National Human Genome Research Institute

Penn Program in Single Cell Biology

ITMAT Institute for Translational Medicine and Therapeutics

Single Cell Transcriptomics Workshop

May 4 – 8, 2015

Penn Program in Single Cell Biology
University of Pennsylvania

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THE PENN PROGRAM IN SINGLE CELL BIOLOGY

The Penn Program in Single Cell Biology began operating in 2014. The mission of the program is to promote interdisciplinary collaboration in single cell analyses, while providing access to resources and technologies. In addition, the program seeks to develop new technologies that will have application in the understanding of single cell function.

The Penn Program in Single Cell Biology offers:

- Workshops on multiple aspects of single cell biology techniques
- Advice for investigators wishing to incorporate single cell biology into their research program

Leadership:

- **James Eberwine, Co-Director**
  Elmer Holmes Bobst Professor, Department of Pharmacology, University of Pennsylvania Perelman School of Medicine
- **Junhyong Kim, Co-Director**
  Patricia M. Williams Professor, Department of Biology, University of Pennsylvania School of Arts and Sciences
- **Catherine Moorwood, Assistant Director for Programs**
  Research Associate, Department of Pharmacology, University of Pennsylvania Perelman School of Medicine

WORKSHOP KEYNOTE SPEAKER

Nancy Allbritton, MD, PhD, Professor and Chair, UNC/NCState Department of Biomedical Engineering

Dr. Allbritton obtained her B.S. in physics from Louisiana State University, her Ph.D. in Medical Physics/Medical Engineering from the Massachusetts Institute of Technology, and her M.D. from the Johns Hopkins University. Upon completion of a postdoctoral fellowship in cell biology at Stanford University, she joined the faculty of the University of California at Irvine in 1994 where she held joint appointments in the Departments of Physiology and Biophysics, Biomedical Engineering, Chemistry, and Chemical Engineering & Materials Science. She has received multiple awards including a Beckman Investigator Award and a Searle Scholar Award and is a Fellow in the American Institute for Medical & Biological Engineering and the AAAS. She is also a member of the National Academy of Inventors. She joined the University of North Carolina at Chapel Hill (UNC) as the Debreczeny Distinguished Professor in the Department of Chemistry in July, 2007 followed by a joint appointment with the School of Medicine in the Department of Pharmacology. In 2009, she was also appointed Professor and Chair of the Department of Biomedical Engineering, a joint department between North Carolina State University and UNC. Dr. Allbritton's research studies, described in over 130 publications, are directed at the development of new technologies by bringing to bear methods from engineering, chemistry, physics and biology to address biomedical problems. This research program has been heavily funded by the National Institutes of Health with over $40 M in grant funding since 1994. Dr. Allbritton is the scientific founder of three companies, Protein Simple (valued at $308 M), Cell Microsystems, and Alltiss Biosystems and has 11 issued patents with >8 more pending.

Workshop Schedule

Lab work will take place in room 328, Carolyn Lynch Laboratories, unless otherwise indicated. Lectures will take place in room 318, Carolyn Lynch Laboratories. Timings for experimental work are approximate.

Day 1 – Monday 4th May

08:30am – 09:00am	Breakfast (coffee, tea and pastries)
09:00am – 10:00am	Lecture – Why work with single cells? *James Eberwine, Elmer Holmes Bobst Professor of Pharmacology and Co-Director, PPSCB*
10:00am – 12:00pm	Single cell isolation techniques (patch pipette and Isoraft) Rooms 24 and 37, John Morgan building *Isoraft: Marcela Garcia, Postdoctoral Researcher, Pharmacology*
12:00pm – 01:15pm	Lunch break (Pick up temporary Penn cards)
01:15pm – 01:45pm	aRNA: first round, first strand synthesis
01:45pm – 02:30pm	Smart-Seq part 1
02:30pm – 02:45pm	aRNA: first round, second strand synthesis
02:45pm – 04:45pm	Fluidigm C1 demonstration *Olga Smirnova, Research Specialist and Jonathan Schug, Technical Director, Next Generation Sequencing Core*
04:45pm – 05:15pm	aRNA: finish first round, second strand synthesis
05:15pm – 05:30pm	aRNA: first round IVT

Day 2 – Tuesday 5th May

08:15am – 08:45am	Breakfast
08:45am – 10:30am	aRNA: first round cleanup
10:30am – 11:30am	Lecture – Nuts and bolts of single cell transcriptomics techniques *Catherine Moorwood, Assistant Director for Programs, PPSCB*
11:30am – 12:30pm	aRNA: finish first round cleanup
12:30pm – 01:00pm	aRNA: second round, first strand synthesis
01:00pm – 01:45pm	Lunch break (Catherine will transfer samples)
01:45pm – 02:15pm	aRNA: second round, second strand synthesis
02:15pm – 03:00pm	Smart-Seq part 2
03:00pm – 04:00pm	Lecture – Single cell analysis in development: robustness and temporal coordination in the face of molecular noise *John Murray, Assistant Professor of Genetics*
04:15pm – 05:00pm	aRNA: finish second round, second strand synthesis and cDNA cleanup
05:00pm – 05:15pm	aRNA: second round IVT

Day 3 – Wednesday 6th May

08:30am – 09:00am	Breakfast
09:00am – 10:45am	aRNA: second round cleanup
10:45am – 11:30am	Smart-Seq part 3: 1st cleanup
11:30am – 12:15pm	aRNA: finish second round cleanup
12:15pm – 12:45pm	aRNA: third round, first strand synthesis
12:45pm – 01:30pm	Lunch break (Catherine will transfer samples)
01:30pm – 02:00pm	aRNA: third round, second strand synthesis
02:00pm – 03:00pm	TIVA demonstrations (in 2 groups) *Jai-Yoon Sul, Research Assistant Professor of Pharmacology* Room 89, John Morgan building
03:00pm – 04:00pm	Keynote talk – Microengineered Tools for Single Cells *Nancy Allbritton, Professor and Chair, UNC/NCState Department of Biomedical Engineering*
04:00pm – 04:45pm	aRNA: finish third round, second strand synthesis and cDNA cleanup
04:45pm – 05:00pm	aRNA: third round IVT

