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Standardized and Tested Library Construction – Miniaturization of SMART-Seq HT Kit and SMART-Seq HT PLUS Kit

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Abstract

This standardized and tested NGS method provides instructions for automation and miniaturization of the Takara SMART-Seq HT Kit and SMART-Seq HT PLUS Kit using the I.DOT Non-Contact Dispenser and G.PURE NGS Clean-up Device to prepare single-cell RNA samples for next-generation sequencing (NGS) in 384-well format at 1/5th volume.

Materials

- **Instrumentation**
  - DISPENDIX G.PREP (DISPENDIX PN D16110026838)
    - I.DOT Non-Contact Dispenser
    - G.PURE NGS Clean-up Device
  - P-1000 Single, P-100 Single and P-10 Multichannel Pipettes and Tips
  - Alpaqua 384-compatible magnet plate
  - Vortex Mixer
  - Plate Centrifuge
  - Microtube Centrifuge
  - Thermal Cycler (384-well) with Heated Lid
  - Thermo Fisher Scientific Qubit 3.0 Fluorometer
  - Agilent Technologies 4200 TapeStation

- **Reagents and Consumables**
  - Takara SMART-Seq HT Kit (PN 634791)
  - Takara SMART-Seq HT PLUS Kit (PN R400748, R400749)
  - Ethanol
  - Molecular Grade Water
  - 1.5 mL Microcentrifuge Tubes

- **Samples**
  - 384x Single cells

Methods

Step 1: Create Assay Plate containing RNA

Required Reagents

- Single Cells
- Molecular Biology Grade Water
- From Takara SMART-Seq HT Kit:
  - Control RNA
  - RNase Inhibitor

Before You Begin

- Thaw on ice:
  - Control RNA
  - RNase Inhibitor

Prepare the Samples and Reagents

1.1 Ensure the Control RNA and RNase inhibitor is thawed on ice.

1.2 Manually dilute RNase inhibitor with water. Add 396µl water to 4µl RNase inhibitor.

1.3 Dilute Control RNA using RNase inhibited water to 250pg/µl. Add 1µl of diluted control RNA into the 384-well plate.

1.4 Isolate single cells in RNase inhibited water into the 384-well plate as well.

1.5 Spin the plate to make sure all the solutions are at the bottom of the plate and keep on ice.

Step 2: Prepare Reaction Buffer and Primer

Required Reagents

- Single Cells and diluted control RNA from step 1
- Molecular Biology Grade Water
- From Takara SMART-Seq HT Kit:
  - RNase Inhibitor
  - 10X Lysis Buffer
  - CDS Primer IIA

Before You Begin

- Thaw on ice:
  - RNase Inhibitor
  - 10X Lysis Buffer
  - CDS Primer II
- Power on the I.DOT Non-Contact Dispenser and open Assay Studio Software

Prepare the Thermal Cycler, Samples and Reagents

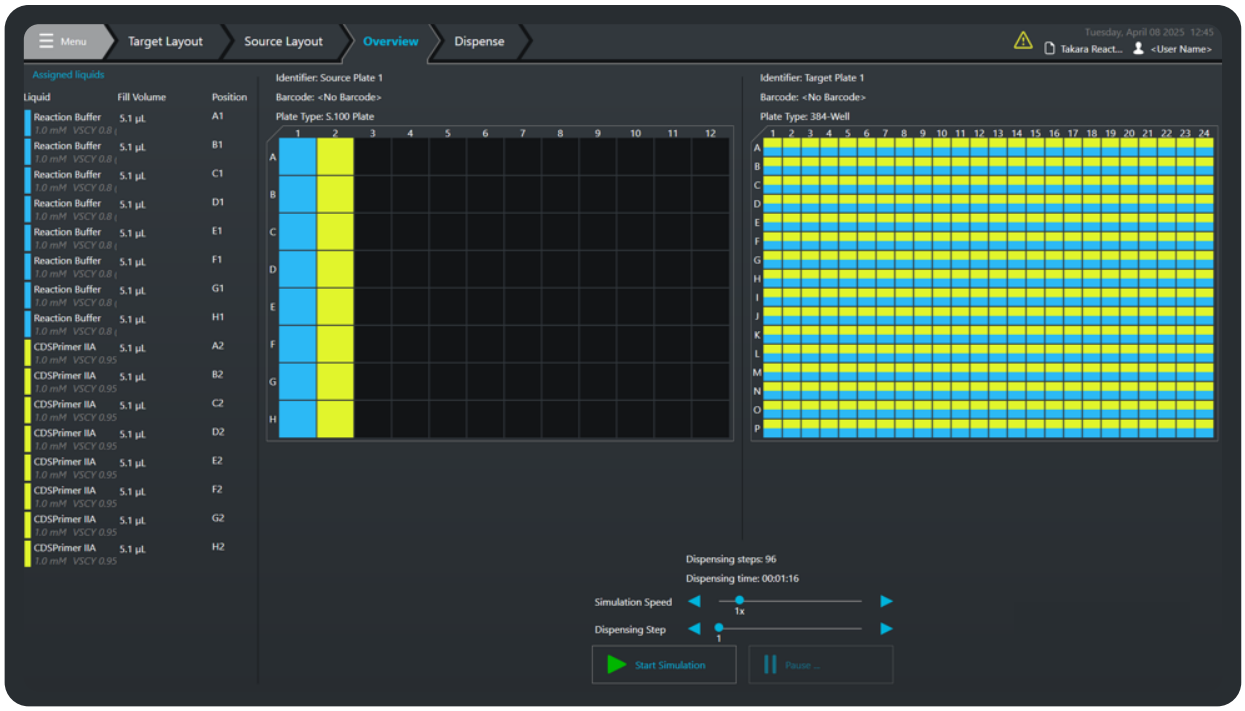
2.1 Ensure the RNase inhibitor, 10X Lysis Buffer and CDS Primer IIA are thawed on ice.

2.2 Vortex the RNase inhibitor, 10X Lysis buffer and CDS Primer IIA for 5 sec. Pulse spin to collect all the liquid in the bottom of the tube.

2.3 Manually add RNase inhibitor (2.5µl) to 10X Lysis Buffer (47.5µl). Vortex for 5 sec and pulse spin to collect all the liquid in the bottom of the tube. Label as Reaction Buffer.

