Protocol Summary

1. Reverse transcription 1
    - Produces single-stranded cDNA
    - Adds an in-line barcode and PCR handle to the 5'-end of the cDNA
    - Adds untemplated nucleotides (C) to the 3'-end of the cDNA upon reaching the 5'-end of the RNA molecule
2. Pool libraries (optional)
    - Given you used different barcodes in the above step, libraries can be pooled at this stage and de-convoluted by trimming 5' nucleotides from the start of Read 2 after sequencing
    - Pooling here enables all subsequent steps to be conducted in singlet form - the primer document includes 24 unique barcodes for this step
3. AMPure XP bead cleanup
    - Removes primers from reverse transcription 1 to prevent a "short-circuit" of random primer acting directly on the template switching oligo to yield an empty PCR amplicon without cDNA inserts
4. Reverse transcription 2
    - Untemplated C's from the first RT reaction anneal to untemplated G's in a template switching oligo
    - RT transcribes the template switching oligo, adding a 3'-PCR handle to the cDNA
5. AMPure XP bead cleanup
    - Remove unreacted template switching oligos
6. Amplify libraries
    - Can check for correct cycle number by gel or qPCR.
    - As with typical Illumina libraries, i7 barcodes can be added at this stage as the Read 2 / i7 index region of the final library uses compatible sequences with standard primers. The primer document includes 12 primers for step; combined with barcoding during the first reverse transcription step, this enables 24 × 12 = 288 unique barcodes
7. Size selection
    - Double-sided magnetic bead size selection removes both primer-dimers and large fragments that can interfere with sequencing
8. Sequence
    - Note sequencing requires use of a custom oligo for the Read 1 side that matches the template switching oligo

Protocol

I. Reverse Transcription 1

See worksheet for calculating multiple reactions. If using our homebrew rRNA depletion, the 'input RNA' here can just be the supernatent off of the streptavidin beads. Since the typical reaction size is 15 uL for the V1.1 depletion, you should be able to get 2 typical libraries (5 uL each) out of each depletion reaction if you want. I have routinely gone as low as ~200 ng input total RNA using the V1.1 depletion method and a 5 uL input into this step. If you're inputting total RNA into this step, you're likely to get plenty of signal with as little as 25 ng of input total RNA into this step. Lower inputs should be possible in both cases, I just haven't tried them.

1. Prepare master mix containing all components except input RNA, SmartSeq2 buffer, and reverse transcription primer.

Reagent	Amount (uL)
10 mM dNTPs	1
100 mM dC	0.4
RNase Inhibitor	1
100 mM DTT	4
5M Betaine	4
Reverse Transcriptase	0.5
Water	1.6

2. Mix input RNA, SmartSeq2 buffer, and primer in striptubes or 96-well plate. The RT primers are oPCf107-112 or oPCf121-138. These primers constitute the 24 different inline barcodes that can be integrated at this step. See spreadsheet for sequences.

Reagent	Amount (uL)
Input RNA	5
SS2 Buffer	2
RT Primer (100 uM)	0.5

3. If fragmenting, heat at 95C on a heat block for 3-10 minutes. If using a plate, completely seal it or your reactions will evaporate. Place on ice after fragmentation.
4. Add master mix to each well (12.5 uL MM into 7.5 uL reaction → 20 uL final volume), mix by pipetting.
5. Place on thermocycler set for:
    - 25C x 10 minutes
    - 50C x 50 minutes
    - 85C x 5 minutes
    - hold at 4C
6. Freeze reactions (-20C should be adequate for short-term storage, -80C preferred) or proceed to next step.

II. Pool Libraries (recommended for many libraries)

This will depend on your application. If you used uniquely barcoded primers in the previous step, reactions can be mixed here. If you don't mix here, the subsequent steps need to be conducted separately on each sample - depending on the number of samples this may be prohibitive. If you choose to pool here (I typically use 5 uL of each RT reaction), the final read count from each input sample will vary depending on the amount of input RNA and the efficiency of library construction in the previous step in each sample. If you are just preparing a few libraries, this probably doesn't matter.