Day 4 – Thursday 7th May

08:30am – 09:00am	Breakfast
09:00am – 10:45am	aRNA: third round cleanup
10:45am – 11:30am	Smart-Seq part 3: 2nd cleanup
11:30am – 12:15pm	aRNA: finish third round cleanup
12:15pm – 01:30pm	Lunch break.
01:30pm – 02:30pm	Lecture – Single cell analysis of gene expression by imaging *Arjun Raj, Assistant Professor of Bioengineering*
02:30pm – 04:00pm	TruSeq library prep: first and second strand synthesis
04:00pm – 05:00pm	Optional Bioanalyzer and pipette pulling demonstrations Rooms 24 and 37, John Morgan building
05:00pm – 05:45pm	TruSeq library prep: cleanup

Day 5 – Friday 8th May

08:30am – 09:00am	Breakfast
09:00am – 12:45pm	TruSeq library prep: A-tailing, adapter ligation, cleanup, PCR.
12:45pm – 01:30pm	Lunch break
01:30pm – 02:30pm	Lecture – RNA-Seq data analysis *Junhyong Kim, Edmund J. and Louise W. Kahn Term Endowed Professor of Biology and Co-Director, PPSCB*
02:30pm – 04:30pm	TruSeq library prep: cleanup

BEFORE YOU START

Advice on working with single cell RNA

Working with single cell RNA is similar to working with RNA in other contexts, except that you are starting with much smaller quantities. Therefore, you have to be more precise and careful (even paranoid!) than usual, to avoid loss of material and contamination.

Tips for working with single cell RNA:

- **ALWAYS** wear gloves. Don't touch things on your bench without wearing gloves.
- Change your gloves often. Throw them away when you take them off and get a new pair when you are ready to start again.

- Be careful in how you handle tubes. Don't touch the rim or the inside of the lid.
- Try not to breathe over your tubes when they are open.

- **ALWAYS** use filter tips.
- **ALWAYS** change pipette tips between each sample to prevent cross-contamination.
- Pay attention to your pipetting. You cannot afford to lose material, especially during the first round. Before discarding your tip, make sure no liquid is stuck on or inside it, and be careful not to leave anything behind when transferring between tubes.

- Make working aliquots of reagents such as nuclease-free water and ethanol each day.
- Make fresh dilutions of your primers each time.

- You do not need to worry too much about working quickly or keeping things cold. Working carefully to avoid contamination is more important.

- You can use products like RNaseZAP to clean your bench, but wash it down afterwards with 70% ethanol, because such products contain detergents that will inhibit enzymatic reactions.

Other tips for these protocols:

- For aRNA and library prep, you need to go through five days of work without making any serious mistakes. It will be 2 ½ days before you find out how well your samples are doing. So, work carefully, and ask for help if you make a mistake.
    - Double-check the reagent you are adding before you add it in.
    - Double-check the temperature of your incubation before putting your tubes in.

- When making mastermixes, add components in the order they are listed.

- Don't throw away reagent aliquots after use; you may need them again.

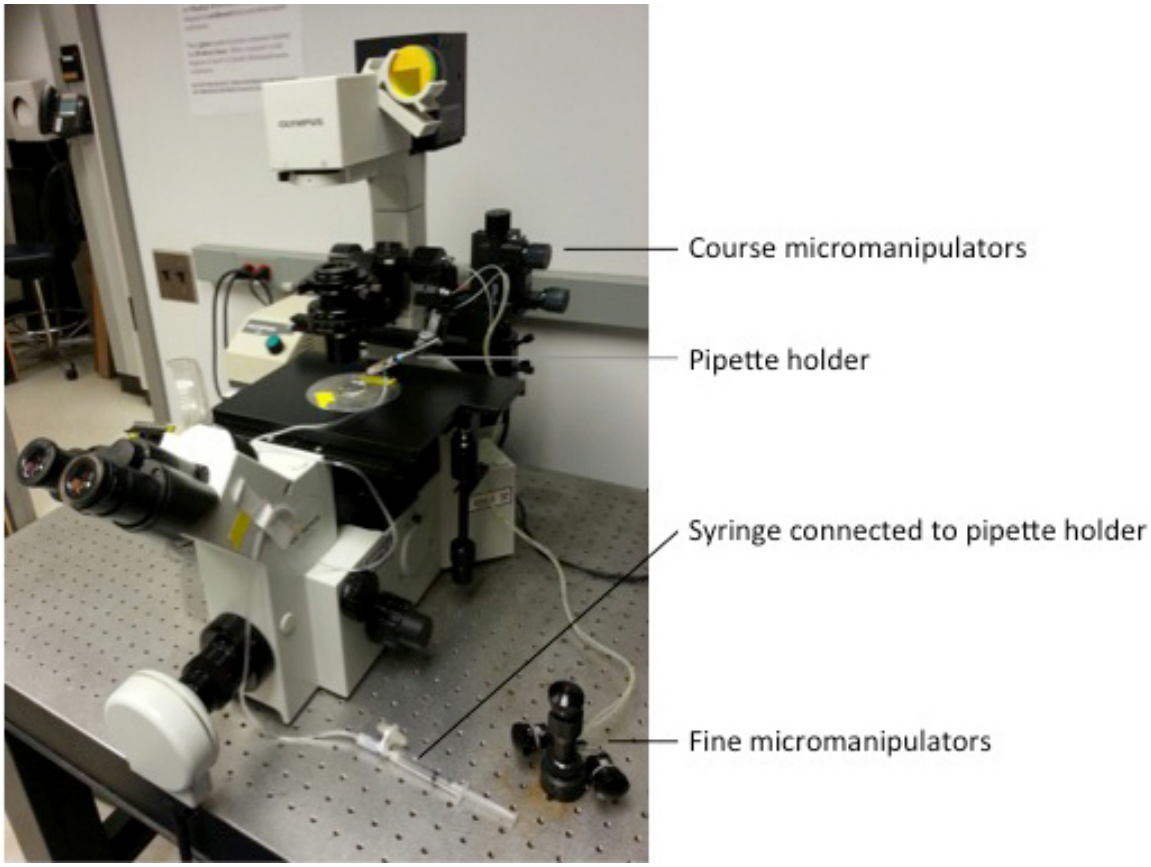
- Use nuclease-free water that has not been DEPC treated, as DEPC inhibits enzymes.