2.4 Dispense 100nl of Reaction Buffer to each well using I.DOT Non-Contact Dispenser with LQC VSCY 0.8 and 100nl of CDS Primer IIA to each well using I.DOT Non-Contact Dispenser with LQC VSCY 0.95.:

2.4.1 Open the "Takara Reaction Buffer Protocol".



2.5 Go to the Dispense screen and load 7µl of Reaction Buffer to column 1 and 7µl of CDS Primer IIA to Column 2 of S.100 source plate.

2.6 Load the S.100 I.DOT source plate into the source tray of the I.DOT Non-Contact Dispenser and load the target plate into the target tray of the I.DOT Non-Contact Dispenser.

2.7 Click dispense to add 100nl of each Reaction Buffer and CDS Primer IIA to each well of your 384-sample plate.

2.8 Pulse spin the sample plate and transfer to the thermal cycler.

2.9 Immediately run the thermal cycling program “Takara Pre-heated Incubation”.

Step 3: Prepare One-Step Reaction and Create cDNA

Required Reagents

- 384-well plate after thermo cycling from step 2
- Molecular Biology Grade Water (Nuclease-Free water)
- From Takara SMART-Seq HT Kit:
  - One-Step Buffer
  - SMART-Seq HT Oligonucleotide
  - RNase Inhibitor

Before You Begin

- Thaw on ice:
  - One-Step Buffer
  - SMART-Seq HT Oligonucleotide
  - RNase Inhibitor
  - SeqAmp DNA Polymerase
  - SMARTScribe Reverse Transcriptase (100U/µl)
- Power on the I.DOT Non-Contact Dispenser and open Assay Studio Software

Prepare the Thermal Cycler, Samples and Reagents

3.1 Ensure the One-Step Buffer, SMART-Seq HT Oligonucleotide, RNase Inhibitor, SeqAmp DNA Polymerase, SMARTScribe Reverse Transcriptase are thawed on ice.

3.2 Vortex the One-Step Buffer, SMART-Seq HT Oligonucleotide, RNase Inhibitor, SeqAmp DNA Polymerase, SMARTScribe Reverse Transcriptase for 5 sec. Pulse spin to collect all the liquid in the bottom of the tube.

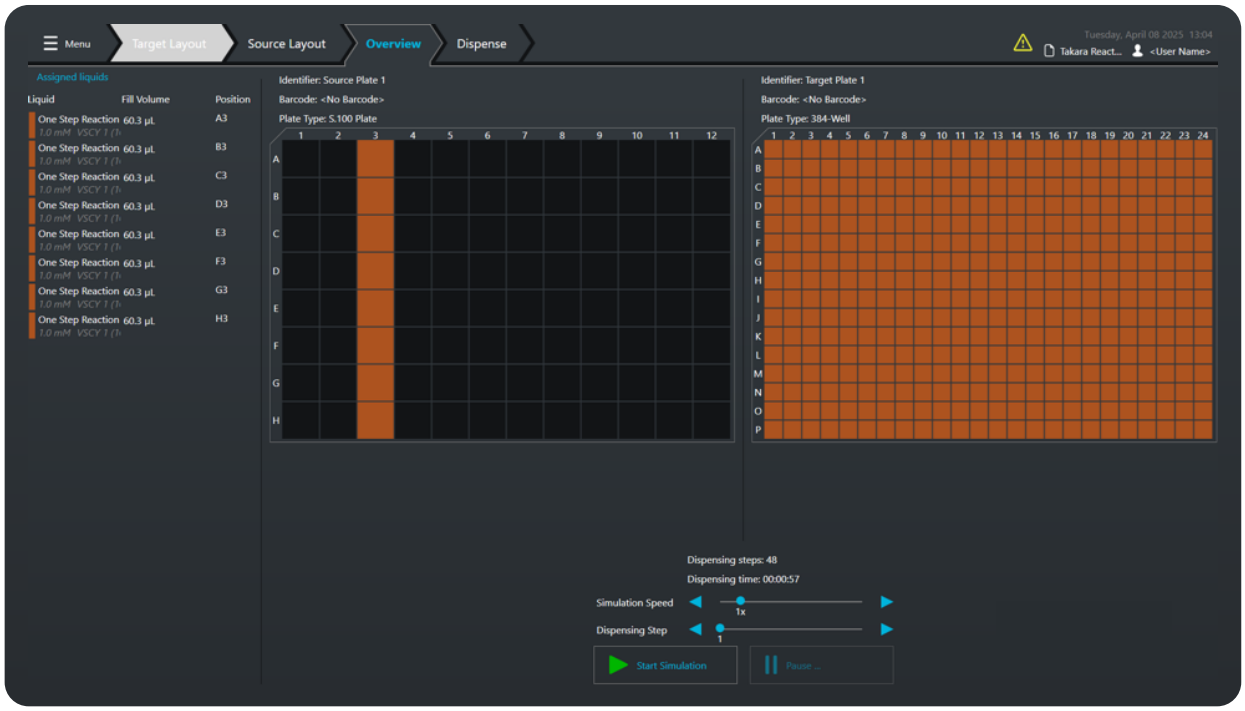
3.3 Manually prepare One-Step Master Mix:

Reagent	Volume
Nuclease-Free Water	27.3 µl
One-Step Buffer	312 µl
SMART-Seq HT Oligonucleotide	39 µl
RNase Inhibitor	19.5 µl
SeqAmp DNA Polymerase	11.7 µl
SMARTScribe Reverse Transcriptase	78 µl

3.4 Vortex for 5 sec and pulse spin to collect all the liquid in the bottom of the tube. Label as One-Step Reaction Buffer.

3.5 Dispense 1.25µl of One-Step Reaction Buffer to each well using I.DOT Non-Contact Dispenser with LQC VSCY 1.

3.5.1 Open the "Takara One-Step Reaction Buffer Protocol".



3.6 Go to the Dispense screen and load 62µl of One-Step Reaction Buffer to column 3 of S.100 source plate.

3.7 Load the S.100 I.DOT source plate into the source tray of the I.DOT Non-Contact Dispenser and load the target plate into the target tray of the I.DOT Non-Contact Dispenser.

3.8 Click dispense to add 1.25µl of One-Step Reaction Buffer to each well of 384 sample plate (Plate from step 2 after thermal cycler is complete).

