

SOLiD Total RNA-Seq Kit
SOLiD RNA Barcoding Kit

life
technologies™



Agenda

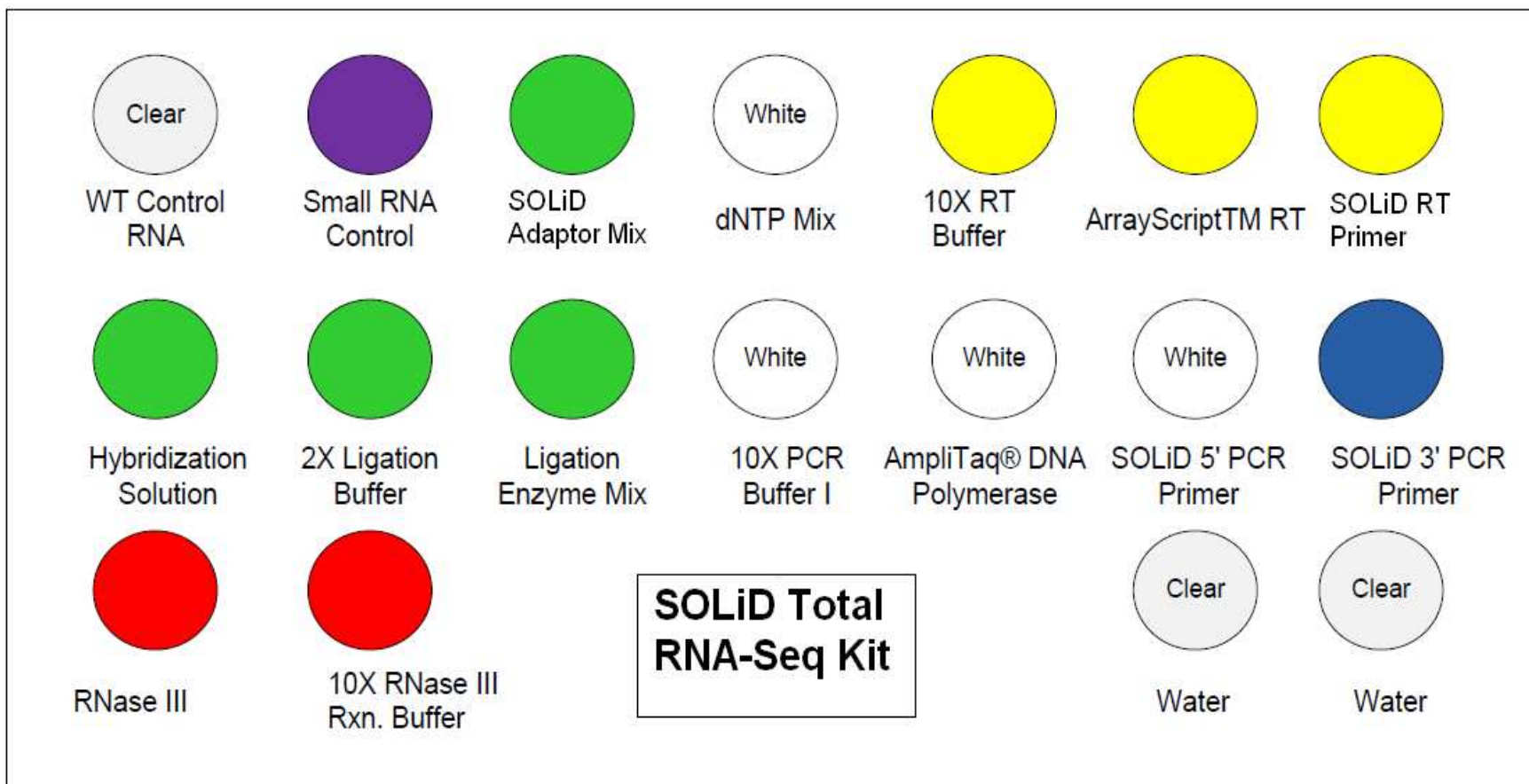
SOLiD Total RNAseq Kit Overview

- Kit Configurations
- Barcoding Kit Introduction

New Small RNA and WT Workflow

- Small RNA Workflow
 - Step-by-step Workflow
 - Sequencing Data
- Whole Transcriptome Workflow
 - Protocol Changes
 - Sequencing Data

New Kit Configuration



Small RNA and WT Workflow SOLiD Total RNA Seq Kit

Preparation of whole transcriptome RNA

0.2-0.5ug rRNA-depleted total RNA or poly(A) RNA

Fragment the RNA

Clean up the RNA

Preparation of small RNA

Obtain total RNA then determine the quality

Purify/enrich small RNA if necessary

Quantitate small RNA sample and determine input amount

SOLiD™ amplified library construction

Hybridize and ligate the RNA adapters over night

Perform reverse transcription

Purify the cDNA

Size select the cDNA

Amplify the cDNA

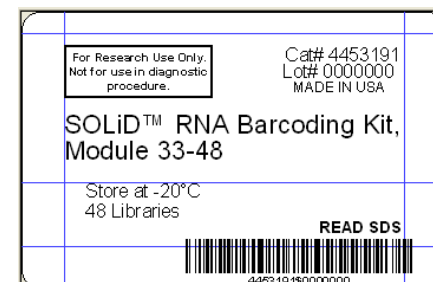
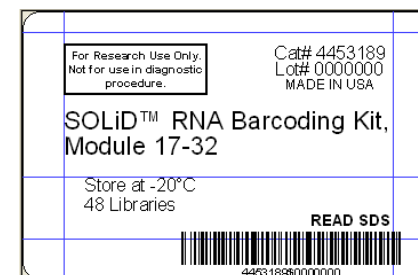
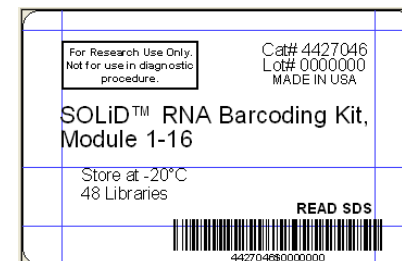
Purify the amplified DNA

Assess the yield and size distribution of the amplified DNA

SOLiD™ System templated bead preparation and sequencing

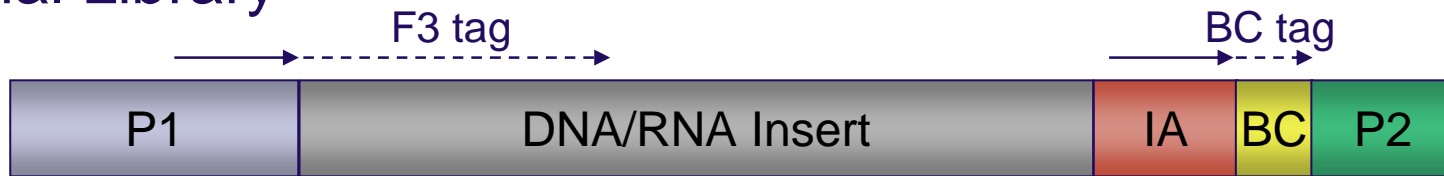
RNA Barcodes Introduction

- The RNA barcodes are sold in 3 modules for a total of 48 barcodes.
- Barcodes in kits that include 16 barcoded 3' PCR primers with one 5' PCR primer
- Provide 5 X100 uL PCR rxns for each barcode (2 libraries/BC, 32 libraries/kit)
- Available Now
 - **SOLiD™ Transcriptome Multiplexing Kit (Set 1-16), pn 4427046**
- COMING SOON:
 - **SOLiD™ RNA Barcoding Kit Module 17-32 pn 4453189.**
 - **SOLiD™ RNA Barcoding Kit Module 33-48 pn 4453191.**
- COMING LATER in 2010
 - **SOLiD RNA Barcode Kit module 49-64,**
 - **SOLiD RNA Barcode Kit module 65-80,**
 - **SOLiD RNA Barcode Kit module 81-96**