So far, there are two ways I have used to ensure an equal read count in pools of many samples:

1. Take a fraction of each library from the previous step, proceed with the following steps, and sequence the resulting final library using a low cost / low yield kit such as a MiSeq Micro or Nano kit. The relative contribution of each inline barcode from Reverse Transcription 1 can be used to generate a new pool to proceed with or, if the current pool is acceptable, the original library can be sequenced with a high yield kit.
2. Take a fraction of each library and proceed through Step 5 (2nd AMPure XP bead cleanup). Next, instead of a typical amplification, conduct a qPCR on the pool using primers matching the template switching region and unique inline barcodes (oPCf33 + oPCf113-118 or oPCf139-156). The Ct values of the various primers on the pool can be used to estimate the yield of RT1 for each sample. The yields for each sample can then be used to plan a final pool.

III. AMPure XP bead cleanup

Note that AMPure XP beads can be pelleted and resuspended in Tris in a pool for multiple reactions to reduce pipetting.

1. Place 50 uL of AMPure XP beads on a magnetic rack. Once beads are pelleted, carefully remove the stock buffer without disturbing the bead pellet.
2. Determine volume needed to bring the pool from **II.** or reaction from **I.** to 200 uL (e.g. for a single reaction, add 180 uL; for 5 uL from 24 reactions, add 80 uL). Remove the bead pellet from the rack and resuspend in the calculated volume of 10 mM Tris pH 8.
3. Mix sample or library pool with resuspended beads to achieve a final volume of 200 uL.
4. Add 200 uL of PEG binding buffer, briefly vortex to mix, and incubate 5 minutes at RT.
5. Place on magnetic rack and allow beads to settle until solution has clarified (~5 minutes).
6. Carefully remove supernatent without disturbing pellet and wash twice with 450 uL fresh 80% ethanol without removing from magnetic rack.
7. To facilitate drying, take tube off of rack and do a ~1 second spin on a benchtop centrifuge to pull remaining ethanol to the bottom of the tube. Place back on magnetic rack and remove remaining ethanol with a 10 uL pipette without disturbing pellet.
8. Dry for ~2 minutes. Do not overdry as this may reduce yields. Overdried pellets are difficult to resuspend and have a cracked surface.
9. Remove from rack, resuspend in 10 uL of 10 mM Tris pH 8, and incubate for 5 minutes at RT.
10. Return to rack, pull off supernatent without disturbing pellet.
11. Freeze reactions (-20C should be adequate for short-term storage, -80C preferred) or proceed to next step.

IV. Reverse Transcription 2

See worksheet for calculating multiple reactions.

1. Add master mix to 10 uL output from the above step, mix by pipetting. Master mix shown below.

Reagent	Amount (uL)
Resuspension	10
5x RT Buffer	4
Template Switch Primer (oPCf42) (100 uM)	0.5
dCTP (100 mM)	0.4
RNase Inhibitor	1
dNTPs (10 mM)	1
RT Enzyme	0.5
Water	2.6

2. Place on thermocycler set for:
    - 25C x 10 minutes
    - 42C x 30 minutes
    - 85C x 5 minutes
3. Freeze reactions (-20C is now acceptable for long term storage as cDNA is complete) or proceed to next step.

V. AMPure XP bead cleanup

1. Prepare 50 uL of AMPure XP beads as in **III**. Resuspend bead pellet in 180 uL 10 mM Tris pH 8.
2. Mix reverse transcription reaction and resuspended beads for a final volume of 200 uL.
3. Proceed with steps 4-10 as in **III.**, except resuspending in 20 uL of 10 mM Tris.
4. Store purified reactions at -20C or proceed to next step.

VI. Amplification

*I try to avoid over-amplifying libraries. Commercial kits have guidelines of input RNA → number of cycles. Given the number of variables in this protocol (e.g. RNA depletion efficiency, amount of each reaction added to pool in **II.**), it's difficult to easily assign a number here. In my experience with reasonable inputs of RNA (see **I.**), I typically get nice library signal from 11-16 cycles. As a QC and to determine the correct number of amplification cycles, I typically run a trial PCR. Below I describe 2 ways to run this trial PCR.*

A. Trial PCR

See worksheet for reaction preparation for multiple samples for both the qPCR and gel version of the protocol.