3.9 Seal and pulse spin the sample plate and immediately transfer to the Thermal cycler.

3.10 Immediately run the thermal cycling program "Takara Pre One-Step Incubation protocol".

3.11 Once completed, run the thermal cycling program "Takara One-Step Reaction protocol".

Step 4: Purify cDNA

Required Reagents

- 384-well plate after One-Step reaction thermal cycling from step3
- Molecular Biology Grade Water (Nuclease-free water)
- DNA Purification AMPure Beads

Before You Begin

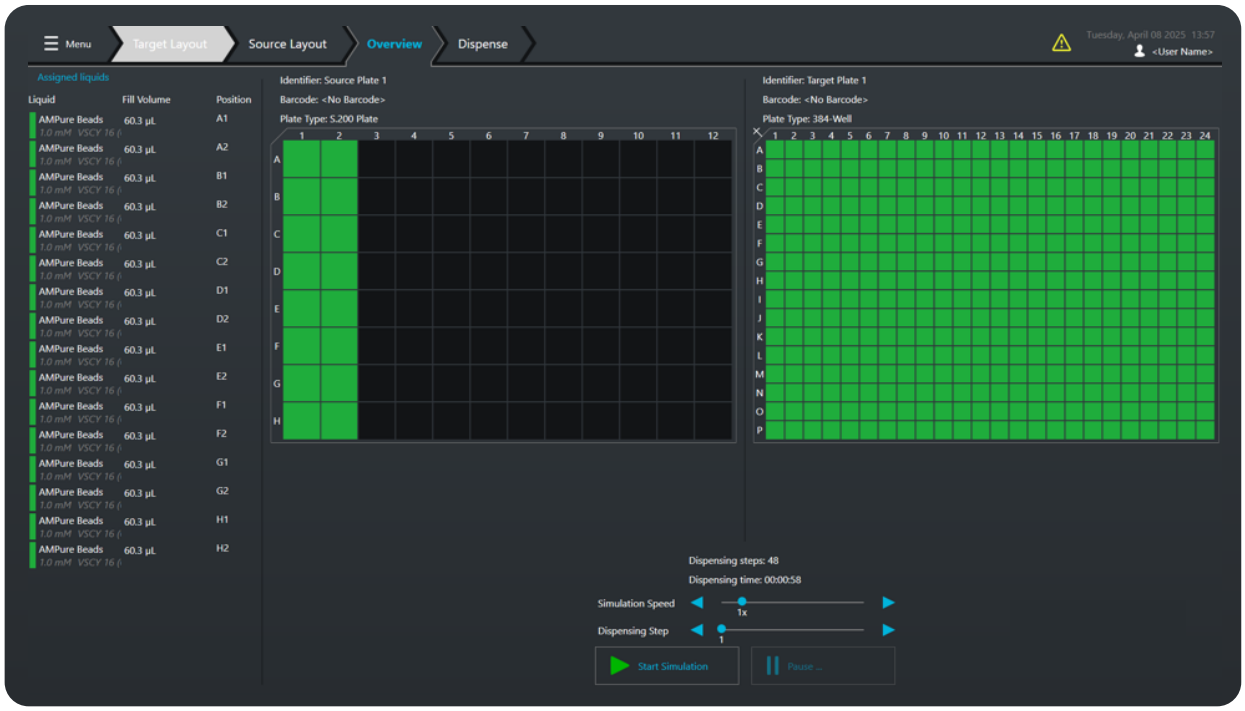
- Bring to Room Temperature:
  - 384-well plate having cDNA from step 3 One-Step Buffer
  - DNA Purification AMPure Beads
- Power on the I.DOT Non-Contact Dispenser and open Assay Studio Software
- Power on G.PURE

Prepare the Samples and Reagents

4.1 Vortex the pre-equilibrated room temperature DNA Purification AMPure beads until well mixed.

4.2 Add 2.5µl of homogenized DNA Purification AMPure Beads to each cDNA sample from Step 3.10 using the I.DOT Non-Contact Dispenser with LQC VSCY 16.

4.2.1 Open the "Takara cDNA Purification" Protocol.



4.3 Go to the Dispense screen and load 62µl of DNA Purification AMPure beads to column 1 and 2 of S.200 source plate.

4.4 Load the S.200 I.DOT source plate into the source tray of the I.DOT Non-Contact Dispenser and load the target plate into the target tray of the I.DOT Non-Contact Dispenser.

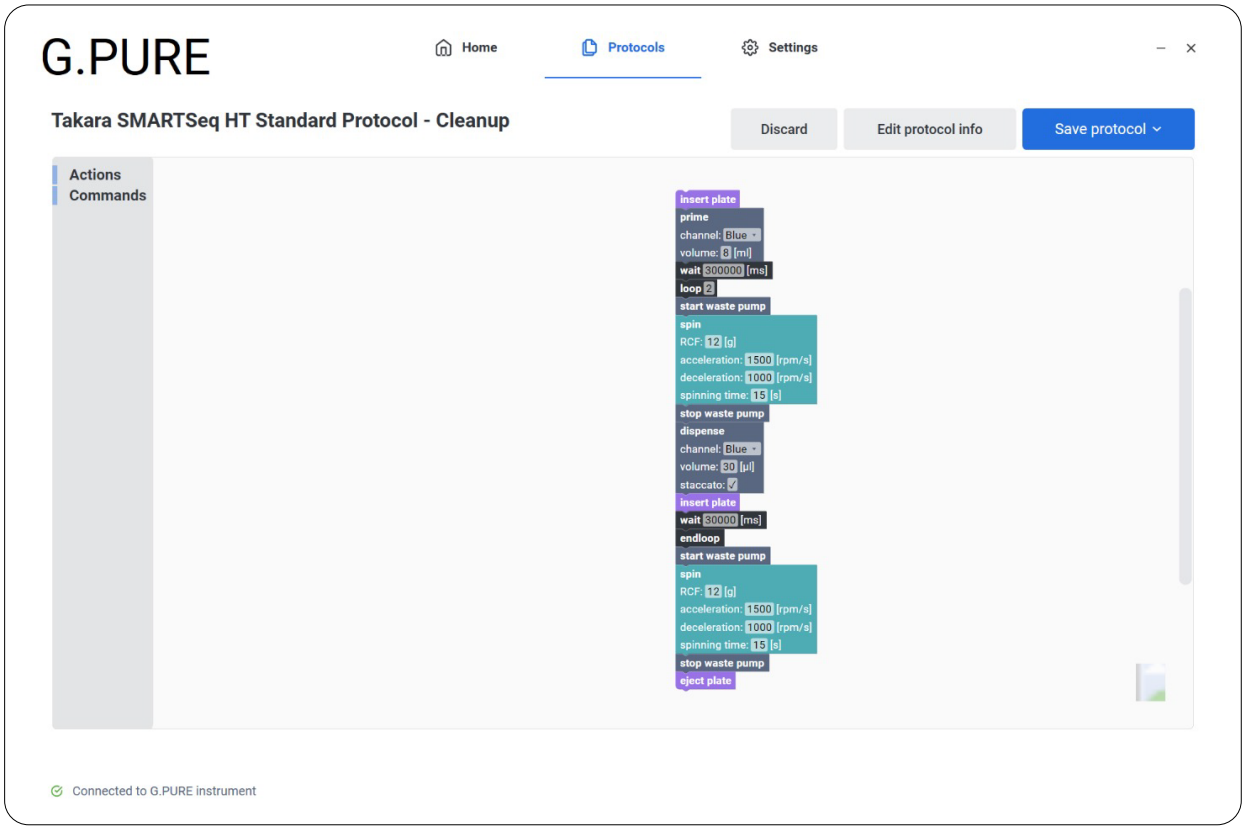
4.5 Click dispense to add 2.5µl of DNA Purification AMPure Beads to each well of the 384-sample plate (Plate from step 3 after thermal cycler is complete).