P2 Barcodes

Final Library



DNA Method – Adaptor ligation



RNA Prep – Barcode PCR – for all RNA applications





Agenda

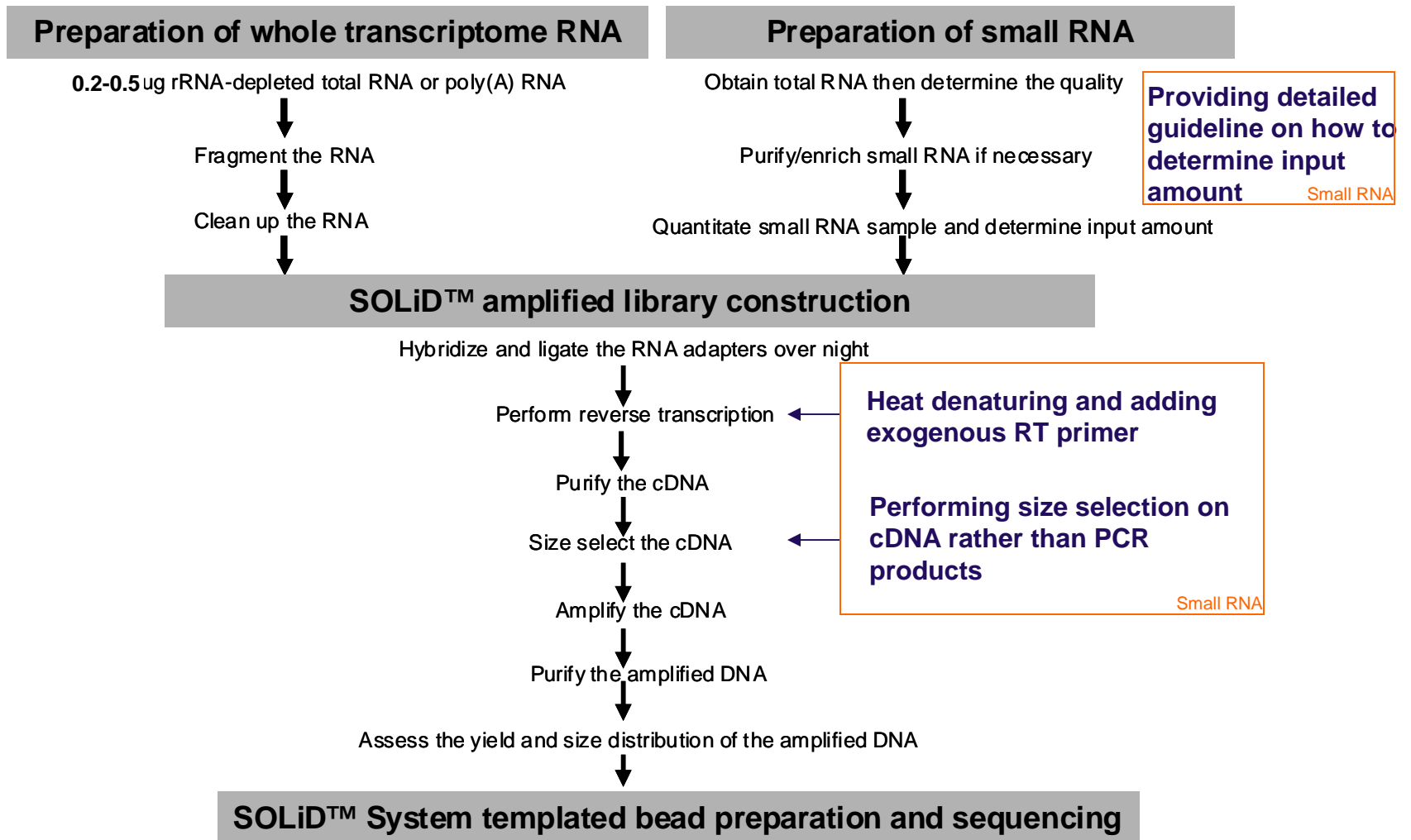
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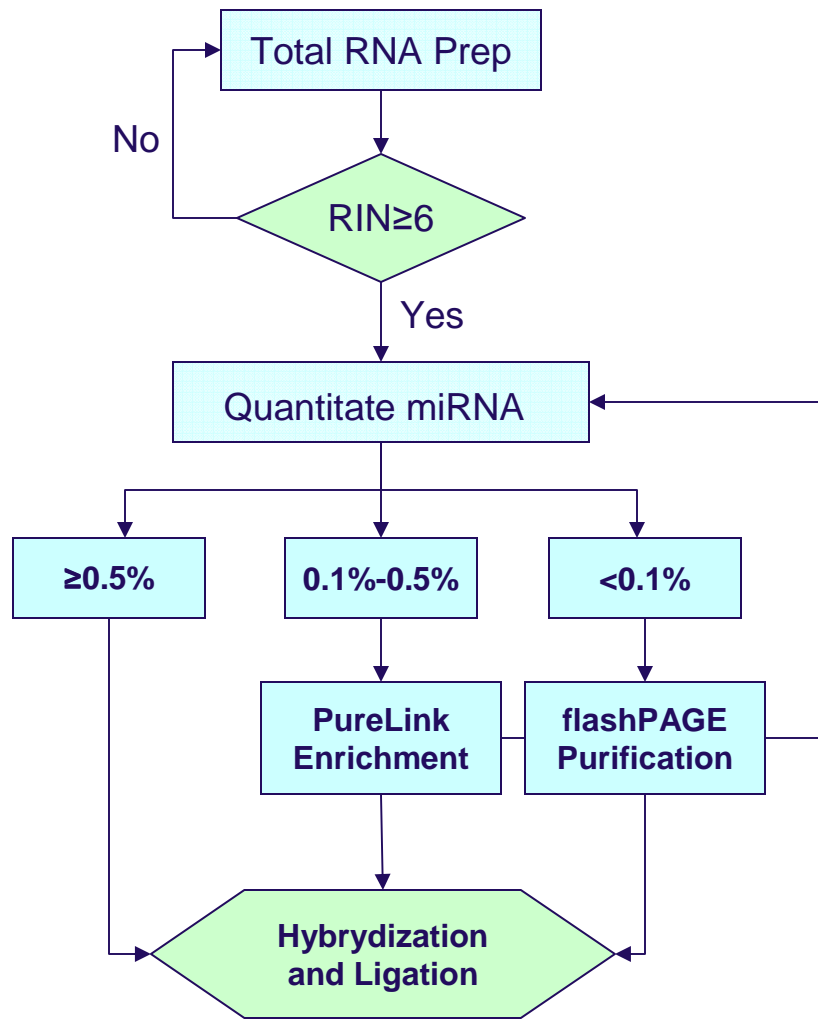
Small RNA Workflow



Before you start...

