIP** First transfer the DNA Standards and NTC into an 8-tube strip. Then use an 8-channel pipette to transfer 4  $\mu$ L of each into the wells of the plate.

- 6 Tightly and carefully seal the plate with an optical plate sealer without any creases, vortex thoroughly for 20 sec at a high speed, and centrifuge the plate at 2,200–3,000  $\times g$  for 1–2 min.

### Run the qPCR Reaction for Normalization

- 1 Run qPCR with the following thermal cycle program **qPCR for Normalization**. Select **Absolute Quantification** in the instrument software, and adjust run parameters as required:

Temperature	Time	Cycles
95 °C	5 min	1
95 °C	30 sec	35
55 °C	15 sec	
60 °C	45 sec (data collection)	

- 2 Annotate the DNA Standards as follows:
 

DNA Standard 1	20 pM
DNA Standard 2	2 pM
DNA Standard 3	0.2 pM
DNA Standard 4	0.02 pM
DNA Standard 5	0.002 pM
DNA Standard 6	0.0002 pM
- 3 Select **autothreshold** for analysis at the end of the qPCR run.
- 4 Export the data from the qPCR run and save it in Microsoft® Excel® format to get pooled library preparation information. In the Advanta RNA-Seq Library Normalization Workbook (101-9819), use the **Retrieve to extract** feature as a starting point. (Alternatively, enter the data manually.)

### Prepare the Normalized Pooled Libraries

- 1 Print the pooling information from the **Main** tab of the Normalization Workbook (101-9819) and use it for normalization of 6 pooled libraries per 1 IFC run.

- 2 Adjust the volume of each pool with Nuclease-Free Water according to the Normalization Workbook.
- 3 Use the calculated amount of Agencourt AMPure XP beads listed in the Normalization Workbook for the first purification step.

### Prepare Reagents for Cleanup

- 1 **Agencourt AMPure XP magnetic beads.** Remove the Agencourt AMPure XP magnetic beads from 4 °C and warm the beads to room temperature for 30 min before use. Vortex the Agencourt AMPure XP magnetic beads vigorously to ensure that they are fully suspended.
- 2 **Fresh 80% ethanol.** Using a new graduated tube, prepare 15 mL of fresh 80% ethanol for 6 pooled samples:
  - Pipet 3 mL of Nuclease-Free Water into the tube.
  - Add 12 mL of absolute ethanol to bring the volume to 15 mL.
  - Close the tube and invert to mix.


### First Cleanup

Before you begin, see the steps in **Prepare the Normalized Pooled Libraries** in this document.

- 1 Vortex to mix, followed by a brief centrifugation.
- 2 Ensure that the Agencourt AMPure XP magnetic beads are at room temperature and vortex the beads at high speed for 20 sec or until the beads are fully resuspended.
- 3 Pipet the equivalent of 0.7x amount of beads to the volume of pooled libraries (as defined by the Normalization Workbook, 101-9819) into each pooled library. Expel any beads left in the pipette tip by pipetting the suspension up and down 5 times.
- 4 Vortex the suspension at high speed for 20 sec.
- 5 Incubate the suspension at room temperature for 8 min.
- 6 Briefly centrifuge the tubes and place them on a magnetic stand for 5 min or until the solution is clear.
- 7 Without disturbing the beads and keeping each tube on the magnetic stand, remove and discard the supernatant.
- 8 Use a P10 pipette to remove as much residual supernatant from the tubes as possible without disturbing the beads.
- 9 Wash the beads 2 times with 80% ethanol:
  - Keeping the tubes on the magnetic stand, pipet 200  $\mu$ L of 80% ethanol.
  - Incubate the tubes at room temperature for 30–60 sec.
  - Without disturbing the beads and keeping the tubes on the magnetic stand, remove and discard the ethanol.
  - Repeat Steps a–c one more time for a total of 2 washes.

**TIP** If the beads are not covered by 80% ethanol in Step 9a, remove the tubes from the magnetic stand and mix by inverting.
- 10 Briefly centrifuge the sample tubes to collect the remaining ethanol at the bottom, place the tubes on the magnetic stand for 30 sec, and use a P10 pipette to remove any residual supernatant from the tube.
- 11 Remove any remaining ethanol by incubating the open sample tubes at room temperature for 3 min.