4.6 Seal the plate, vortex and pulse spin down.

4.7 Incubate the samples for 5 min at room temperature off magnet.

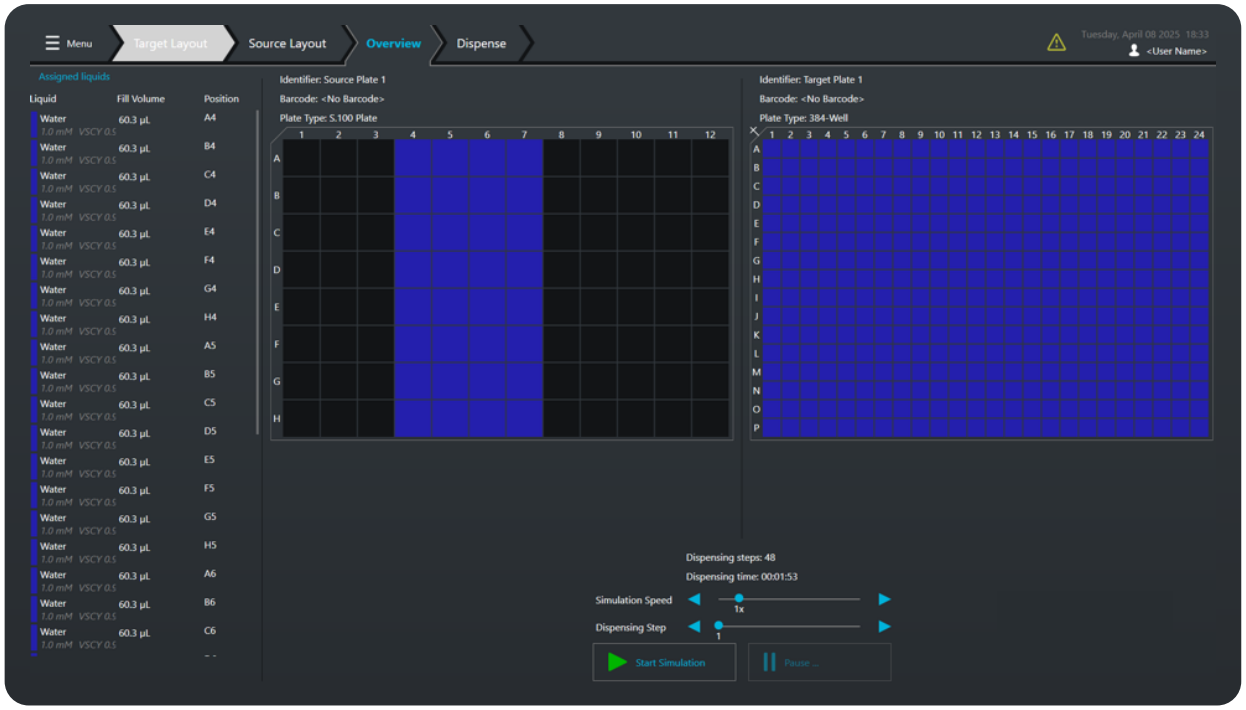
4.8 Place the samples on the G.PURE 384-well magnetic carrier of the G.PURE. Remove the plate seal

4.9 Run the G.PURE protocol "Takara SMARTSeq HT Standard Protocol" by clicking "Run Protocol" on the "Protocol" screen.



4.10 Remove the plate off the magnetic carrier and dispense 5µl nuclease-free water to each sample using the I.DOT Non-Contact Dispenser with LQC VSCY0.95.

4.10.1 Open the "Takara cDNA Elution" Protocol



4.11 Go to the Dispense screen and load 62µl of nuclease-free water to column 4-7 of S.100 source plate.
4.12 Load the S.100 I.DOT source plate into the source tray of the I.DOT Non-Contact Dispenser and load the target plate into the target tray of the I.DOT Non-Contact Dispenser.
4.13 Click dispense to add 5µl of nuclease-free water to each well of the 384-sample plate.
4.14 Seal the plate, vortex and pulse spin down.
4.15 Incubate the samples for 2 min at room temperature.
4.16 Place the plate on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
4.17 Transfer 4.5µl of the clear supernatant containing the purified cDNA to a 384-well thermal cycling plate (Sample plate 2) using a manual pipette, making sure not to disturb the bead pellet.
4.18 Use 1µl of the Purified cDNA on the Qubit HS DNA to determine the cDNA yield.

Step 5: Create Assay Plate containing cDNA

Required Reagents

- Purified cDNA (Sample Plate 2)

5.1 Manually dispense 1µl of cDNA into 384-well plate (Sample Plate 3).
5.2 Pulse spin the plate to collect all the liquid at the bottom of the plate. Plate can be stored at 20C for Library preparation. **(Safe stopping point)**.