- Plan your experiments to include multiples of 4 different barcoded libraries in every multiplex sequencing pool to preserve color balance.
- It is not recommended to mix Small RNA and WT libraries in one multiplex pool.
 - 35 bp for smRNA runs vs 50 bp (& optional 25 paired end) for WT
 - 5-10 million recommended for smRNA vs 30-100 million mapped reads recommended for WT

Step 1: Guideline for Small RNA Input



Isolate total RNA that contains small RNA, dilute to 50-100 ng based on nanodrop

Evaluate RNA Integrity on Agilent Nano Chip

Evaluate %miRNA on Agilent Small RNA Chip

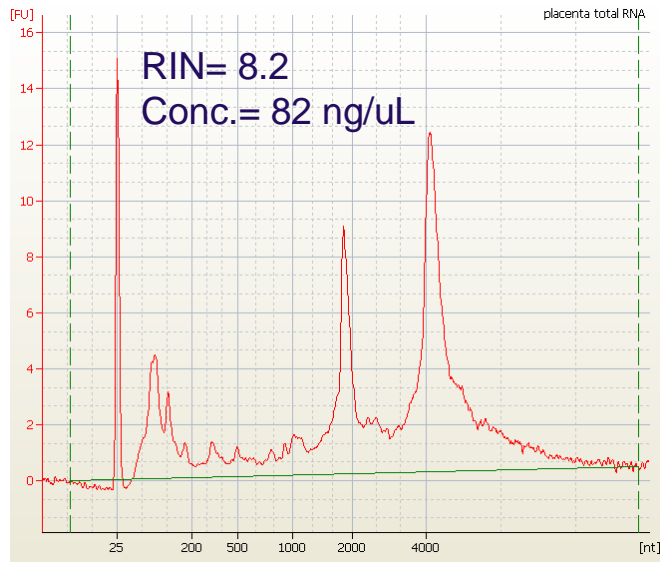
$$\% \text{ miRNA} = \left(\frac{\text{mass of miRNA (10-40 nts) from the Small RNA Chip}}{\text{mass of total RNA from the RNA 6000 Nano Chip}} \right) \times 100$$

Evaluate miRNA on Agilent Small RNA Chip

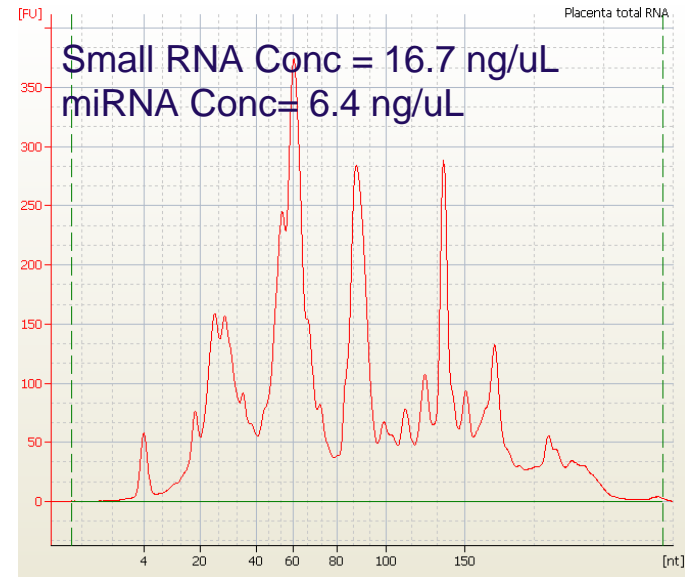
Input sample type	Amount of miRNA (10 to 40 nt) in 1 µL on the Small RNA Chip	Total RNA input†
Total RNA	5 to 100 ng	≤1 µg
Enriched small RNA	1 to 100 ng	≤1 µg
Purified small RNA	1 to 100 ng	100 ng

smRNA workflow cont...

Input smRNA, example 1



Total RNA on nano chip



Total RNA on small RNA chip

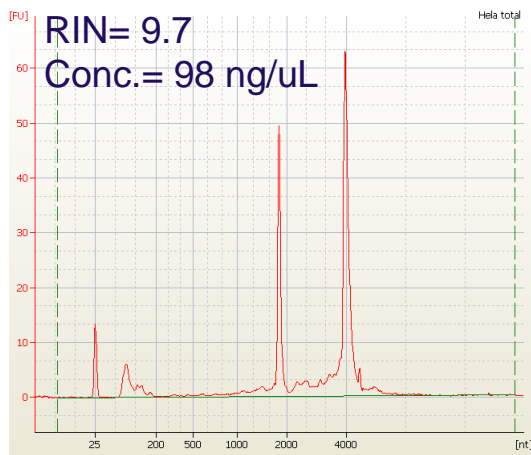
$$\% \text{ miRNA} = \left(\frac{\text{mass of miRNA (10–40 nts) from the Small RNA Chip}}{\text{mass of total RNA from the RNA 6000 Nano Chip}} \right) \times 100$$

$$\% \text{ miRNA} = 6.4 / 82 * 100\% = 7.8\%$$

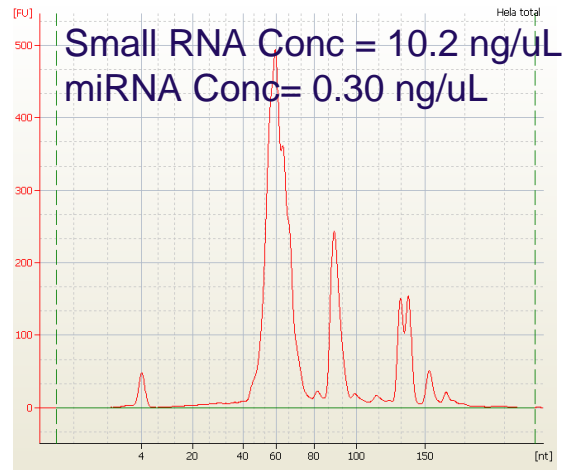
You can use from 64 – 1000 ng total RNA (5 ng- 78 ng miRNA) for ligation.

smRNA workflow cont...

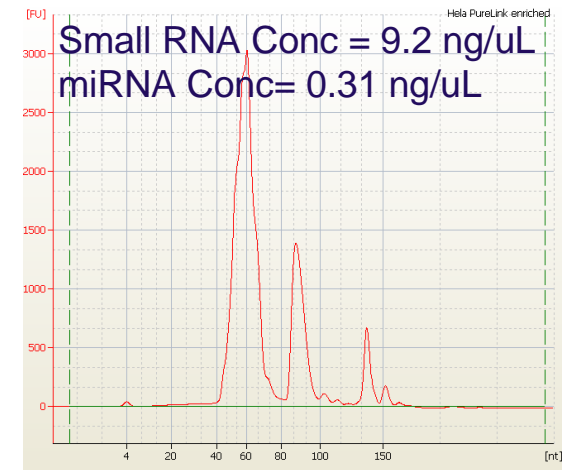
Input smRNA, example 2



Total RNA on nano chip



Total RNA on small RNA chip



PureLink enriched small RNA on small RNA chip

$$\% \text{ miRNA} = \left(\frac{\text{mass of miRNA (10-40 nts) from the Small RNA Chip}}{\text{mass of total RNA from the RNA 6000 Nano Chip}} \right) \times 100$$

%miRNA= 0.30 / 98 * 100% = 0.3% so you need to use enriched small RNA for ligation.

After PureLink enrichment, you can use 1- 33.7 ng miRNA (~30-1000 ng enriched small RNA) for ligation.

Input Type and Amount - Tips

- Input amount should always be determined by small RNA chip quantitation. Total input mass should be $\leq 1 \mu\text{g}$.
- **PureLink enriched small RNA is highly recommended.** FlashPAGE purified small RNA is only recommend when a sample contains extremely low miRNA (<0.1%). Protocol for miRNA enrichment from total RNA using PureLink is in the appendix of the manual.
- For fixed input type, the more input, the better the end results.
- For fixed input amount (miRNA, 10-40 nt), different input type will have comparable mapping statistics. However, purer small RNA sample will give higher yield.

smRNA workflow cont...