- **12** Add 52.0 µL of Nuclease-Free Water (101-9100) to the dry beads.
- **13** Close the sample tubes, remove them from the magnetic stand, and vortex the suspension at high speed for 20 sec or until all the beads have been washed off the sides of the tubes.
- **14** Incubate at room temperature for 5 min.
- **15** Briefly centrifuge the sample tubes and place the tubes on the magnetic stand for 5 min or until the solution is clear.
- **16** Keeping the tube on the magnetic stand, pipet 50.0 µL of the eluate into new tubes.
- **17** Pipet 40.0 µL of Agencourt AMPure XP magnetic beads to each sample. Expel any beads left in the pipette tip by pipetting the suspension up and down 5 times.
- **18** Vortex the suspension at high speed for 20 sec.
- **19** Incubate the suspension at room temperature for 8 min.
- **20** Briefly centrifuge the tubes and place the tubes on a magnetic stand for 5 min or until the solution is clear.
- **21** Without disturbing the beads and keeping the tube on the magnetic stand, remove and discard the supernatant.
- **22** Wash the beads 2 times with 80% ethanol:
  - a Keeping the tube on the magnetic stand, pipet 200 µL of 80% ethanol.
  - 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 one more time for a total of 2 washes.

**TIP** If the beads are not covered by 80% ethanol in Step 22a, remove the tube from the magnetic stand and mix by inverting.
- **23** Briefly centrifuge the sample tubes to collect the remaining ethanol at the bottom, place the tubes on the magnetic stand for 30 sec, and use a P10 pipette to remove any residual supernatant from the tube.
- **24** Remove any remaining ethanol by incubating the open sample tubes at room temperature for 3 min.
- **25** Add 22.0 µL of Nuclease-Free Water (101-9100)  to the dry beads.
- **26** Close the sample tubes, remove them from the magnetic stand, and vortex the suspension at high speed for 20 sec or until all the beads have been washed off the sides of the tubes.
- **27** Incubate the suspension at room temperature for 5 min.
- **28** Briefly centrifuge the sample tubes and place the tubes on the magnetic stand for 5 min or until the solution is clear.
- **29** Keeping the tubes on the magnetic stand, pipet 20.0 µL of the purified library to a new 8-well strip tube for PCR amplification.

## Second Amplification





- **1** Preheat the thermal cycler using thermal cycle program

### PCR2:

Temperature	Time	Cycles
94 °C (Preheat block and lid before use.)		
94 °C	1 min	1
98 °C	15 sec	6–9*
55 °C	15 sec	
68 °C	30 sec	
4 °C	Hold	

\* The number of PCR cycles to be used can be determined using the Advanta RNA-Seq Library Normalization Workbook (101-9819).

- **2** Prepare PCR2 Master Mix for 6 pools of 8 samples per pool:


Component	Vol per 6 Pools (6.6 Rxn) (µL)*
Nuclease-Free Water (101-9100) 	171.6
PCR Buffer (101-9097) 	330.0
PCR2 Primer (101-9099) 	13.2
DNA Polymerase (101-9096) 	13.2
<b>Total</b>	<b>528.0</b>

\* Includes 10% overage.


- **3** Vortex the PCR2 Master Mix and centrifuge to collect the contents to the bottom.
- **4** Add 80.0 µL of PCR2 Master Mix to the tubes containing the 20.0 µL of RNA-seq library in Nuclease-Free Water.
- **5** Close the tubes, vortex 5 sec at medium speed, and centrifuge for 10 sec.
- **6** Place the tubes in a preheated hot-lid thermal cycler and perform PCR using the program **PCR2**.

**STOPPING POINT** Samples can be left overnight in the thermal cycler at 4 °C. If not processed within the next day, freeze the PCR products at –20 °C for up to 2 weeks.