Step 6: Library Preparation

Required Reagents

- Purified cDNA (Sample Plate 3)
- Unique dual index kit
- FE Dilution Buffer
- Library Prep Buffer
- Rxn Enhancer
- Stem-Loop Adapters

Before You Begin

- Thaw on ice:
  - Purified cDNA (Sample Plate 3)
- From Takara SMART-Seq HT PLUS Kit
  - FE Dilution Buffer
  - Library Prep Buffer
  - Rxn Enhancer
  - Stem-Loop Adapters
- Power on the I.DOT Non-Contact Dispenser and open Assay Studio Software

Prepare the Thermal Cycler, Samples and Reagents

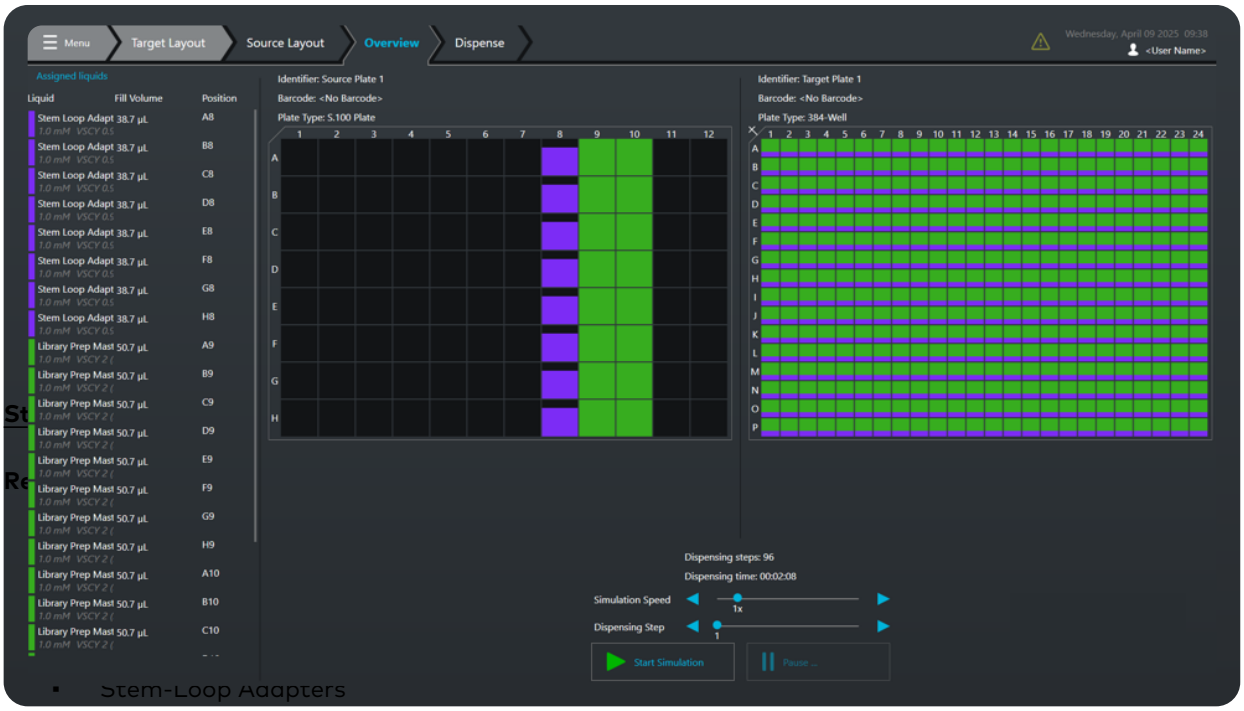
6.1 Ensure the Purified cDNA, FE Dilution Buffer, Library Prep Buffer, Rxn Enhancer and Stem-Loop Adapters are thawed on ice.
6.2 Vortex the FE Dilution Buffer, Library Prep Buffer, Rxn Enhancer and Stem-Loop Adapters for 5 sec. Pulse spin to collect all the liquid in the bottom of the tube.
6.3 Manually prepare 1X FE Buffer by adding 90µl of FE Dilution Buffer to 10µl of 10X FE. Vortex for 5 sec and pulse spin to collect all the liquid in the bottom of the tube. Label as 1X FE.
6.4 Manually prepare Library Prep Master Mix by adding:

Reagent	Volume
Library Prep Buffer	320 µl
Rxn Enhancer	280 µl
Library Prep Enzime	160 µl
1XFE	80 µl

6.5 Vortex the Library Prep Master Mix for 5 sec and pulse spin to collect all the liquid in the bottom of the tube. Label as 1X FE.

6.6 Dispense 800nl of Step-Loop adapter to each well using I.DOT Non-Contact Dispenser with LQC VSCY0.95 and 2.1µl of Library Prep Master Mix to each well using I.DOT Non-Contact Dispenser with LQC VSCY2.

6.7 Open the "Takara Library Prep Protocol".



- Stem-Loop Adapters

6.8 Go to the Dispense screen and load 40µl of Reaction Buffer to column 8 and 52µl Library Prep Master Mix from Column 9 to 10 of S.100 source plate.

6.9 Load the S.100 I.DOT Source plate into the Source Tray of the I.DOT Non-Contact Dispenser and load the target plate into the Target Tray of the I.DOT Non-Contact Dispenser.

6.10 Click dispense to add 800nl of Stem-Loop Adapter and 2.1µl of Library Prep Master Mix to each well of the 384-sample plate.

6.11 Seal and Pulse spin the sample plate and immediately transfer to the Thermal cycler.

6.12 Immediately run the thermal cycling program "Takara Library Prep protocol".

6.13 Once the thermal cycling is complete, Pulse spin the samples and place on ice.

Step 7: Library Amplification

Required Reagents

- Prepared Library from Step 6.13
- Unique dual index kit
- Amplification Buffer
- PrimeSTAR HS DNA Polymerase (5U/ul)

Before You Begin

- Thaw on ice:
- From Takara SMART-Seq HT PLUS Kit
  - Unique dual index kit (PN 634752, 634753, 634754, 634755)
  - Amplification Buffer
  - PrimeSTAR HS DNA Polymerase (5U/ul)
- Power on the I.DOT Non-Contact Dispenser and open Assay Studio Software

Prepare the Thermal Cycler, Samples and Reagents

7.1 Ensure the Prepared Libraries, Unique dual index kit, Amplification Buffer, and PrimeSTAR HS DNA Polymerase are thawed on ice.

7.2 Vortex the Unique dual index kit, Amplification Buffer, and PrimeSTAR HS DNA Polymerase for 5 sec. Pulse spin to collect all the liquid in the bottom of the tube.