Step 2: Hybridization and Ligation

Component	Volume (µL)
Small RNA sample: <ul style="list-style-type: none">• 5 to 100 ng of miRNA in ≤1 µg of total RNA• 1 to 100 ng of miRNA in ≤1 µg of enriched small RNA• 1 to 100 ng of purified small RNA	3
Hybridization Solution	3
SOLiD™ Adaptor Mix	2
Total volume per reaction	8

Temperature	Time
65 °C	10 min
16 °C	5 min

Component (add in order shown)	Volume (µL)
2X Ligation Buffer	10
Ligation Enzyme Mix	2

16 °C for 16 hours.



IMPORTANT! You may observe a white precipitate in the 2X Ligation Buffer. If so, warm the tube at 37 °C for 2–5 minutes or until the precipitate is dissolved. 2X Ligation Buffer is very viscous; pipet slowly to dispense it accurately.

smRNA workflow cont...

Step 3: Perform Reverse Transcription

1. On ice, prepare RT master mix (*without* the ArrayScript™ Reverse Transcriptase):

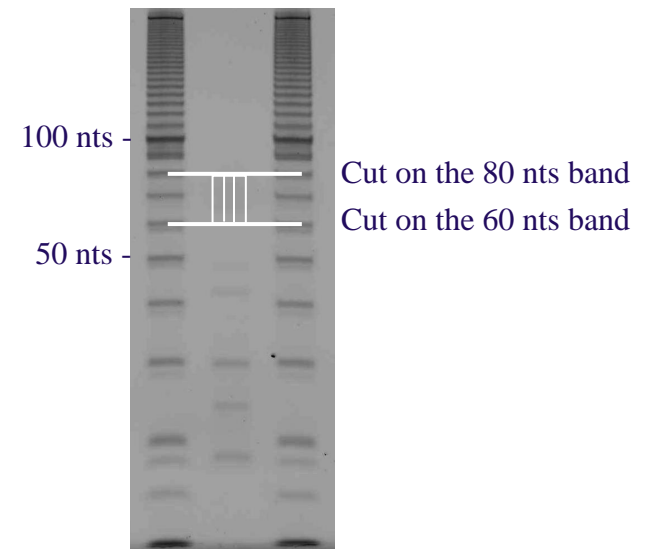
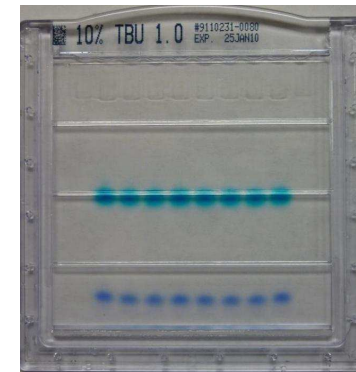
Component	Volume for each reaction (μL) [†]
Nuclease-free Water	11
10X RT Buffer	4
dNTP Mix	2
SOLiD™ RT Primer	2
Total volume per reaction	19

2. Incubate the RT master mix with the ligated RNA sample:
 - a. Add 19 μL of RT master mix to each 20-μL ligation reaction.
 - b. Pipet up and down a few times to mix, then spin briefly.
 - c. Incubate in a thermal cycler with a heated lid at 70 °C for 5 minutes, then snap-cool on ice.
3. Perform the reverse transcription reaction:
 - a. Add 1 μL ArrayScript™ Reverse Transcriptase to each ligated RNA sample.
 - b. *Gently* vortex to mix thoroughly, then spin briefly.
 - c. Incubate in a thermal cycler with a heated lid at 42 °C for 30 minutes.

smRNA workflow cont...

Step 4: Purify and Size-select cDNA

- 5 μ L Qiagen minElute purified cDNA on **10% Novex TBU**
- SYBR Gold is highly recommended
- Size select 60-80 nts, use the two middle gel slices for PCR (each is around 1x 6 mm)



Excised cDNA length (nt)	Insert length (bp)	PCR product length (bp)
42	~0	~93
60	~18	~110
80	~38	~130

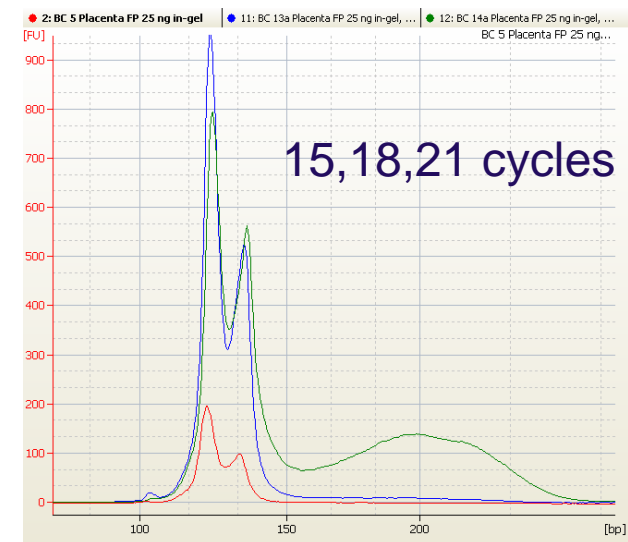
smRNA workflow cont...

Step 5: PCR and Purification

Component	Volume (μL)	
	One 100-μL reaction	Two 100-μL reactions†
Nuclease-free Water	76.8	169.0
10X PCR Buffer	10.0	22.0
2.5 mM dNTP Mix	8.0	17.6
SOLiD™ 5' PCR Primer	2.0	4.4
AmpliTaq® DNA Polymerase	1.2	2.6
Total volume	98.0	215.6

Add 2 μL SOLiD 3' PCR Primer to each tube. ← Barcoding kit

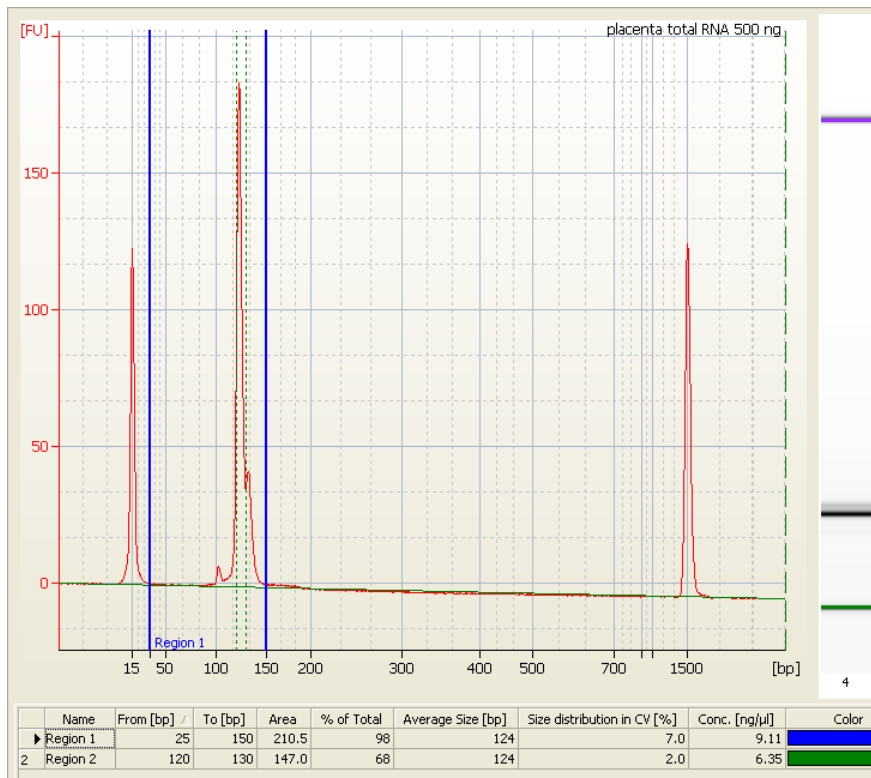
Stage	Temp	Time
Hold	95 °C	5 min
Cycle (15 cycles)	95 °C	30 sec
	62 °C	30 sec
	72 °C	30 sec
Hold	72 °C	7 min



Note: If you started with total RNA and input 1–25 ng, run 18 cycles. If you started with enriched/purified small RNA and input 1–10 ng, run 18 cycles.

smRNA workflow cont...