## Second Cleanup

- **1** Thaw Tris Buffer (100-9098)  and keep it at room temperature prior to use.
  - **2** Ensure that each pool of sample libraries is at room temperature.
  - **3** Ensure that the Agencourt AMPure XP magnetic beads are at room temperature and vortex them at high speed for 20 sec or until the beads are fully resuspended.
- NOTE** If the samples were left overnight in the 4 °C thermal cycler using the **PCR2** program, allow 30 min for the beads to thaw to room temperature.
- **4** Pipet 100.0 µL of beads into each pool of sample libraries. Expel any beads left in the pipette tip by pipetting the suspension up and down 5 times.
  - **5** Vortex the suspension at high speed for 20 sec.
  - **6** Incubate the suspension at room temperature for 8 min.
  - **7** Briefly centrifuge the tubes and place them on a magnetic stand for 5 min or until the solution is clear.
  - **8** Without disturbing the beads and keeping the tubes on the magnetic stand, remove and discard the supernatant.

- **9** Wash the beads 2 times with 80% ethanol:
  - a Keeping the tubes on the magnetic stand, pipet 200  $\mu\text{L}$  of 80% ethanol to cover the beads.
  - b Incubate the tubes 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 once more for a total of 2 washes.

**TIP** If the beads are not covered by 80% ethanol in Step 9a, remove the tubes from the magnetic stand and mix by inverting.
- **10** Briefly centrifuge the sample tubes to collect the remaining ethanol at the bottom, place the tubes on the magnetic stand for 30 sec, and use a P10 pipette to remove any residual supernatant from the tube.
- **11** Remove any remaining ethanol by incubating the open sample tubes at room temperature for 3 min.
- **12** Add 22.0  $\mu\text{L}$  of Tris Buffer (101-9098)  to the dry beads.
- **13** Close the sample tubes, remove them from the magnetic stand, and vortex the suspension at high speed for 20 sec or until all the beads have been washed off the sides of the tubes.
- **14** Incubate the suspension at room temperature for 5 min.
- **15** Briefly centrifuge the sample tubes and place the tubes on the magnetic stand for 5 min or until the solution is clear.
- **16** Keeping the tubes on the magnetic stand, pipet 20.0  $\mu\text{L}$  of the eluate into new tubes.

## Perform qPCR to Quantify Final Libraries for Sequencing

### Dilute the Final Libraries for the qPCR Run

- **1** Label a new 96-well plate **qPCR Dilutions**.
- **2** Pour 2 mL of the library dilution buffer (Teknova, T1485) into a new reagent reservoir. (Keep the volume at 2 mL for 2 IFCs.)
- **3** Prepare 10-fold serial dilutions of each final library in library dilution buffer as follows:

Final Library Dilution	Final Library Input Volume ( $\mu\text{L}$ )	Library Dilution Buffer Input Volume ( $\mu\text{L}$ )
10-fold dilution (see footnote)	2.0*	18.0
100-fold dilution	2.0	18.0
1,000-fold dilution	2.0	18.0
10,000-fold dilution	2.0	18.0

\* To preserve samples, the input volumes for the 10-fold dilution of the final library can be 1.0  $\mu\text{L}$  for the final library (Step 3b) and 9.0  $\mu\text{L}$  for the library dilution buffer (in column 1 of Step 3a).

- a Pipet 18  $\mu\text{L}$  per well of the library dilution buffer from the reservoir into the first 4 columns of the qPCR Dilutions plate to prepare for 10-fold, 100-fold-, 1,000-fold, and 10,000-fold dilutions.

- b Transfer 2  $\mu\text{L}$  of the final libraries into the 10-fold dilution (column 1) of the qPCR Dilutions plate.
- c Seal the plate, vortex thoroughly for 20 sec at a high speed, and centrifuge the plate at 2,200–3,000  $\times g$  for 1–2 min.
- d Transfer 2  $\mu\text{L}$  of the 10-fold dilution samples to the 100-fold dilution (column 2) of the qPCR Dilutions plate.
- e Seal the plate, vortex thoroughly for 20 sec at a high speed, and centrifuge the plate at 2,200–3,000  $\times g$  for 1–2 min.
- f Transfer 2  $\mu\text{L}$  of the 100-fold dilution samples to the 1,000-fold dilution (column 3) of the qPCR Dilutions plate.
- g Seal the plate, vortex thoroughly for 20 sec at a high speed, and centrifuge the plate at 2,200–3,000  $\times g$  for 1–2 min.
- h Transfer 2  $\mu\text{L}$  of the 1,000-fold dilution samples to the 10,000-fold dilution (column 4) of the qPCR Dilutions plate.
- i Seal the plate, vortex thoroughly for 20 sec at a high speed, and centrifuge the plate at 2,200–3,000  $\times g$  for 1–2 min.