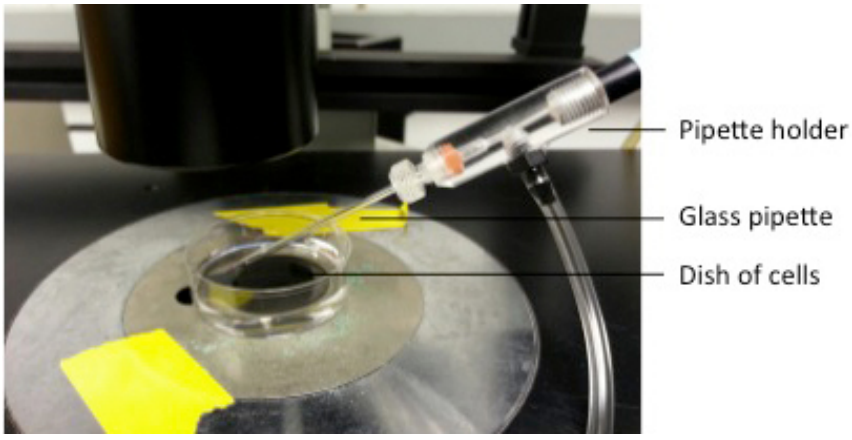
- Some days this week will be long, so use your time efficiently. Whenever you have an incubation step, look ahead to the next step and start to get things ready.

ISOLATION USING A PATCH PIPETTE

- Cells should be plated at a low enough density that there is space around each cell.
- For isolation, cells should be in a solution such as HBSS, that has a low concentration of phosphates and no calcium or magnesium.

Pipette harvesting setup:





To isolate cells:

- Secure a glass pipette in the pipette holder. For more information about pipettes, refer to the Sutter Pipette Cookbook.
- Position the cell you want to harvest in the center of the field of view.
- Use the course micromanipulators to position the pipette in the field of view (you will see a 'shadow' because it is not in the plane of focus).
- Lower the pipette into the solution. Use the syringe to apply positive pressure.
- Adjust the focus so you can see the pipette tip. Check that it looks as you expect and you can see the meniscus near the tip.
- Focus back on the cells. Use the course micromanipulators to carefully lower the pipette towards the cells. Before the tip comes fully into focus, switch to using the fine micromanipulators. Stop when the tip touches the cell body (you will see the cell deform slightly).
- Release the positive pressure on the pipette and use the syringe to aspirate the cell into the pipette. The shear forces on the cell will cause it to lyse in the process.
- Drag, and simultaneously rotate, the pipette around the inner wall of a tube containing first strand buffer and nuclease-free water, in order to break off the tip. Spin the tube briefly in a microcentrifuge to collect the tip and its contents in the bottom of the tube. Keep on ice until ready to start first-strand synthesis.

Illustration of pipette isolation (from Morris *et al* 2011 *J Vis Exp*)

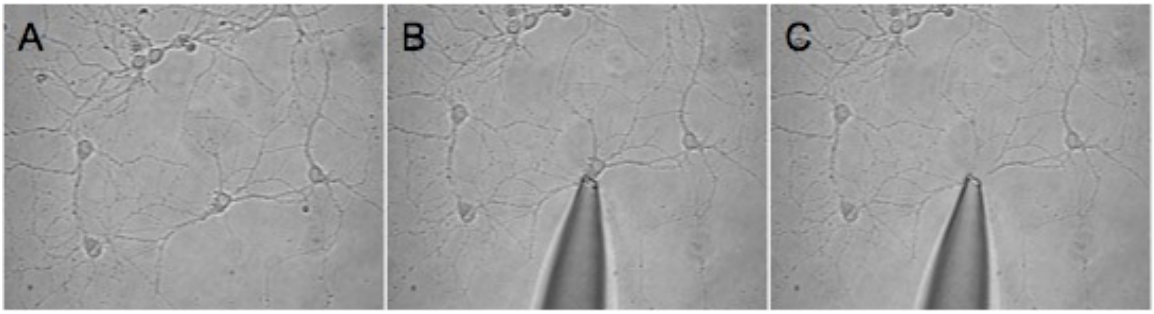


Figure 1. Shown is an example of a successful harvest of an isolated neuron. We selected a relatively low-density region (A) and advanced the pipette tip toward the desired cell (B). The third image (C) shows the field of view after the cell had been harvested. Note that the surrounding processes remain on the coverslip.