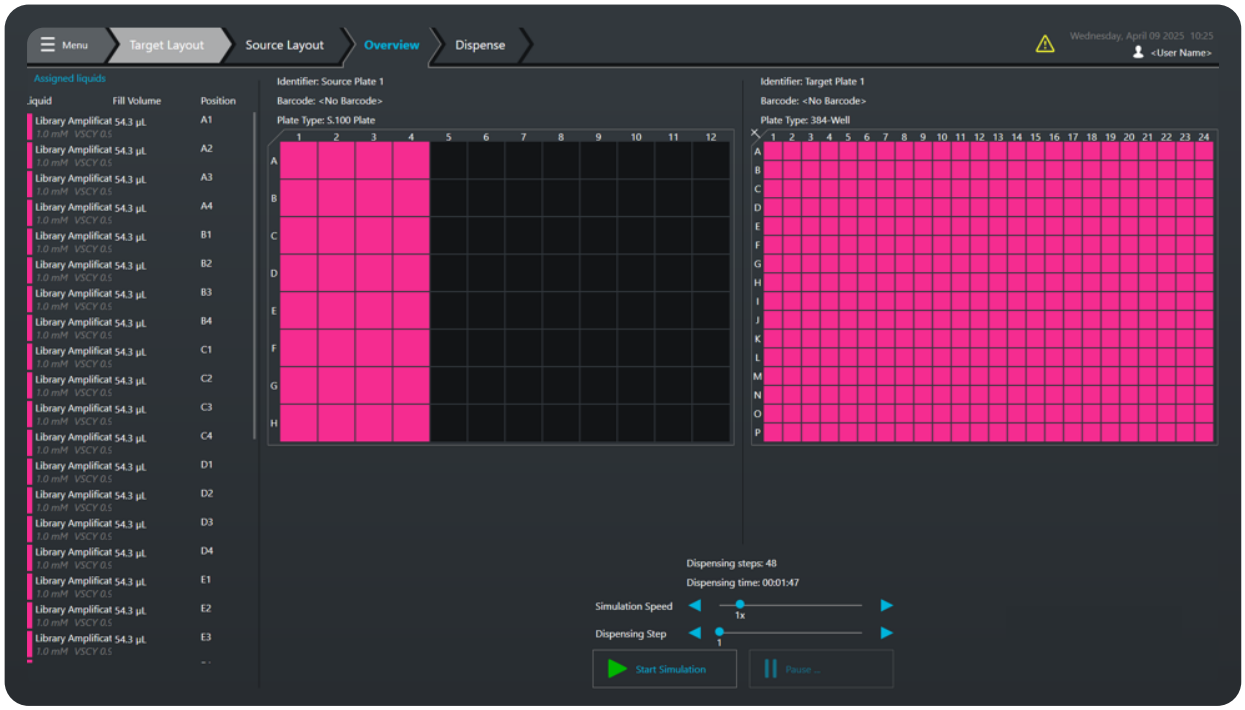
7.3 Manually prepare Library Amplification Master Mix by adding:

Reagent	Volume
Amplification Buffer	1720 µl
PrimeSTAR HS DNA Polymerase	80 µl

7.4 Vortex the Library Amplification Master Mix for 5 sec and pulse spin to collect all the liquid in the bottom of the tube. Label as Library Amplification Master Mix.

7.5 Dispense 4.5µl of Library Amplification Master Mix to each well using I.DOT Non-Contact Dispenser with LQC VSCY0.95.

7.6 Open the "Takara Library Amplification Protocol".



7.7 Go to the Dispense screen and load 56µl of Library Amplification Master Mix to Column 1 to 4 of S.100 source plate.

7.8 Load the S.100 I.DOT Source plate into the Source Tray of the I.DOT Non-Contact Dispenser and load the target plate into the Target Tray of the I.DOT Non-Contact Dispenser.

7.9 Click dispense to add 4.5µl of Library Amplification Master Mix to each well of the 384 sample plate.

7.10 Manually add 1µl of a different index from the Unique dual index kit.

7.11 Seal and pulse spin the sample plate and immediately transfer to the Thermal cycler.

7.12 Immediately run the thermal cycling program "Takara Library Amplification protocol".

7.13 Once the Thermal cycling is complete, Pulse spin the samples and place at room temperature to Purify.

Step 8: Purify Amplified Libraries

Required Reagents

- 384-well plate after Library Amplification thermal cycling from step 7
- Molecular Biology Grade Water (Nuclease-free water)
- DNA Purification AMPure Beads

Before You Begin

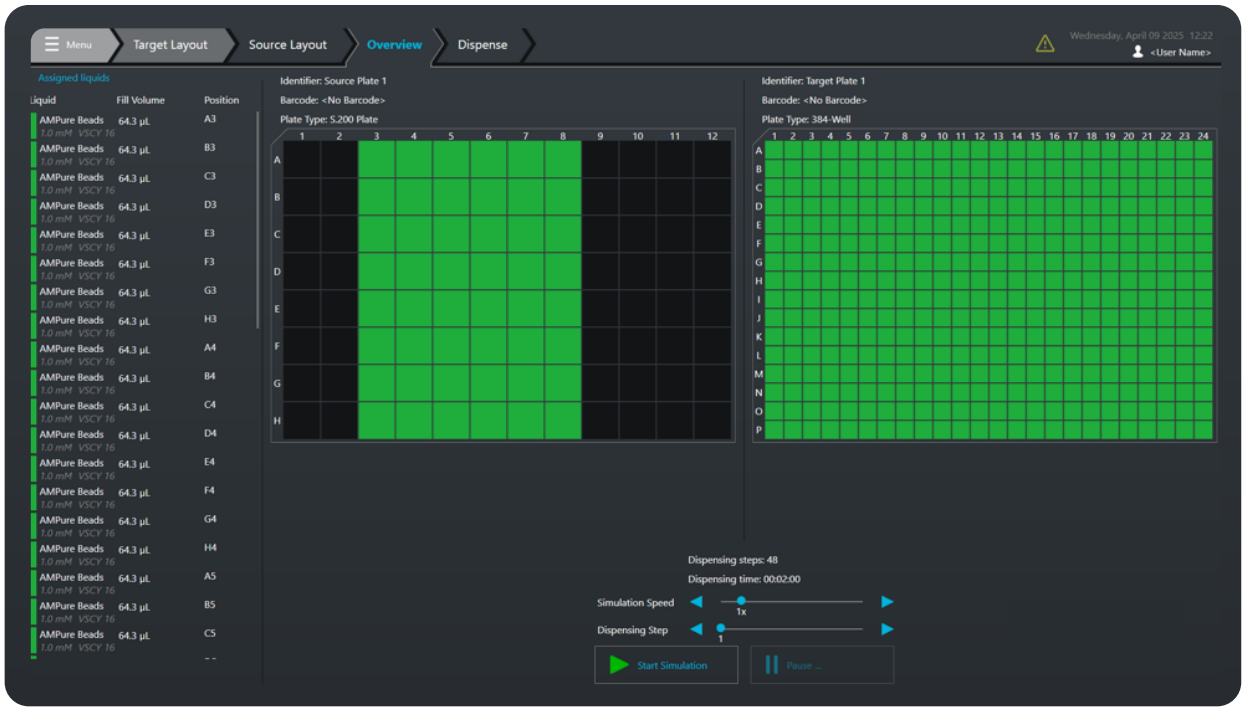
- Bring to Room Temperature:
  - 384-well plate having cDNA from step 3 One-Step Buffer
  - DNA Purification AMPure Beads
- Power on the I.DOT Non-Contact Dispenser and open Assay Studio Software
- Power on G.PURE