### Prepare qPCR Master Mix for the Final Library

- **1** Ensure that all components of the KAPA Library Quantification Kit are completely thawed and thoroughly mixed.
- **2** Prepare the qPCR Master Mix in a 1.5 mL tube as follows:

#### NOTE

- Ensure that the correct KAPA SYBR FAST qPCR Master Mix is used in accordance with the reference dye requirements (if any) of the qPCR instrument to be used for library quantification. (See KAPA Library Quantification Kit Technical Data Sheet, KR0405.)
- If the qPCR Master Mix does not contain ROX reference dye, reduce the water volume to accommodate the ROX volume.

Component	1 Sample Well ( $\mu\text{L}$ )	1 IFC with 96-Well Plate ( $\mu\text{L}$ )	2 IFCs with 96-Well Plate ( $\mu\text{L}$ )
Nuclease-Free Water (not included)	4.0	184.0	294.4
KAPA SYBR FAST qPCR Master Mix (2X)*	10.0	460.0	736.0
qPCR Primer Premix (10X from the KAPA kit)	2.0	92.0	147.2
<b>Total (Vortex 10 sec, centrifuge 10 sec, keep tube on ice.)</b>	<b>16.0</b>	<b>736.0</b>	<b>1,177.6</b>

\* The KAPA SYBR FAST qPCR Master Mix must not contain primers.

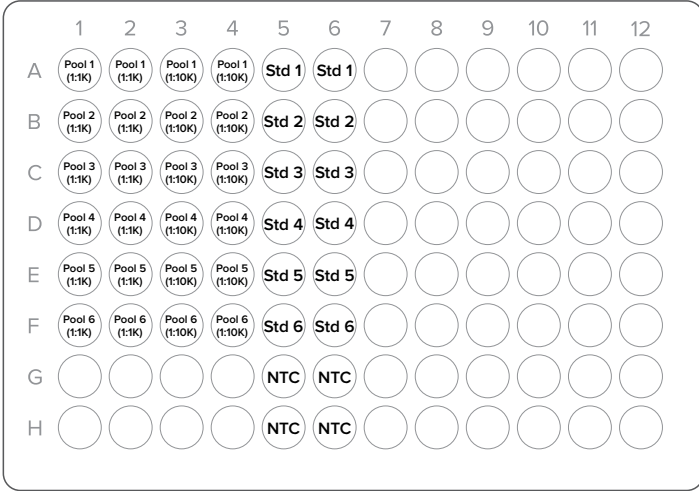
### Prepare the qPCR Reaction Plate for the Final Library

- **1** Label a new optical 96-well plate **qPCR Reaction**.
- **2** Using a 96-well qPCR plate, pipet qPCR Master Mix into each of 6 tubes of a new 8-tube strip as shown for the number of IFCs in use:

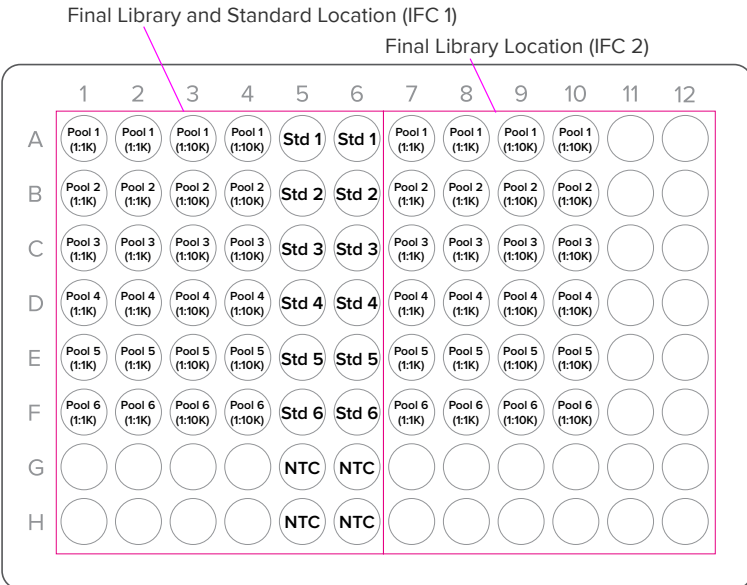
Using a 96-Well Plate	qPCR Master Mix Volume ( $\mu\text{L}$ )
For 1 IFC	106.0
For 2 IFCs	178.0