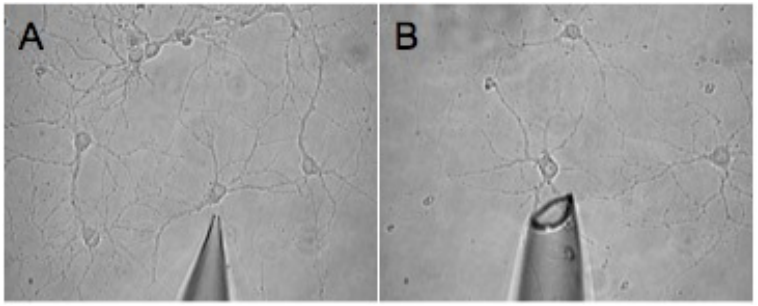


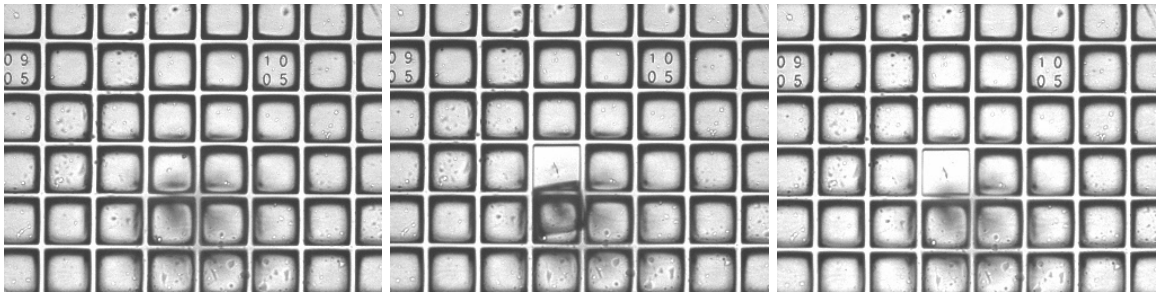
Figure 2. Shown are two images of pipette tips, which are an inappropriate size for effective harvesting. These tips will lead to incomplete harvest (A) and harvest of surrounding milieu (B) respectively.

ISOLATION USING THE ISORAFT SYSTEM

- Cells should be plated at a low enough density that many rafts have only one cell (4000 – 6000 cells per array is generally recommended).
- Prime the collection wand in lysis buffer, or HBSS with 0.01% Tween, for 10 min. Keep the wand in this solution when not in use. Rinse in HBSS before each use.
- Set up the release device as described in the Isoraft manual, marking the point where the release device needle punctures the array.

From left to right: release device viewed from below stage, release device viewed from above stage, dish of cells positioned above release device.

- Locate a raft with a single cell growing on it. Position the array so that the raft of interest is centered above the needle, and use the release device to dislodge the raft.



From left to right: Isoraft grid before raft release, after raft has been dislodged by release device needle, and after raft has been collected by magnetic wand.

- Rinse the collection wand in HBSS. Lift the lid of the Isoraft array and bring the collection wand into the area of the dislodged raft. You will see the raft float towards the collection wand. **IMPORTANT**: wait for 5 sec after you see the raft float to the wand, to make sure it is attached.
- Lift the wand out and replace the lid on the Isoraft array. **Without touching the sides of the tube**, carefully bring the tip of the wand to the bottom of a tube containing first strand buffer, nuclease-free water and a low concentration of Tween to aid lysis.
- Hold the wand at the bottom of the tube and bring the tube onto the magnet in the collection plate. You will see the magnet rise inside the wand. Jiggle the wand up and down a little in the liquid at the bottom of the tube, then lift it out, while still holding the tube on the magnet.
- Cap the tube and use a dissecting scope to check that the raft is at the bottom of the tube. Keep on ice until ready to start first-strand synthesis.

DAY 1 (MONDAY)

aRNA: FIRST ROUND, FIRST STRAND SYNTHESIS

In this stage we use the mRNA in a single cell as a template to make cDNA (first strand synthesis). Synthesis is primed by the T7-oligo(dT) primer. The poly(dT) tract at the 3' end of the T7-oligo(dT) primer binds the poly(A) tail of mRNAs; the 5' end of the T7-oligo(dT) primer contains the T7 RNA polymerase promoter sequence. The primer is extended by Superscript III reverse transcriptase, using the dNTPs, to create a complementary cDNA strand bound to the RNA template. To avoid RNA degradation by RNases, we add the RNase inhibitor RNasin. We add DTT to stabilize Superscript III reverse transcriptase and RNasin, and to prevent RNase release from RNasin.

- Reagents are stored at -20°C unless otherwise noted.

1. Each group will be provided with:
    a. Four single neurons isolated by patch pipette, each in a tube containing 2.4 µl 5x First Strand Buffer and 0.9 µl diluted ERCC RNA spike-in (unless you are using your own cells).
    b. Two tubes of control total RNA, one at 2 pg/µl and one at 20 pg/µl (stored at -80°C).
    c. An aliquot of diluted (1:4000000) ERCC RNA spike-in mix.

    Make controls as shown below. The control 'C10' contains 10 pg of total RNA, which is roughly the amount of total RNA in a single cell. The control 'C100' contains 100 pg of total RNA, equivalent to 10 cells.

	Vol. for C10 control (µl)	Vol. for C100 control (µl)
Control RNA (2 pg/µl)	5	-
Control RNA (20 pg/µl)	-	5
First strand buffer (5x)	2.4	2.4
Diluted ERCC RNA spike-in mix	0.9	0.9
Total	8.3	8.3

2. Make the mastermix below, in the order listed.
    - Make sure you use the correct concentration of dNTPs (2.5 mM)!
    - **Dilute the 1 µg/µl stock of T7-oligo(dT) primer 1:100 in nuclease-free water to give 10 ng/µl.** Discard the diluted primer after use, but keep the stock.

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
dNTPs (**2.5 mM each**)	1.2	7.92
T7-oligo(dT) (**10 ng/µl**)	0.3	1.98
DTT (100 mM)	0.45	2.97
Total	1.95	

3. Add 1.95 µl mastermix to each tube (single neurons and controls) and mix by pipetting.

4. Incubate 5 min at 70°C, then immediately place on ice. Spin briefly and return to ice.

5. Check the volume of your single cell samples with a pipette. If needed, bring volume up to 10.25 µl with nuclease-free water. You do not need to do this for the controls.