Step 6: Final Library QC on Bioanalyzer



$$\%miRNA = 147.0/210.5 * 100\% = 69.8\%$$

Ratio of 120–130-bp DNA:25–150-bp DNA	Next steps
Greater than 50%	"Proceed with SOLiD™ System templated bead preparation" on page 47
Less than 50%	"Second-round size selection of amplified cDNA" on page 59

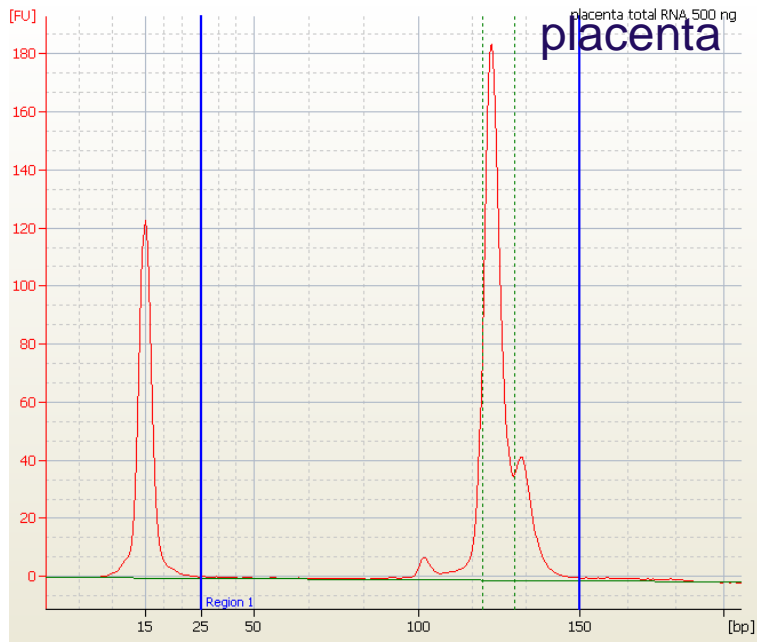
Measure the area for the DNA that is 25–150 bp (the size range for ligation products with no insert and ligation products with short inserts) and 120–130 bp (the size range for the desired miRNA ligation products).

Calculate the ratio of 120–130-bp DNA: 25–150-bp DNA:

$$[\text{Area (120–130 bp)}] \div [\text{Area (25–150 bp)}]$$

smRNA workflow cont...

Why 120-130 bp?

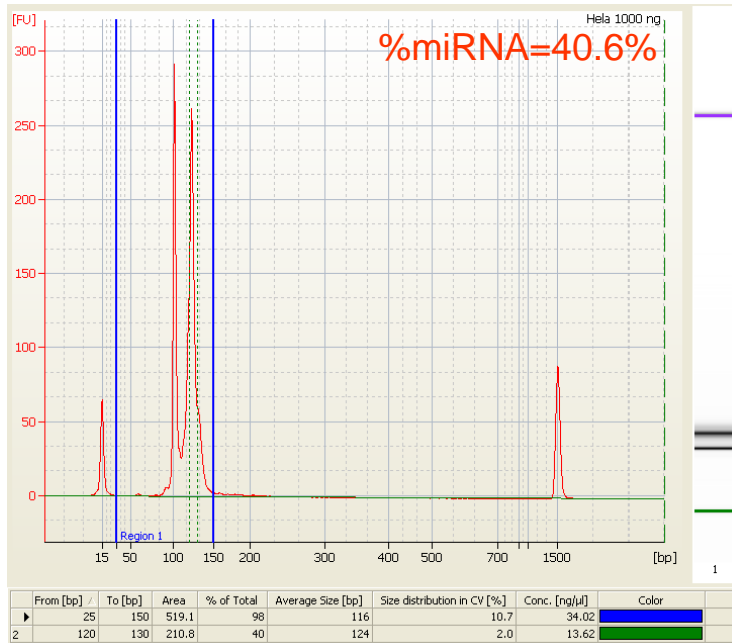


Region	RNA Inserts
25-120 bp	Ligation by-products (102-105 nt)
120-130 bp	miRNA ligation products (122-126 nt)
130-150 bp	Other RNA (degraded tRNA?, 132-135 nt)

% 120-130 bp (%miRNA) on bioanalyzer is a good indicator of library quality.

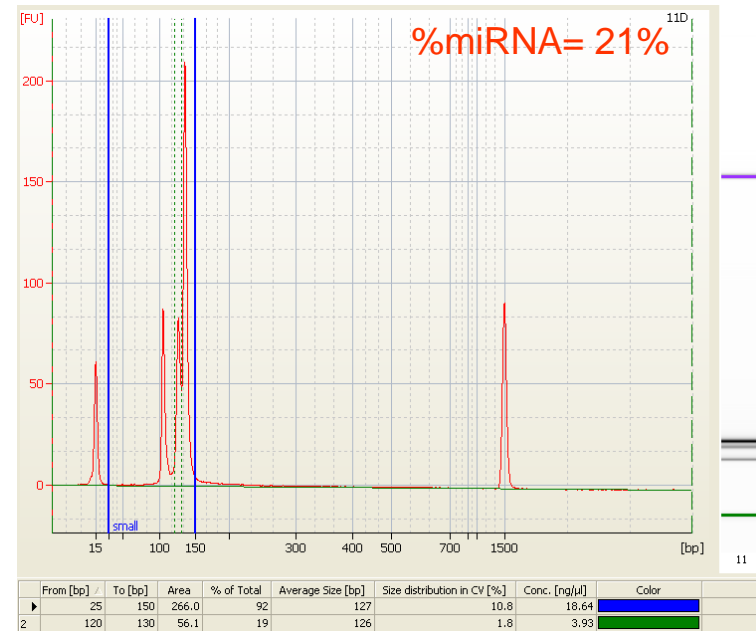
smRNA workflow cont...

Library QC



Small by-products are dominant

- Normally happens when the input amount is low or cut too low during size selection
- Repeat size selection on cDNA or perform extra native gel purification on PCR products

