TruSeq Small RNA Library Prep Checklist

Ligate Adapters

- ☐1 Combine the following volumes in a 200 µl PCR tube on ice:
  - RA3 (1 µl)
  - 1 µg total RNA in nuclease-free water (5 µl)
- ☐2 Pipette to mix, and then centrifuge briefly.
- ☐3 Place on the thermal cycler.
- ☐4 Incubate at 70°C for 2 minutes.
- ☐5 Remove from the thermal cycler and place on ice.
- ☐6 Combine the following volumes in a new 200 µl PCR tube on ice. Multiply each volume by the number of samples. Make 10% extra reagent for multiple samples.
  - HML (2 µl)
  - RNase Inhibitor (1 µl)
  - T4 RNA Ligase 2, Deletion Mutant (1 µl)
- ☐7 Pipette to mix, and then centrifuge briefly.
- ☐8 Add 4 µl to the RA3/total RNA mixture.
- ☐9 Pipette to mix.
- ☐10 Place on the thermal cycler.
- ☐11 Incubate at 28°C for 1 hour.
- ☐12 Add 1 µl STP and pipette to mix.
- ☐13 Continue incubating at 28°C for 15 minutes.
- ☐14 Remove from the thermal cycler and place on ice.
- ☐15 Add 1.1 × N µl RA5 to a 200 µl PCR tube.
- ☐16 Place on the thermal cycler.
- ☐17 Incubate at 70°C for 2 minutes.
- ☐18 Remove from the thermal cycler and place on ice.
- ☐19 Add 1.1 × N µl 10mM ATP to the RA5.
- ☐20 Pipette to mix.
- ☐21 Add 1.1 × N µl T4 RNA Ligase to the RA5/ATP mixture.
- ☐22 Pipette to mix.
- ☐23 Add 3 µl to the RA3 mixture.
- ☐24 Pipette to mix.
- ☐25 Place on the preheated thermal cycler.
- ☐26 Incubate at 28°C for 1 hour.
- ☐27 Remove from the thermal cycler and place on ice.

Reverse Transcribe and Amplify Libraries

- ☐1 Combine the following volumes in the 12.5 mM dNTP Mix tube to dilute to 12.5 mM. Multiply each volume by the number of samples. Prepare 10% extra reagent for multiple libraries.
  - 25 mM dNTP Mix (0.5 µl)
  - Ultrapure water (0.5 µl)
- ☐2 Pipette to mix, and then centrifuge briefly.
- ☐3 Set aside on ice.
- ☐4 Add 6 µl each RNA library to a 200 µl PCR tube.
- ☐5 Add 1 µl RNA RT Primer to the RNA.
- ☐6 Pipette to mix, and then centrifuge briefly.
- ☐7 Place on the thermal cycler.
- ☐8 Incubate at 70°C for 2 minutes.
- ☐9 Remove from the thermal cycler and place on ice.
- ☐10 Combine the following volumes in a 200 µl PCR tube on ice. Multiply each volume by the number of libraries. Make 10% extra reagent for multiple libraries.
  - 5X First Strand Buffer (2 µl)
  - 12.5 mM dNTP Mix (0.5 µl)
  - 100 mM DTT (1 µl)
  - RNase Inhibitor (1 µl)
  - SuperScript II Reverse Transcriptase (1 µl)
- ☐11 Pipette to mix, and then centrifuge briefly.
- ☐12 Add 5.5 µl to the RNA/primer mix.
- ☐13 Pipette to mix, and then centrifuge briefly.
- ☐14 Incubate at 50°C for 1 hour.
- ☐15 Remove from the thermal cycler and place on ice.
- ☐16 Combine the following reagents in a 200 µl PCR tube on ice. Multiply each volume by the number of libraries. Make 10% extra reagent for multiple libraries with the same index.
  - Ultrapure water (8.5 µl)

- PML (25 µl)
- RP1 (2 µl)
- RPIX (2 µl)

☐17 Pipette to mix, and then centrifuge briefly.
☐18 Place on ice.
☐19 Add 37.5 µl PCR master mix to the adapter-ligated RNA mixture.
☐20 Pipette to mix, and then centrifuge briefly.
☐21 Place on ice.
☐22 Place on the thermal cycler.
☐23 Incubate using the following program on the thermal cycler:

- Choose the preheat lid option and set to 100°C.
- 98°C for 30 seconds
- 11 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 15 seconds
- 72°C for 10 minutes
- 4°C hold

☐24 Run each library on a High Sensitivity DNA chip.