6. On ice, make the following mastermix in the order listed:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
RNasin (40U/µl)	0.3	1.98
Superscript III (200U/µl)	0.45	2.97
Nuclease-free water*	1	6.6
Total	1.75	

*Stored at room temp. Never use DEPC-treated water, as DEPC can inhibit enzymes.

7. Add 1.75 µl mastermix to each tube and mix by pipetting.

8. Incubate 30 min at 42°C, 15 min at 70°C, spin briefly and place on ice. Store at -20 or -80°C or continue on to the next stage.

aRNA FIRST ROUND, SECOND STRAND SYNTHESIS

RNase H nicks the RNA strand of the RNA-cDNA hybrid, creating priming sites for DNA polymerase I, which extends the short RNA fragments using dNTPs, creating a second cDNA strand complementary to the first (second strand synthesis). This includes creating the complementary strand to the T7 RNA polymerase promoter sequence that was incorporated during first strand synthesis. All but the most 5' RNA fragments are displaced by the 5'-3' exonuclease activity of DNA polymerase I. T4 DNA polymerase fills in 5' overhangs with its 5'-3' polymerase activity, and removes 3' overhangs with its 3'-5' exonuclease activity, creating blunt-ended, double-stranded cDNA.

1. On ice, make the following mastermix in the order listed.
   - Make sure you use the correct concentration of dNTPs (0.69 mM)! This is the only time you will use 0.69 mM dNTPs.

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
Second strand buffer (5x)	7.5	49.5
dNTPs **(0.69 mM each)**	0.75	4.95
DNA polymerase I (10U/µl)	1	6.6
Nuclease-free water	8.25	54.5
RNase H (2U/µl)	0.25	1.65
Total	17.75	

2. Add 17.75 µl mastermix to each tube and mix by pipetting up and down 10 times.

3. Incubate 2 hours at 16°C.

4. Add 1 µl T4 DNA polymerase (5 U/µl). Mix by pipetting. Incubate 10 min at 16°C.

5. Incubate 10 min at 75°C.

FIRST ROUND, IVT (MEGASCRIPT T7 KIT)

In this stage, we use the double-stranded cDNA made in the previous two stages as a template for in vitro transcription (IVT). Using the T7 RNA polymerase promoter that has been 'built in' to our cDNA, T7 RNA polymerase synthesizes thousands of copies of antisense RNA from each cDNA template. We add the RNase inhibitor SUPERaseIn to protect the newly synthesized RNA from degradation.

1. At room temp, make the mastermix below, in the order listed.
   - **Important: make sure the reaction buffer does not contain any precipitate.** If it does, warm gently and/or vortex until the precipitate dissolves.
   - Make sure you use NTPs and not dNTPs!

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
NTP mix (18.75 mM each)	24	158
Reaction buffer (10x)	6	39.6
Enzyme mix (10x)	6	39.6
SUPERaseIn (20 U/µl)	3	19.8
Total	39	

2. For each sample, add 39 µl mastermix to a PCR tube.

3. Add each of your samples (30 µl) to a PCR tube containing mastermix. Be careful to transfer everything!

4. Incubate in a thermocycler with a heated lid overnight, using the program below. Your total volume will be 69 µl.

   37°C for 14 hours
   4°C hold

DAY 2 (TUESDAY)

IVT CLEANUP (MEGACLEAR KIT)

In this stage, we use the Ambion MEGAclear kit to purify the antisense RNA produced during IVT, removing unused nucleotides, primers, enzymes and other proteins, and salts.

1. Label the following sets of tubes (one for each sample):
    a. Regular 1.5 ml microcentrifuge tubes
    b. MEGAclear kit tubes with filter cartridges placed in them
    c. MEGAclear kit tubes without filter cartridges

2. Add elution solution (stored at 4°C) to the regular 1.5 ml tubes as follows:
    Day 2 / Tuesday (first round cleanup): 31 µl
    Days 3 and 4 / Wednesday and Thursday (second and third round cleanup): 80 µl

3. Transfer your first round aRNA from the PCR tubes to the tubes containing elution solution and mix gently but thoroughly by pipetting, bringing the volume to 100 µl.

4. Add 350 µl Binding Solution Concentrate (stored at 4°C). Mix gently by pipetting.

5. Ask for a demonstration of this step. Set pipette to 750 µl. Draw up 250 µl 100% ethanol (stored at room temp), add it to your sample, mix by pipetting and then pipette the entire contents of the tube onto the filter cartridge.

6. Centrifuge for 1 min at 10 000 x g at room temp.

7. With pipette set to 750 µl, add 500 µl Wash Solution (with ethanol added; stored at 4°C) to the filter cartridge, then lift up the filter cartridge and use the pipette to remove and discard the flow-through from the previous step.

8. Centrifuge for 1 min at 10 000 x g at room temp.

9. Set pipette to 500 µl, add another 500 µl Wash Solution to the filter cartridge, then use the pipette to remove and discard the flow-through from the previous step.

10. Centrifuge for 1 min at 10 000 x g at room temp.

11. Use the pipette to remove and discard the flow-through.

12. Centrifuge for 30 sec at 10 000 x g at room temp.

13. Transfer filter cartridges to fresh MEGAclear tubes. Add 50 µl Elution Solution. Incubate at 70°C for 10 min. *Meanwhile, prepare a fresh set of regular tubes (i.e. not from the MEGAclear kit)*.