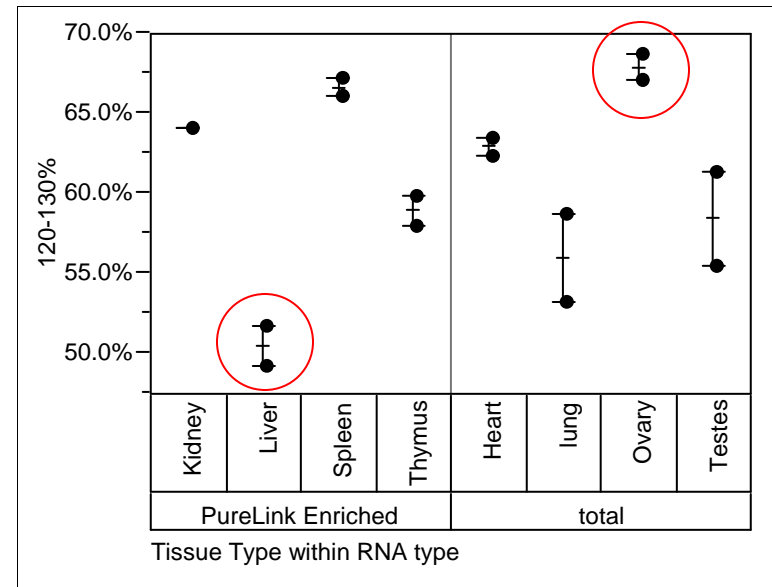
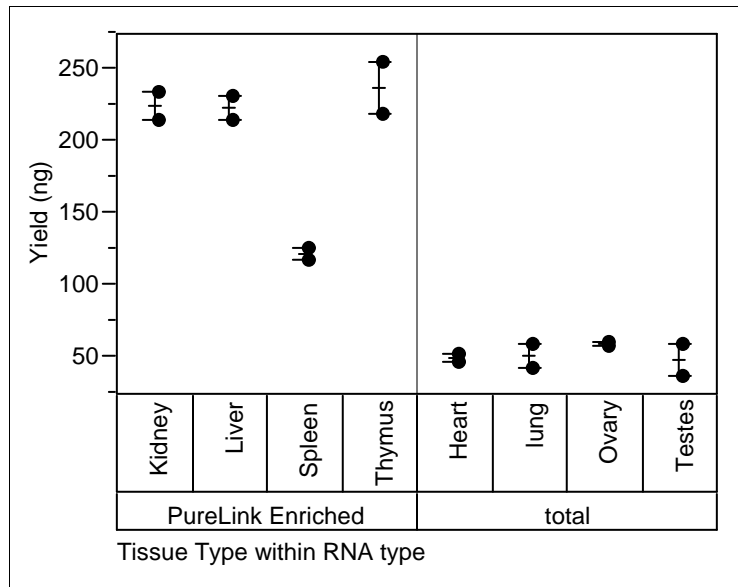


Larger ligation products (~135 bp) are dominant

- real small RNA (~35 nts)
- tRNA degradation products
- Repeat size selection on cDNA by cutting 60-70 nts cDNA

smRNA workflow cont...

Small RNA Tissue Panel



Input amount: RNA (total/enriched) containing 25ng of small RNA (10-40 nts). 15 cycles of PCR was performed on all samples. Yield is above 50 ng for all the samples. Enriched RNA generates more cDNA than total RNA.



Agenda

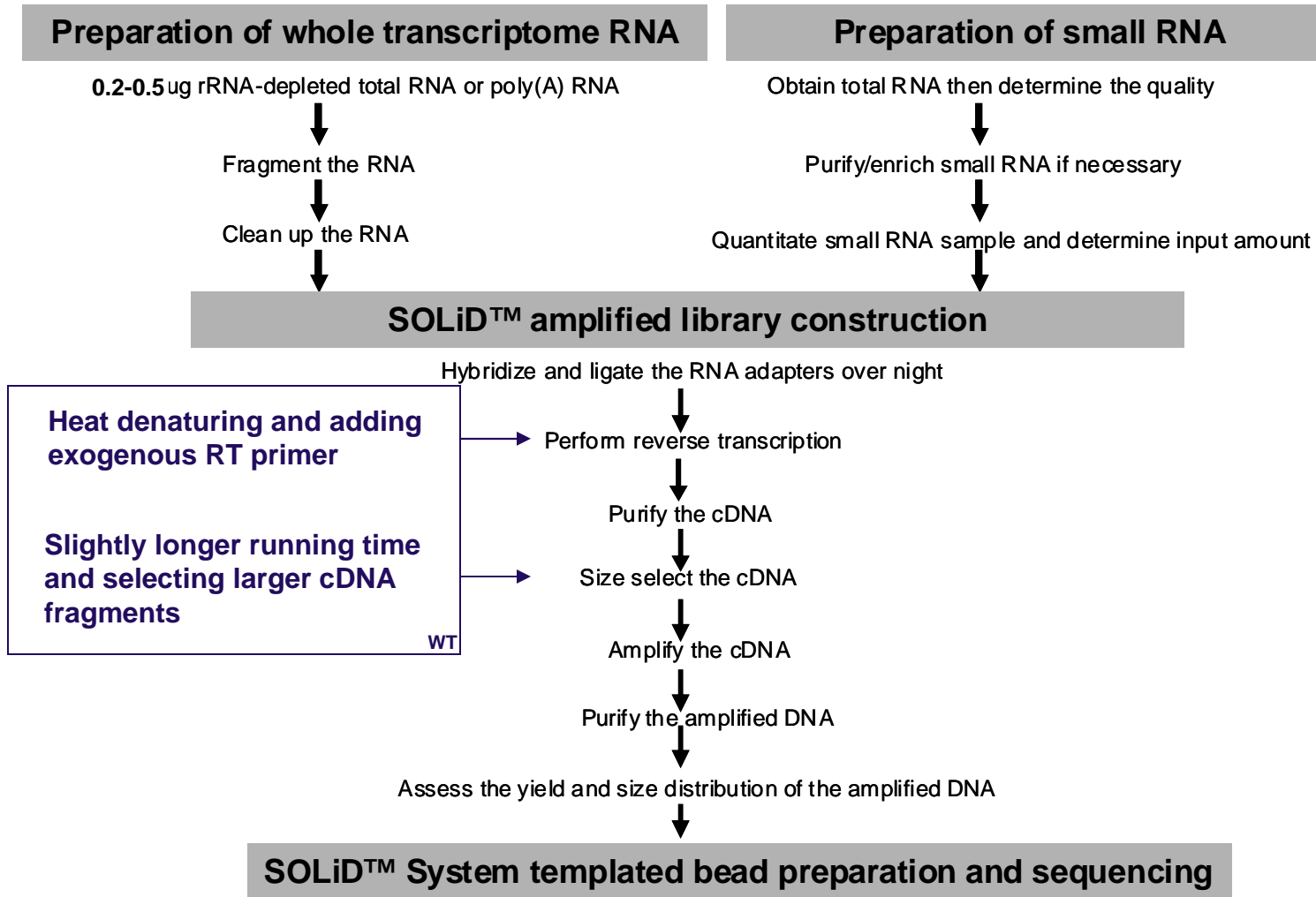
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WT Workflow



WT workflow cont...

Change 1: DNA Fragmentation Elution Vol

Component	Volume
RNA sample and Nuclease-free Water: <ul style="list-style-type: none">• Poly(A) RNA: 100–500 ng• rRNA-depleted total RNA: 200–500 ng• WT Control RNA: 500 ng	8 μ L
10X RNase III Buffer	1 μ L
RNase III	1 μ L
Total volume	10 μL

RiboMinus Concentration Module Cleanup

5. Elute the RNA in a clean Recovery Tube:
 - a. Place the Spin Column in a clean Recovery Tube.
 - b. Add 12 μ L of RNase-Free Water to the center of the Spin Column.
 - c. Wait 1 minute, then spin the column at maximum speed for 1 minute.

Notice: Elution volume change, no need to speed vac if starting from 500 ng of RNA.

WT workflow cont...

Change 2. Perform Reverse Transcription

1. On ice, prepare RT master mix (*without* the ArrayScript™ Reverse Transcriptase):

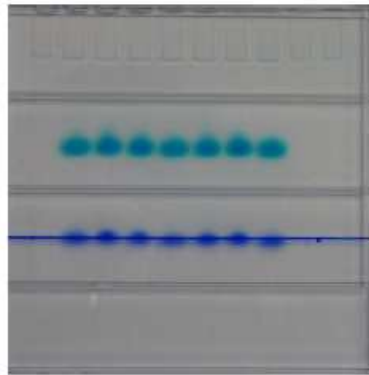
Component	Volume for each reaction (μL) [†]
Nuclease-free Water	11
10X RT Buffer	4
dNTP Mix	2
SOLiD™ RT Primer	2
Total volume per reaction	19

2. Incubate the RT master mix with the ligated RNA sample:
 - a. Add 19 μL of RT master mix to each 20-μL ligation reaction.
 - b. Pipet up and down a few times to mix, then spin briefly.
 - c. Incubate in a thermal cycler with a heated lid at 70 °C for 5 minutes, then snap-cool on ice.
3. Perform the reverse transcription reaction:
 - a. Add 1 μL ArrayScript™ Reverse Transcriptase to each ligated RNA sample.
 - b. *Gently* vortex to mix thoroughly, then spin briefly.
 - c. Incubate in a thermal cycler with a heated lid at 42 °C for 30 minutes.