Using qPCR

This is my typical choice as it's faster to prep and uses less library than testing multiple cycle numbers on a gel.

1. Prepare trial reactions using 1/10 of clean up resuspension (2 uL) and 2x qPCR master mix (I use iTaq Universal SYBR Green Supermix) in a 10 uL reaction loaded on a 384 well plate. Prepare 1 trial reaction per cleaned up RT2 reaction. Primers used here are oPCf39 (adding the i5 side) and oPCf161-172 (adding the i7 side and i7 barcode).

Reagent	Amount (uL)
2x qPCR Master Mix	5
oPCfx (100 uM)	0.2
oPCf39 (100 uM)	0.2
Template	2

Reagent	Amount (uL)
Water	2.6
Total	10

2. Set to run at least 25 cycles on a qPCR machine. On a ViiA 7, I use a 2 step reaction:
    - 95C x 5 minutes
    - (95C x 30s, 60C x 45s) x 25 cycles
3. The cycle number where the signal breaks ~1/2 maximum signal is typically adequate for the final PCR.

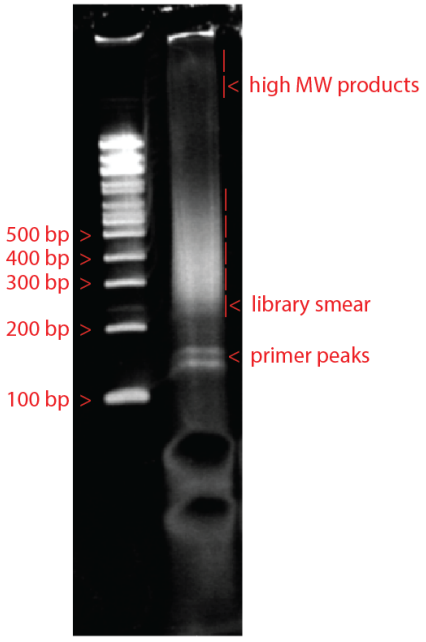
Using end point PCR and gels

Gels are also a great QC check if the library is not sequencable, fails library quantification, or fails sequencing QC.

1. Prepare trial reaction as in qPCR, but using KAPA HiFi HotStart ReadyMix (2x). Since this is an endpoint assay, prepare multiple reactions per sample - I typically do 3 reactions trying 11, 13 and 16 cycles. Primers used here are oPCf39 (adding the i5 side) and oPCf161-172 (adding the i7 side and i7 barcode).

Reagent	Amount (uL)
2x KAPA	5
oPCfx (100 uM)	0.2
oPCf39 (100 uM)	0.2
Template	2
Water	2.6
Total	10

2. Load reactions into striptubes and cycle on a standard thermocycler:
    - 95C x 45s
    - (98C x 15s, 60C x 30s, 72C x 30s) x cycles (see above)
    - 72C x 1 minute
    - hold at 10C
3. After cycling, run reactions out on a non-denaturing acrylamide gel and stain for ~5 minutes with SYBR gold stain for highest resolution and lowest detection limit. Agarose gels + EtBr are also acceptable, but note that an adequately amplified library will just barely be visible by agarose gel due to the lower sensitivity.
4. A good library at the correct cycle number will have medium signal in the library smear on a acrylamide gel. If the smear is just barely visible, another cycle or two is probably a good idea. If there is laddering or the appearance of high MW products, fewer cycles should be used. See below image for example of a high quality library run on a 8% TBE Gel alongside 5 uL of NEB 100 bp ladder. If I saw this library on a gel, I'd likely subtract 1 cycle number as the signal is perhaps a little too strong.



B. Final PCR

See worksheet for reaction preparation for multiple reactions. At this stage, you can integrate barcodes via PCR primer to the i7 side to further multiplex samples.