14. Centrifuge for 1 min at 10 000 x g at room temp.

15. Do not discard the eluate; it contains RNA! Transfer it to a fresh regular tube and keep on ice.

16. Add another 50 µl Elution Solution to the filter cartridge and incubate at 70°C for 10 min. *Meanwhile, prepare 5M ammonium acetate with glycogen; see Step 20.*

17. Centrifuge for 1 min at 10 000 x g at room temp.

18. Do not discard the eluate! Add it to the eluate from the first elution.

19. **Day 3 / Wednesday (second round cleanup) <u>ONLY</u>: remove 3 µl and save for Bioanalyzer analysis (RNA 6000 Pico chip).**
    On days 2 and 4, skip step 19 and proceed to step 20.

20. Add 12 µl (0.12 volumes) of ammonium acetate with glycogen, prepared as shown:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
Ammonium acetate (5M)	10	66
Glycogen (5 mg/ml)	2	13.2
Total	12	

21. Add 275 µl (2.5 volumes) of 100% ethanol. Mix well and incubate at -80°C for 30 min. *Meanwhile, turn centrifuge down to 4°C.*
    - Samples may be kept at -80°C for several days

22. Centrifuge for 15 min at 17 000 x g at 4°C. Position each tube with the hinge uppermost, so you know where the pellet will be after centrifugation.

23. Remove tubes from centrifuge and place on ice at the same angle as when they were in the centrifuge, to avoid disturbing the pellet (ask for an explanation if unclear).

24. Look to make sure you can see the pellet. Carefully remove supernatant and discard.

25. Wash with 500 µl cold 70% ethanol (stored at -20°C). Pipette up and down until you dislodge the pellet from the side of the tube.

26. Centrifuge for 10 min at 17 000 x g at 4°C.

27. Check that you can still see the pellet. Then, carefully remove the 70% ethanol and discard, without dislodging the pellet.

28. Centrifuge for a further 30 sec at 17 000 x g at 4°C, then use a pipette with the smallest size tip to remove the remaining 70% ethanol. This is important so that the pellets don't take hours to dry.

29. Look at the pellet. You will notice it is white in color. Lie tubes on their sides with lids open on a clean paper towel and allow to dry for 10 – 15 min. When the pellet is dry, it will look clear.

30. Ask for a demonstration of this step. Resuspend RNA pellet in nuclease-free water, using the volumes below:
    Days 2 and 3 / Tuesday and Wednesday (first and second round cleanup): 4 µl
    Day 4 / Thursday (third round cleanup): 20 µl

31. **Day 4 / Thursday (third round) only**: Heat samples to 70°C for 2 min. Spin briefly. Remove 3 µl for Nanodrop and Bioanalyzer analysis. Once you have sample concentrations from the Nanodrop, proceed to library prep (p16).

SECOND / THIRD ROUND, FIRST STRAND SYNTHESIS

In this stage, we use the antisense RNA produced during IVT as a template for first strand cDNA synthesis by Superscript III reverse transcriptase. Synthesis is primed using random hexamers. Note that antisense RNA has a poly(U) tract at its 5' end.

1. Incubate resuspended aRNA at 70°C for 2 min, then return to ice.

2. Spin tubes briefly. Dilute the 500 ng/µl stock of random primers 1:10 in nuclease-free water to give 50 ng/µl, and add 1 µl to each tube.

3. Incubate 10 min at 70°C. Immediately place on ice for at least 2 min, spin briefly, then return to ice.

4. On ice, make the following mastermix, in the order listed.
   - Make sure you have the right concentration of dNTPs (2.5 mM)!

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
First strand buffer (5x)	2	13.2
dNTPs **(2.5 mM each)**	0.5	3.3
RNasin (40U/µl)	0.5	3.3
Superscript III (200U/µl)	1	6.6
DTT	1	6.6
Total	5	

5. Add 5 µl mastermix to each tube.

6. Incubate as shown below (all groups start together; Catherine will transfer samples).
   10 min at 20°C
   30 min at 42°C
   5 min at 95°C

7. Place on ice for at least 2 min, spin briefly, then return to ice.

SECOND / THIRD ROUND, SECOND STRAND SYNTHESIS

In this stage, the RNA-cDNA hybrid is denatured, and the first strand cDNA is used as a template for second strand synthesis by DNA polymerase I. Because antisense RNA has a 5' poly(U) tract, the first strand cDNA has a 3' poly(A) tail. Therefore, second strand cDNA synthesis is primed using T7-oligo(dT).

1. Make a fresh 1:100 dilution of T7-oligo(dT). Add 2 µl of diluted (10 ng/µl) T7-oligo(dT) to each tube and incubate 6 min at 70°C. Discard diluted primer after use.

2. Immediately place on ice for at least 2 min. Spin briefly, then return to ice.

3. On ice, make the following mastermix, in the order listed:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
Nuclease-free water	43.5	287
Second strand buffer (5x)	15	99
dNTPs (2.5 mM)	1.5	9.9
DNA polymerase I (10U/µl)	2	13.2
Total	62	

4. Add 62 µl mastermix to each tube. Mix thoroughly by pipetting.

5. Incubate 2 hours at 16°C.

6. Add 2 µl T4 DNA polymerase (5U/µl). Mix thoroughly by pipetting.

7. Incubate 10 min at 16°C.

SECOND/THIRD ROUND cDNA CLEANUP (MINELUTE KIT)

In this stage, we purify our double-stranded cDNA using the Qiagen MinElute kit, removing enzymes and other proteins, dNTPs, primers, and salts. Note that we do not perform this step during the first round of aRNA amplification.

1. Add 300 µl Buffer ERC (stored at room temp) to each tube, mix, and apply the sample to a MinElute column (stored at 4°C).

2. Centrifuge at 12 000 x g for 1 min.

3. Use a pipette to remove and discard the flow-through.

4. To wash, add 750 µl Buffer PE (stored at room temp) to the MinElute column.

5. Centrifuge at 12 000 x g for 1 min.

6. Use a pipette to remove and discard the flow-through.

7. Centrifuge at 12 000 x g for a further 1 min.

8. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.