Prepare the Samples and Reagents

8.1 Vortex the pre-equilibrated room temperature DNA Purification AMPure beads until well mixed.

8.2 Add 8µl of homogenized DNA Purification AMPure Beads to each sample from Step 7.13 using the I.DOT Non-Contact Dispenser with LQC VSCY16.

8.3 Open the "Takara Library Purification" Protocol.



8.4 Go to the Dispense screen and load 66µl of DNA Purification AMPure Beads from column 3 to 8 of S.200 source plate.

8.5 Load the S.200 I.DOT Source plate into the Source Tray of the I.DOT Non-Contact Dispenser and load the target plate into the Target Tray of the I.DOT Non-Contact Dispenser.

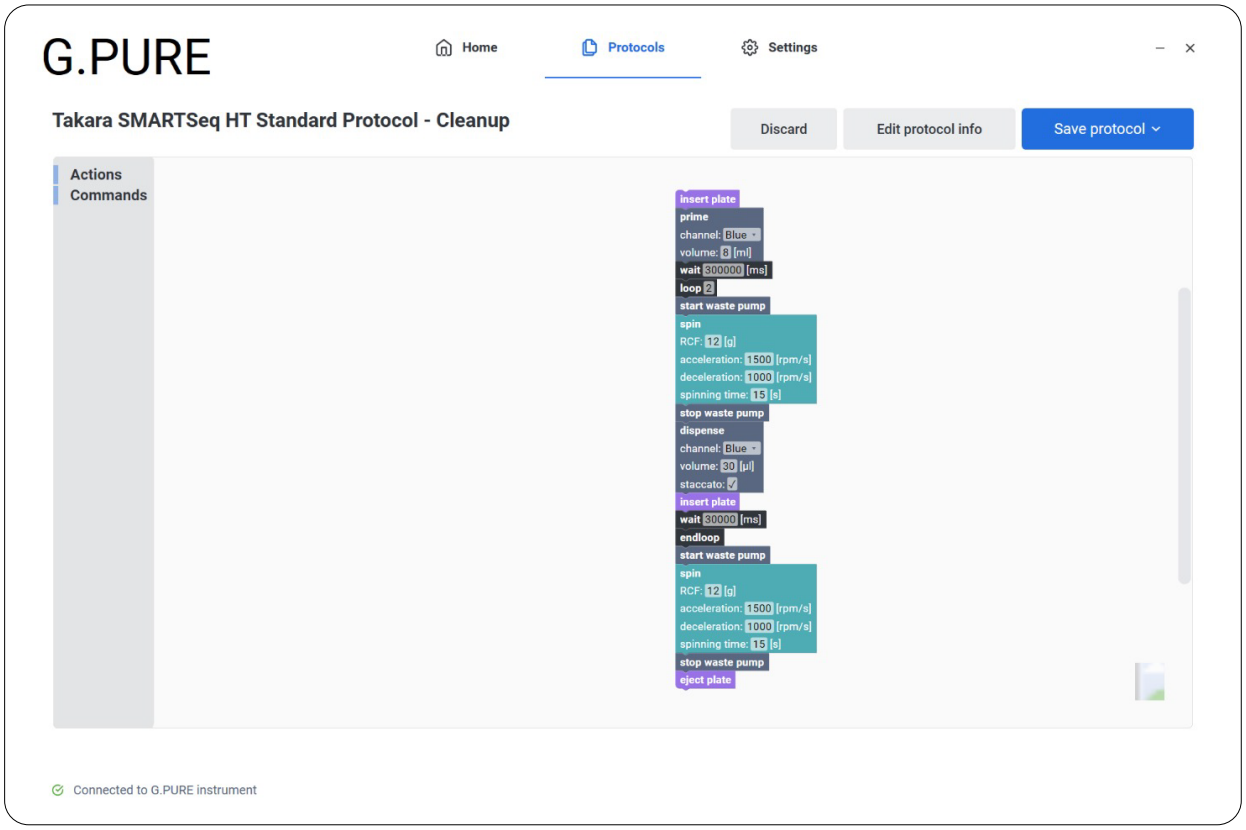
8.6 Click dispense to add 8µl of DNA Purification AMPure Beads to each well of the 384-sample plate (Plate from step 7 after thermal cycler is complete).