WT workflow cont...

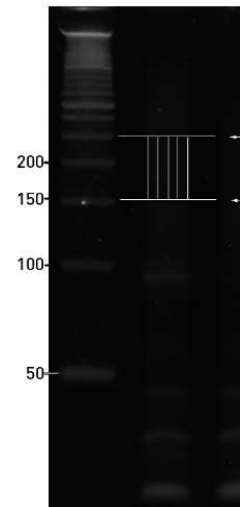
Change 3. Gel Running Time and Cutting Size

6. Run the gel at 180 V until the leading dye front is 1 cm below the middle of the gel (~25 minutes).



Run the gel until the leading dye front is 1 cm below the middle of the gel

- a. Using a clean razor blade, make horizontal cuts to excise the gel containing 150–250 nt cDNA.

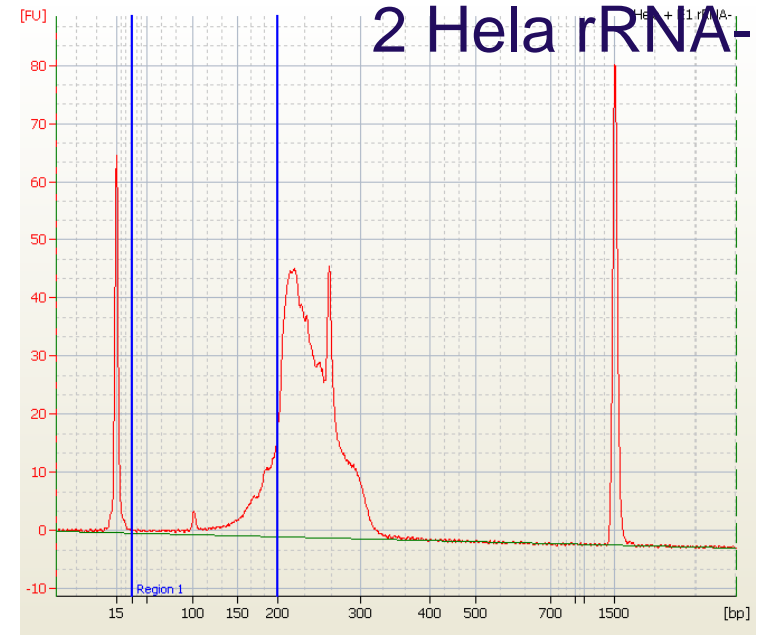
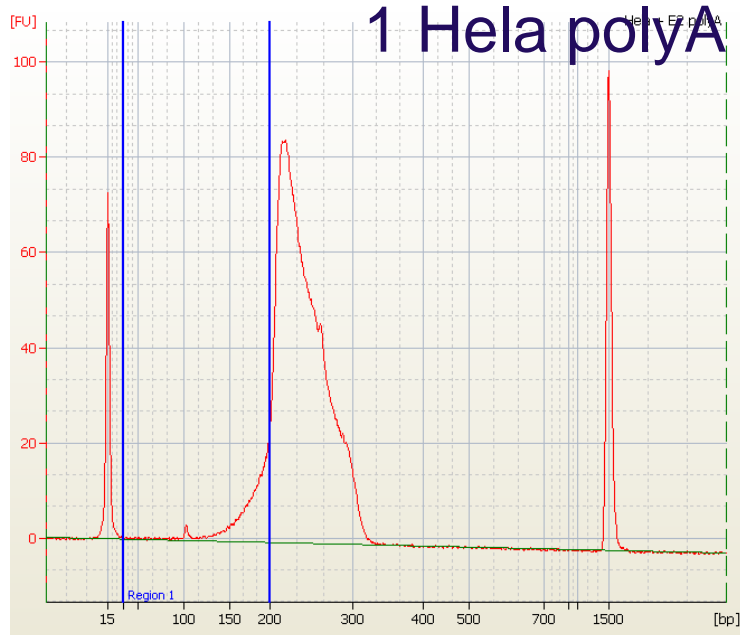


Make the horizontal cuts first to obtain the desired insert length

Excised cDNA length (nt)	Insert length (bp)	PCR product length (bp)
50	~0	~100
100	~50	~150
150	~100	~200
200	~150	~250
250	~200	~300

WT workflow cont...

Change 4. Library QC



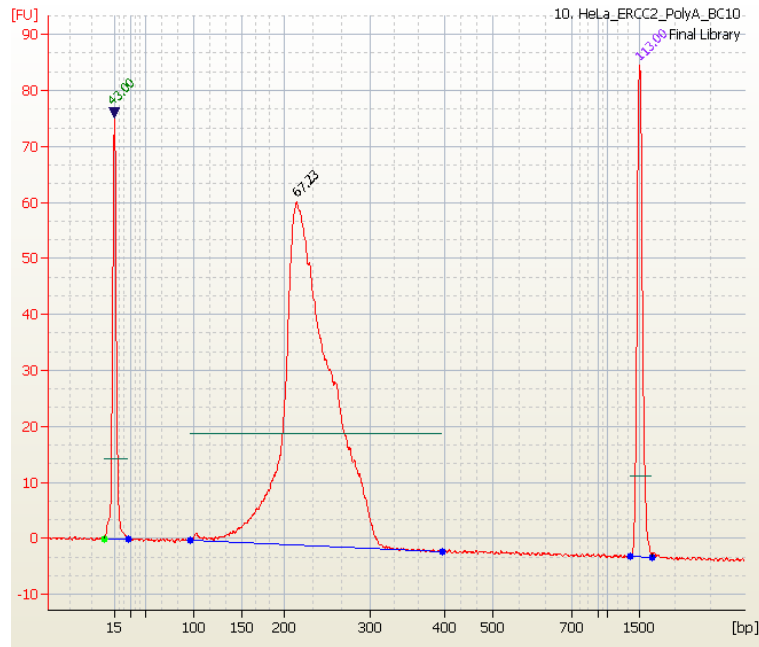
1	Name	From [bp]	To [bp]	Area	% of Total	Average Size [bp]	Conc. [ng/μl]	Molarity [nmol/l]
▶	Region 1	25	200	68.1	10	171	3.39	32.5

2	Name	From [bp]	To [bp]	Area	% of Total	Average Size [bp]	Conc. [ng/μl]	Molarity [nmol/l]
▶	Region 1	25	200	61.2	14	164	3.85	40.6

Percent of DNA in the 25–200 bp range	Next steps
Less than 20%	Proceed with SOLiD™ System templated bead preparation (see page 29).
Greater than 20%	Follow the troubleshooting instructions for “Normal yield and bad size distribution in the amplified library” on page 31 .

WT workflow cont...