- 3 Use an 8-channel pipette to transfer 16  $\mu\text{L}$  per well of the aliquoted qPCR Master Mix from the 6 wells of the 8-tube strip into the qPCR Reaction plate according to the following plate map for either 1 IFC or 2 IFCs. (Use a single-channel pipette to transfer 16  $\mu\text{L}$  from the 1.5 mL tube into the NTC wells.)

#### Columns 1–6 of Optical 96-Well Reaction Plate for 1 IFC



#### Columns 1–10 of Optical 96-Well Reaction Plate for 2 IFCs



- 4 Ensure that the dilutions and standards are mixed well.
- 5 Use an 8-channel pipette to transfer 4  $\mu\text{L}$  of the 1,000- and 10,000-fold sample dilutions into each of the corresponding wells of the qPCR Reaction plate.
- 6 Transfer 4  $\mu\text{L}$  of the DNA Standards and NTC into each of the corresponding wells of the qPCR Reaction plate.

**TIP** First transfer the DNA Standards and NTC into an 8-tube strip. Then use an 8-channel pipette to transfer 4  $\mu\text{L}$  of each into the wells of the plate.

- 7 Seal the plate with an optical plate sealer, vortex thoroughly for 20 sec at a high speed, and centrifuge the plate at 2,200–3,000  $\times g$  for 1–2 min.

#### Run the qPCR Reaction for the Final Library

- 1 Run qPCR according to KAPA Library Quantification Kit Technical Data Sheet (KR0405), selecting the **Absolute Quantification** option in the instrument software. Adjust run parameters (for example reporters, reference dyes, and gain settings) as required.

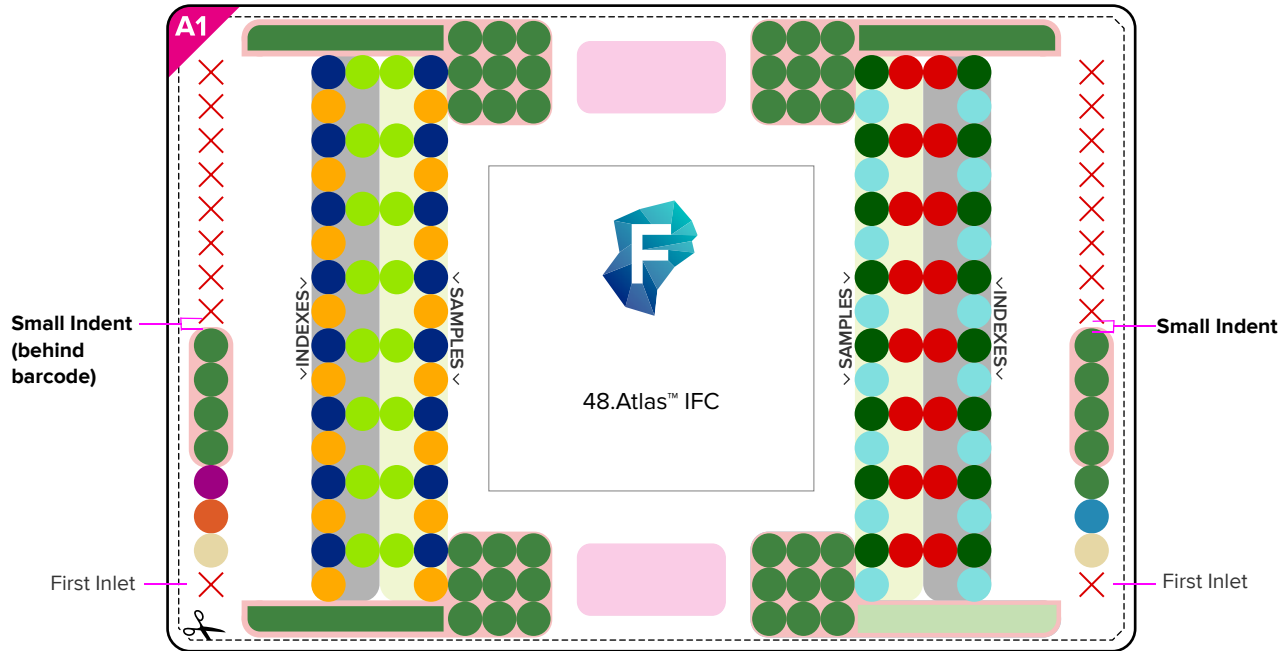
**IMPORTANT** The thermal cycling conditions in this step (shown below) are different from those of Step 1 in **Run the qPCR Reaction for Normalization** in this document.