1. Conduct final PCR using the cycle numbers determined above with KAPA HiFi Hotstart ReadyMix (2x). Typically I do 50 uL reaction volumes (5x trial PCR) though as little as 30 uL has worked fine. Primers used here are oPCf39 (adding the i5 side) and oPCf161-172 (adding the i7 side and i7 barcode).

Reagent	Amount (uL)
2x KAPA	25
oPCfx (100 uM)	1
oPCf39 (100 uM)	1
Template	10
Water	13
Total	50

2. Load reactions into striptubes and cycle on a standard thermocycler:
   - 95C x 45s
   - (98C x 15s, 60C x 30s, 72C x 30s) x cycles (see above)
   - 72C x 1 minute
   - hold at 10C
3. Store reactions at -20C or proceed with next step.

VII. Size Selection

Size selection lower and upper limits can be altered by changing the amount of PEG solution added.

1. Prepare 2 aliquots of 50 uL of AMPure XP beads as described in Step 3. After pelleting and removing supernatent, resuspend the first aliquot in 100 uL of PEG binding buffer and the second aliquot in 40 uL of PEG binding buffer.
2. Bring final PCR to a 200 uL volume using 10 mM Tris pH 8.
3. Add first aliquot of bead / binding buffer mix to achieve a 300 uL volume and allow beads to incubate at room temperature for 5 minutes. This will precipitate fragments that are too large onto the beads given the low concentration of PEG.
4. Place on magnetic rack and allow solution to clarify.

5. Keep supernatent and discard beads. Add the supernatent to a tube containing the second aliquot of 40 uL of PEG binding buffer for a final volume of 340 uL.
6. Allow to incubate at room temperature for 5 minutes. This will precipitate fragments that are large enough to have a cDNA insert onto the beads while avoiding primer dimers and very short unmappable inserts.
7. Place on magnetic rack and allow beads to settle until solution has clarified (~5 minutes).
8. Carefully remove supernatent without disturbing pellet (the sample is now on the beads) and wash twice with 400 uL fresh 80% ethanol without removing from magnetic rack.
9. To facilitate drying, take tube off of rack and do a ~1 second spin on a benchtop centrifuge to pull remaining ethanol to the bottom of the tube. Place back on magnetic rack and remove remaining ethanol with a 10 uL pipette without disturbing pellet.
10. Dry for ~2 minutes. Do not overdry as this may reduce yields. Overdried pellets are difficult to resuspend and have a cracked surface.
11. Remove from rack, resuspend in 12 uL of 10 mM Tris pH 8, and incubate for 5 minutes at RT.
12. Return to rack, pull off supernatent without disturbing pellet.
13. Store libraries at -20C or proceed with next step.

VIII. Sequence

Proceed with a typical library quantification pipeline for Illumina libraries or submit samples to a sequencing facility for quantification and quality control. If multiple libraries were prepared with different i7 indexes, they can be pooled using normal techniques.

For sequencing, you must use a custom Read 1 primer (oPCf40) that binds to the template switch primer sequence. Library diagram shows final structure of reads; to identify which sample each read arose from, use the first 9 nucleotides of Read 2 (the in-line barcode). Note that these libraries require paired-end sequencing to assign sample barcodes.

Reagents

Enzymes, etc

Maxima H Minus Reverse Transcriptase (Thermo) - EP0751
SUPERase-In (Thermo) - AM2694
KAPA HiFi HotStart ReadyMix PCR Kit (Kapa) - KK2600
AMPure XP (Beckman) - A63880

Consumable Recipes

Modified SmartSeq2 Buffer

Use as buffer in Reverse Transcription 1 high magnesium facilitates fragmentation and untemplated addition of Cs. Reverse Transcription 2 uses stock buffer for Maxima H Minus Reverse Transcriptase.

Reagent	Amount
1M Tris pH 8	250 uL
2M KCl	187.5 uL
1M MgCl2	60 uL
Water	2.5 uL

PEG binding buffer (2.5 M NaCl / 20% PEG 8000)

For clean ups and size selection.

Reagent	Amount
5M NaCl	20 mL
PEG 8000 (dry)	8 grams
Water	BTV 40 mL