Change 5. Multiplexing for Sequencing

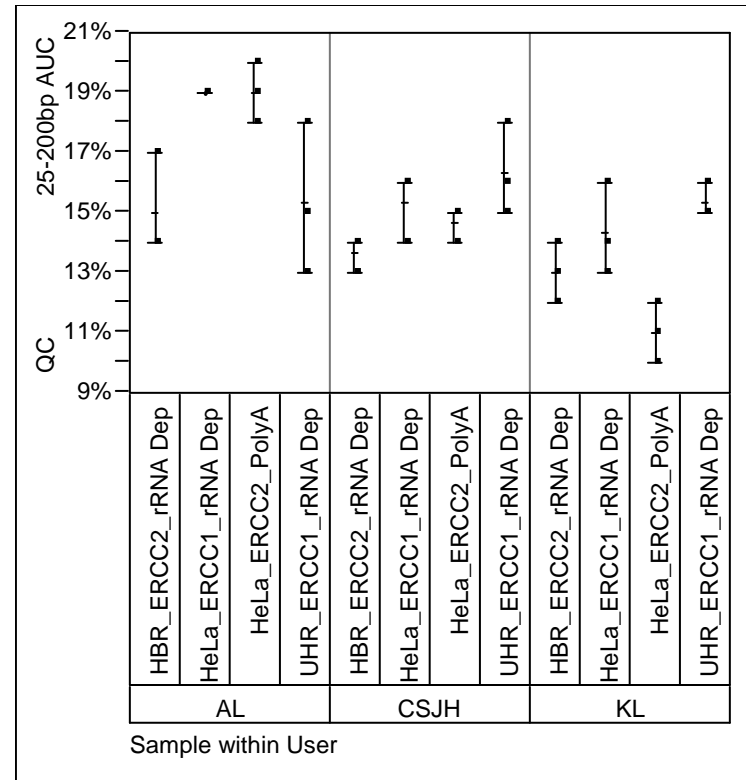
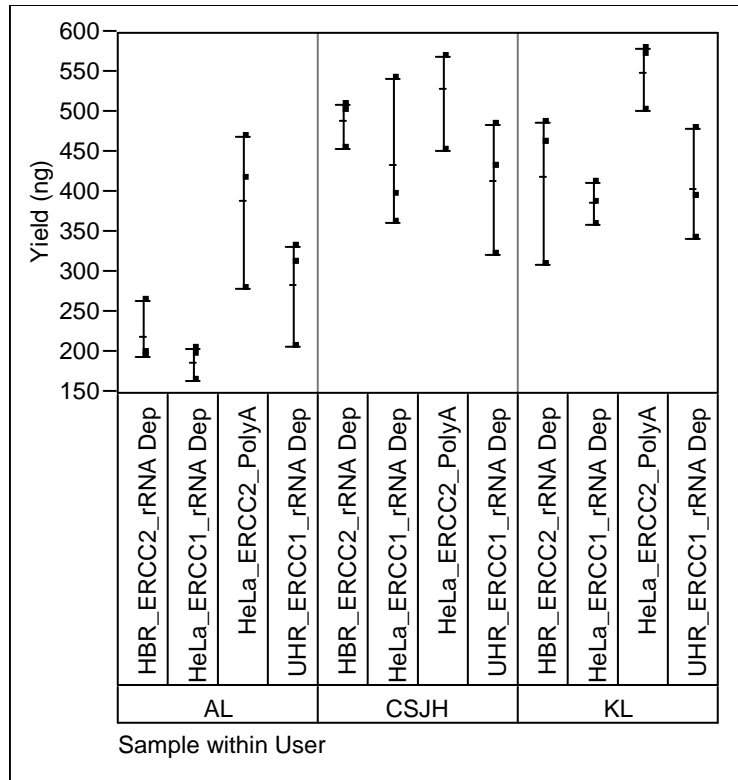


	Size [bp]	Conc. [ng/μl]	Molarity [nmol/l]	Observations
▶ ◀	15	4.20	424.2	Lower Marker
2	214	22.60	159.8	
3 ▶	1,500	2.10	2.1	Upper Marker

- Equal molar mix based on DNA
1000 chip quantitation or,
preferable, QPCR.
- When optimizing library
concentrations by workflow
analysis (WTA), library
concentrations of 0.4 pM and 0.8
pM for ePCR are recommended.

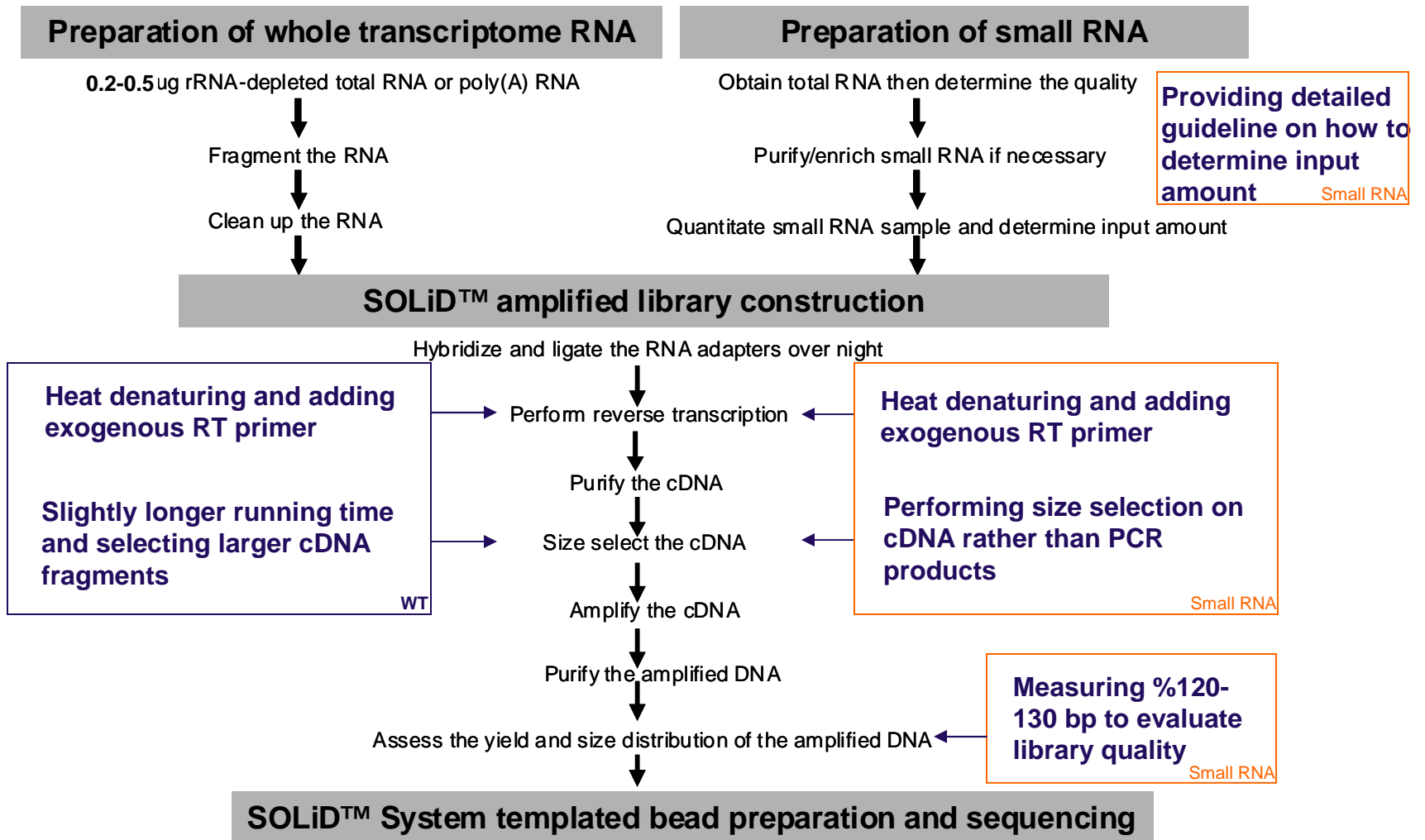
WT workflow cont...

Examples for WT Library Yield and Size



Input- 0.5 μ g Hela polyA, Hela rRNA-, HBR rRNA- and UHR rRNA- RNA

Summary





Thank you!