8.7 Seal the plate, Vortex and pulse spin down.

8.8 Incubate the samples for 5 min at room temperature off magnet.

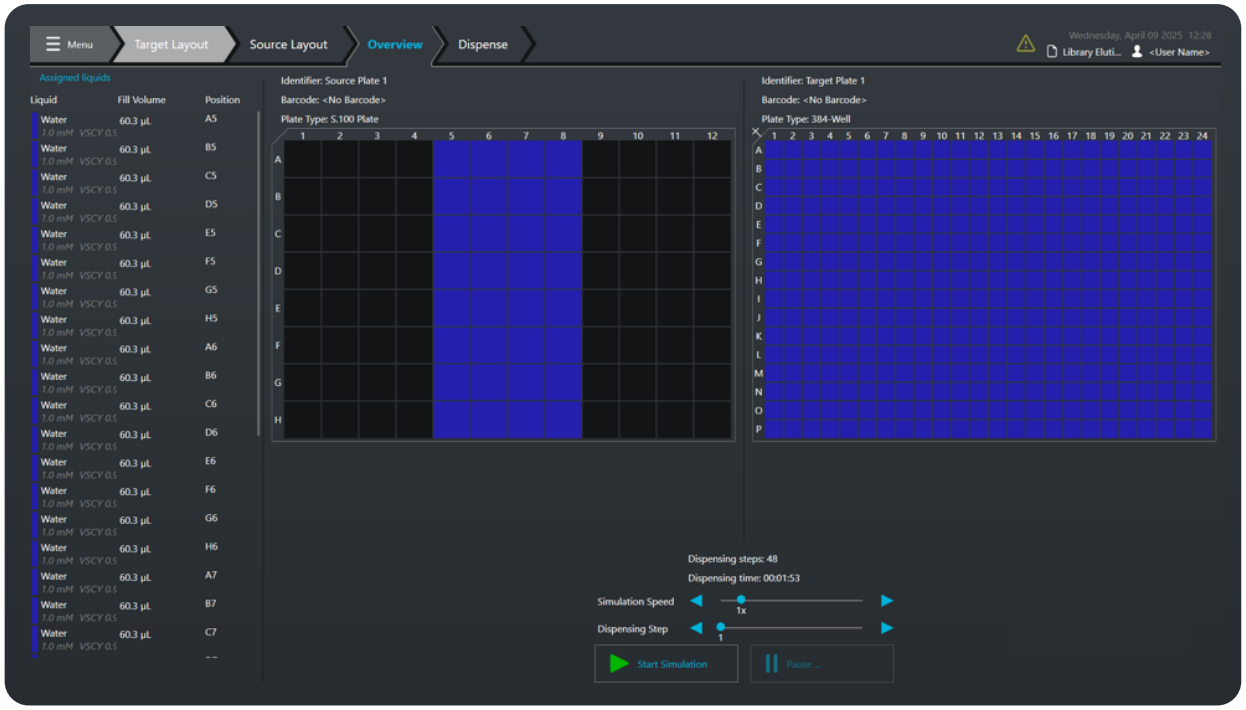
8.9 Place the samples on the G.PURE 384-well magnetic carrier of the G.PURE.

8.10 Run the G.PURE protocol "Takara SMART Seq HT Standard Protocol" by clicking "Run Protocol" on the "Protocol" screen.



8.11 Remove the plate off the magnetic carrier and dispense 5µl nuclease-free water to each sample using the I.DOT Non-Contact Dispenser with LQC VSCY0.95.

8.12 Open the “Takara Library Elution” Protocol.



8.13 Go to the Dispense screen and load 62µl of nuclease-free water to column 5-8 of S.100 source plate.
8.14 Load the S.100 I.DOT Source plate into the Source Tray of the I.DOT Non-Contact Dispenser and load the target plate into the Target Tray of the I.DOT Non-Contact Dispenser.
8.15 Click dispense to add 5µl of nuclease-free water to each well of the 384-sample plate.
8.16 Seal the plate, Vortex and pulse spin down.
8.17 Incubate the samples for 2 min at room temperature.
8.18 Place the plate on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.
8.19 Transfer 4.5µl of the clear supernatant containing the purified cDNA to a 384-well thermal cycling plate (Sample plate 2) using a manual pipette, making sure not to disturb the bead pellet.
8.20 Quantify and validate the library size using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 5000 Assay.

Step 5: Create Assay Plate containing cDNA

250pg of Control RNA and single cells were used to extract RNA, prepare DNA and amplify Libraries using Takara SMART-Seq HT Kit and SMART-Seq HT PLUS Kit. The cDNA and library sizing was done using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 5000 Assay. The quantified cDNAs were in the range of 12ng/µl range for control RNA and the single cells were in the range of 1.5ng/µl. Fig 1 demonstrates the cDNA trace from control RNA (A) and single cell (B).

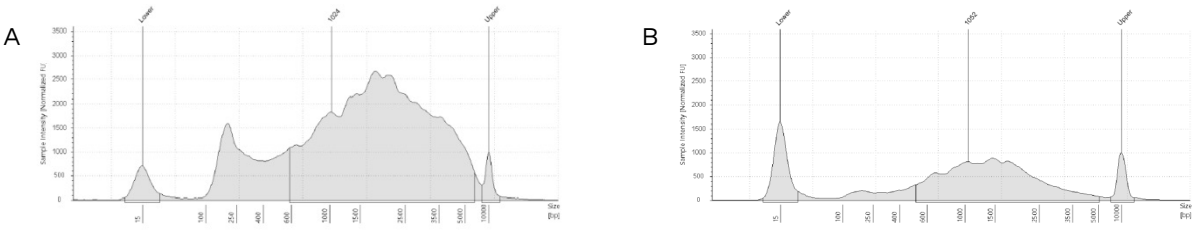


Figure 1: cDNA traces using control RNA (A) and Single cell (B)

The quantified libraries were in the range of 50ng/µl for both control RNA the single cells. Fig 2 demonstrates the Library traces from control cDNA (A) and single cell cDNA (B).

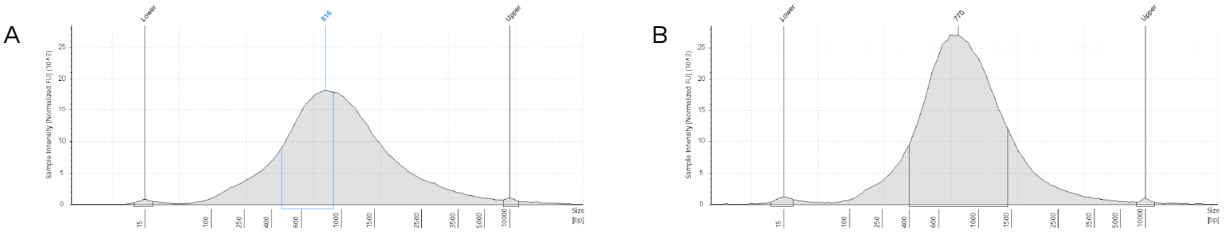


Figure 2: Library traces using cDNA from control RNA (A) and Single cell (B).

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