SAFE STOPPING POINT

If you are stopping, cap the tube and store at -25°C to -15°C for up to 7 days.

Purify cDNA Construct

☐1 Combine the following volumes in the 0.1X Pellet Paint tube. Multiply each volume by the number of libraries. Make 10% extra reagent for multiple libraries.

- 1X Pellet Paint NF Co-Precipitant (0.2 µl)
- Ultrapure water (1.8 µl)

☐2 Pipette to mix, and then centrifuge briefly.
☐3 Combine 2 µl CRL and 2 µl DNA loading dye in a 1.5 ml microcentrifuge tube.
☐4 Pipette to mix.
☐5 Combine 1 µl HRL and 1 µl DNA loading dye in a 1.5 ml microcentrifuge tube.
☐6 Pipette to mix.
☐7 Combine all amplified cDNA construct and 10 µl DNA Loading Dye in a 1.5 ml microcentrifuge tube.
☐8 Pipette to mix.
☐9 Load 2 gel lanes with 2 µl CRL/loading dye mixture.
☐10 Load 1 gel lane with 2 µl HRL/loading dye mixture.
☐11 Load 2 gel lanes with 25 µl each of amplified cDNA construct/loading dye mixture.
☐12 Run the gel for 60 minutes at 145 V or until the blue front dye leaves the gel.
☐13 Remove the gel from the unit.
☐14 Open the cassette and stain the gel with ethidium bromide for 2–3 minutes.
☐15 Place the gel breaker tube into a 2 ml microcentrifuge tube.
☐16 View the gel on a Dark Reader transilluminator or a UV transilluminator.
☐17 Using a razor blade, cut out the bands from the 2 lanes that correspond to the adapter-ligated constructs derived from the 22 nt and 30 nt small RNA fragments.
☐18 Place the band into the 0.5 ml gel breaker tube.
☐19 Centrifuge the nested tubes at 20,000 × g for 2 minutes.
☐20 If you are concentrating the final library, skip the next 4 steps and proceed to adding 300 µl Ultrapure Water to gel debris.
☐21 Add 200 µl ultrapure water to the gel debris.
☐22 Rotate for at least 2 hours.
☐23 Transfer the eluate and gel debris to the top of a 5 µm filter.
☐24 Centrifuge at 10 seconds at 600 × g.
☐25 Add 300 µl ultrapure water to the gel debris.
☐26 Rotate for at least 2 hours.
☐27 Transfer the eluate and gel debris to the top of a 5 µm filter.
☐28 Centrifuge at 600 × g for 10 seconds, and then discard the filter.
☐29 Add the following volumes to the eluate:

- Glycogen (2 µl)
- 3M NaOAc (30 µl)
- [Optional] 0.1X Pellet Paint (2 µl)
- 100% ethanol (975 µl)

☐30 Centrifuge at 20,000 × g at 20 minutes at 4°C.
☐31 Remove and discard the supernatant. Leave the pellet intact.
☐32 If the pellet becomes loose, centrifuge at 20,000 × g for 2 minutes.
☐33 Wash the pellet with 500 µl 70% ethanol.
☐34 Centrifuge at 20,000 × g for 2 minutes.
☐35 Remove and discard the supernatant. Leave the pellet intact.
☐36 With the lid open, place the tube in a 37°C heat block until the pellet is dry.
☐37 Resuspend the pellet in 10 µl 10 mM Tris-HC1, pH 8.5.

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Check Libraries

☐1 Load 1 µl resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip.

☐2 Check the size, purity, and concentration of the library.

Normalize Libraries

☐1 Normalize library concentration to 2 nM using Tris-HCl 10 mM, pH 8.5.

☐2 For storage, add Tween 20 for a final concentration of 0.1% Tween 20.

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Acronyms

Acronym	Definition
cDNA	Complementary DNA
CRL	Custom RNA Ladder
HML	Ligation Buffer
HRL	High Resolution Ladder
PCR	Polymerase Chain Reaction
PML	PCR Mix
RA3	RNA 3' Adapter
RA5	RNA 5' Adapter
RIN	RNA Integrity Number
RP1	RNA PCR Primer
RPI	RNA PCR Primer Index
RTP	RNA RT Primer
STP	Stop Solution
UHR	Universal Human Reference