9. To elute cDNA, add 10 µl nuclease-free water to the MinElute column, let stand at room temp for 1 min, then centrifuge at 12 000 x g for 1 min.

SECOND / THIRD ROUND IVT (MEGASCRIPT T7 KIT)

This stage is identical to first round IVT, except that the volumes are smaller.

1. At room temp, make the following mastermix, in the order listed.
   - **Important: make sure the reaction buffer does not contain any precipitate.** If it does, warm gently and/or vortex until the precipitate dissolves.
   - Make sure you use NTPs and not dNTPs!

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
NTP mix (18.75 mM each)	8	52.8
Reaction buffer (10x)	2	13.2
Enzyme mix (10x)	2	13.2
SUPERaseIn (20 U/µl)	1	6.6
Total	13	

2. For each sample, add 13 µl mastermix to a PCR tube.

3. Add 7 µl of your eluted cDNA from the MinElute cleanup to the PCR tube containing IVT mastermix. Save the remaining 3 µl, labeled as second or third round cDNA, at -80°C, for any future analyses needed.

4. Incubate in a thermocycler with a heated lid overnight, using the program below. Your total volume will be 20 µl.

   37°C for 14 hours
   4°C hold

DAY 3 (WEDNESDAY)

SECOND ROUND, IVT CLEANUP:

Follow the protocol for Day 2 / first round (p11).

THIRD ROUND, FIRST STRAND SYNTHESIS

Follow the protocol for Day 2 / second round (p13)

THIRD ROUND, SECOND STRAND SYNTHESIS

Follow the protocol for Day 2 / second round (p14)

THIRD ROUND, cDNA CLEANUP

Follow the protocol for Day 2 / second round (p14)

THIRD ROUND, IVT

Follow the protocol for Day 2 / second round (p15)

DAY 4 (THURSDAY)

THIRD ROUND, IVT CLEANUP

Follow the protocol for Days 2 and 3 / first and second rounds (p11). Analyze the final product using Nanodrop and Bioanalyzer, then proceed to library preparation (below).

LIBRARY PREPARATION (ILLUMINA TRUSEQ STRANDED mRNA KIT)

- Thaw all reagents to room temp before use

First strand synthesis

In this stage, amplified antisense RNA is used as a template for first strand synthesis by Superscript III reverse transcriptase. We assume that synthesis is primed by random primers contained in the elute, prime, fragmentation mix. Note that we do not actually fragment the RNA, because the lengths are quite short already. At the end of this stage, the 70°C denaturation step separates the first strand cDNA from the RNA template.

1. Start with 200 ng RNA in 5 μl nuclease-free water. If your samples are not concentrated enough, you can reduce the amount of RNA to as little as 50 ng, but ideally you should use the same amount for each sample.

2. Add 13 μl elute, prime, fragmentation mix (FPF).

3. Remove and discard 1 μl.

4. Make the following mastermix:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
First strand actinomycin D mix (FAD)	7.2	47.5
Superscript III (200U/µl)	0.8	5.28
Total	8	

5. Add 8 µl mastermix to each tube, pipette up and down 6 times to mix and incubate as shown below:
   10 min at 25°C.
   15 min at 42°C.
   15 min at 70°C

6. Spin briefly and place on ice.

Second strand synthesis and end repair

In this stage, first strand cDNA is used as a template for second strand cDNA synthesis. Importantly, dUTP is incorporated into the second strand, which prevents it from being amplified during PCR enrichment. This allows the sequencing data to be 'stranded'.

1. Make a mastermix of the following, add 25 µl to each tube and pipette 6 times to mix.

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
Resuspension buffer (RSB)	5	33
Second strand mix (SSM)	20	132
Total	25	

2. Incubate 1 hour at 16°C.
   - Get AMPure XP beads out of the fridge to equilibrate to room temp
   - Do not put RSB away; you will need it for cleanup

3. Allow samples and RSB to equilibrate to room temp.

4. Cleanup:
   a. Vortex beads, add 90 µl to each tube and pipette up and down 10 times.
   b. Incubate at room temp for 15 min.
   c. Open lids and place on magnetic stand, wait 5 min.
   d. Remove and discard 135 µl supernatant.
   e. Add 200 µl freshly prepared (*i.e.* today) 80% ethanol (without disturbing beads). Incubate at room temp for 30 sec, then remove 80% ethanol.
   f. Repeat 80% ethanol wash.
   g. Let stand at room temp for 15 min to dry. Before pellets dry they have a 'glossy' look. After drying they have a 'matte' look.

h. Remove from the magnetic stand and resuspend in 17.5 µl resuspension buffer (RSB). Be careful to keep the beads together so they don't end up smeared all over the inside of the tube.
i. Incubate 2 min at room temp, place on magnetic stand and wait 5 min.
j. Transfer 15 µl supernatant to new tubes.

- Samples may be stored at -80°C for a few days.

DAY 5 (FRIDAY)

3' Adenylation

In this stage, we add an A base to the 3' ends of both cDNA strands, which will allow the adapter complex to bind via its unpaired 3' T bases.

1. Make the following mastermix:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
A-tailing mix (ATM)	12.5	82.5
Resuspension buffer (RSB)	2.5	16.5
Total	15	

2. Add 15 µl mastermix to each tube.

3. Incubate 30 min at 37°C.
   - Get beads out of fridge to ensure they have 30 min to equilibrate to room temp.

4. Incubate 5 min at 70°C. Spin briefly and place on ice.

Ligate adapters

In this stage we ligate the adapter complexes to the cDNA.

1. Add 2.5 µl RNA adapter index to each tube and mix. For more information on indexing, see p26. Note which sample has which index.