Temperature	Time	Cycles
95 °C	5 min	1
95 °C	30 sec	35
60 °C	45 sec (data collection)	

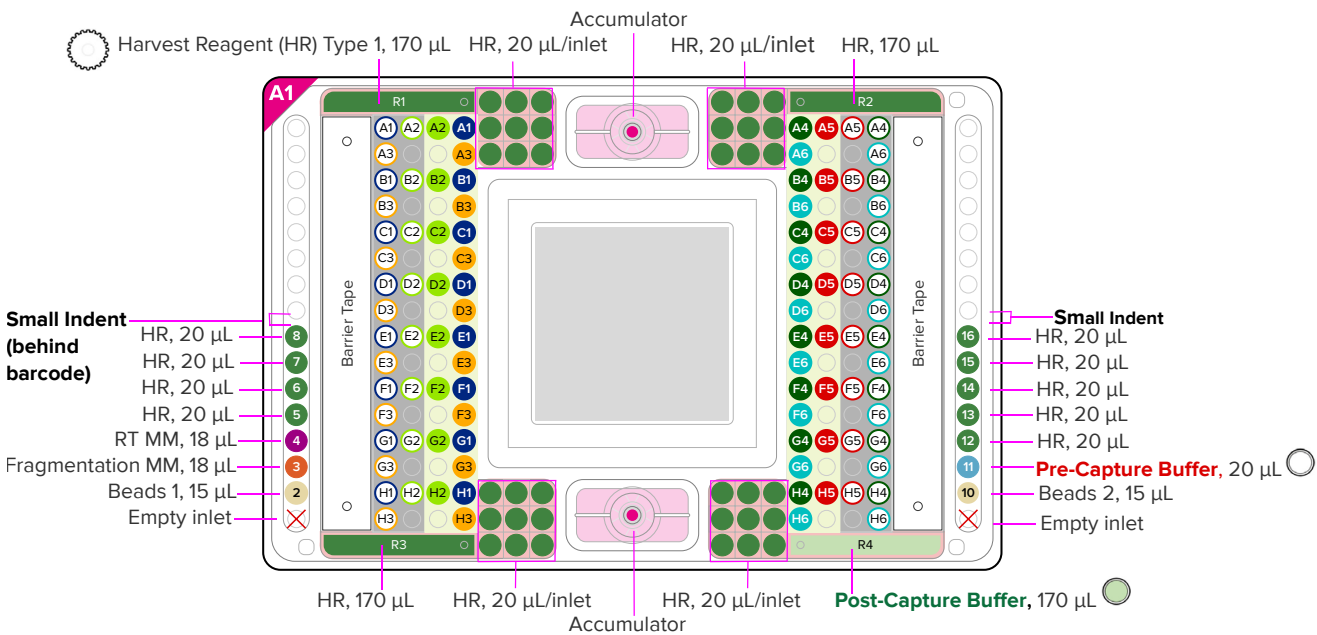
- 2 Select **autothreshold** for analysis at the end of the qPCR run.
- 3 Export the data from the qPCR run and save it in Microsoft Excel format to get pooled library preparation information. In the Advanta RNA-Seq Library Normalization Workbook (101-9819), use the **Retrieve to extract** feature as a starting point. (Alternatively, enter the data manually.)

## Pipetting Map

This document view is set at 108% scale, so before printing this map to help you load, change the scale to 100% to fit the IFC.



## Loading Map



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