2. Make the following mastermix:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
Ligation mix (LGM)	2.5	16.5
Resuspension buffer (RSB)	2.5	16.5
Total	5	

3. Add 5 µl mastermix to each tube.

4. Set a pipette to 40 µl and pipette up and down 10 times to mix.

5. Incubate 10 min at 30°C.

6. Add 5 µl Stop ligation buffer (SLB) and pipette up and down 10 times to mix.

7. Cleanup.
   First wash:
   a. Vortex beads, add 42 µl to each tube and pipette up and down 10 times.
   b. Incubate at room temp for 15 min. *Meanwhile, prepare 8 ml 80% ethanol.*
   c. Place on magnetic stand, wait 5 min.
   d. Remove and discard 79.5 µl supernatant.
   e. Add 200 µl freshly prepared 80% ethanol (without disturbing beads). Incubate at room temp for 30 sec, then remove 80% ethanol.
   f. Repeat 80% ethanol wash.
   g. Let stand at room temp for 10 – 15 min until dry (non-shiny appearance).
   h. Remove from the magnetic stand and resuspend in 52.5 µl RSB.
   i. Incubate 2 min at room temp, place on magnetic stand and wait 5 min.
   j. Transfer 50 µl supernatant to new tubes.

   Second wash:
   a. Vortex beads, add 50 µl to each tube and pipette up and down 10 times.
   b. Incubate at room temp for 15 min.
   c. Place on magnetic stand, wait 5 min.
   d. Remove and discard 95 µl supernatant.
   e. Add 200 µl freshly prepared 80% ethanol (without disturbing beads). Incubate at room temp for 30 sec, then remove 80% ethanol.
   f. Repeat 80% ethanol wash.
   g. Let stand at room temp for 10 – 15 min to dry.
   h. Remove from the magnetic stand and resuspend in 22.5 µl RSB.
   i. Incubate 2 min at room temp, place on magnetic stand and wait 5 min.
   j. Transfer 20 µl supernatant to PCR tubes.

   - Samples may be stored at -80°C for several days.

Enrich DNA Fragments

In this stage, we use PCR to selectively amplify those cDNA fragments that have adapters ligated to them, by using PCR primers that bind to the adapters. We also selectively amplify the forward strand, while the dUTP-containing reverse strand is not amplified. The enzyme is probably Pfu DNA polymerase, as this polymerase stalls, and then falls off, when it encounters dUTP in the template.

1. Make the following mastermix:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
PCR primer cocktail (PPC)	5	33
PCR master mix (PMM)	25	165
Total	30	

2. Add 30 μl mastermix to each tube and mix by pipetting.

3. Run the following PCR program. Your total volume will be 50 μl.

    98°C for 10 sec
    15 cycles of:
        98°C for 10 sec
        60°C for 30 sec
        72°C for 30 sec
    72°C for 5 min
    4°C hold

4. Cleanup.
    First wash:
        a. Vortex beads and add 50 μl to a tube (one per sample). Add each sample to a tube of beads and pipette up and down 10 times.
        b. Incubate at room temp for 15 min.
        c. Place on magnetic stand, wait 5 min.
        d. Remove and discard 95 μl supernatant.
        e. Add 200 μl freshly prepared 80% ethanol (without disturbing beads). Incubate at room temp for 30 sec, then remove 80% ethanol.
        f. Repeat 80% ethanol wash.
        g. Let stand at room temp for 10 – 15 min to dry.
        h. Remove from the magnetic stand and resuspend in 52.5 μl RSB.
        i. Incubate 2 min at room temp, place on magnetic stand and wait 5 min.
        j. Transfer 50 μl supernatant to new tubes.
    Second wash:
        a. Vortex beads and add 50 μl to each tube. Pipette up and down 10 times.
        b. Incubate at room temp for 15 min.
        c. Place on magnetic stand, wait 5 min.
        d. Remove and discard 95 μl supernatant.
        e. Add 200 μl freshly prepared 80% ethanol (without disturbing beads). Incubate at room temp for 30 sec, then remove 80% ethanol.
        f. Repeat 80% ethanol wash.
        g. Let stand at room temp for 10 – 15 min to dry.
        h. Remove from the magnetic stand and resuspend in 22.5 μl RSB.
        i. Incubate 2 min at room temp, place on magnetic stand, wait 5 min.
        j. Transfer 20 μl supernatant to new tubes.

5. Analyze with the Bioanalyzer using the DNA 1000 assay. Libraries should have an average size of 260 bp. Before submitting for sequencing, libraries are diluted to 10 nM in RSB and pooled. The concentration is then checked with the Bioanalyzer High Sensitivity DNA assay.

SMART-SEQ RNA AMPLIFICATION

This protocol uses the SMARTer Ultra Low Input RNA Kit for Sequencing v3 (Clontech).

SMART-SEQ PART 1: FIRST STRAND SYNTHESIS (MONDAY)

First strand synthesis is primed with the 3' SMART CDS Primer II A, which has a poly(dT) tract at its 3' end, allowing it to bind to the poly(A) tail of the mRNA. The 3' SMART CDS Primer II A also contains an 'anchor' sequence at its 5' end. MMLV reverse transcriptase extends the 3' SMART CDS Primer II A, creating a cDNA strand complementary to the mRNA (first strand synthesis). MMLV reverse transcriptase adds a few extra C bases at the 3' end of the first strand cDNA. This allows the SMARTer II A Oligonucleotide to bind to the newly-synthesized cDNA via complementary G bases at its 3' end. The SMARTer II A Oligonucleotide contains the same anchor sequence as is found in the 3' SMART CDS Primer II A, at its 5' end. MMLV reverse transcriptase (RT) continues first strand cDNA synthesis, using the remainder of the SMARTer II A Oligonucleotide as a template. This is known as template-switching, and is a property of MMLV RT. The resulting first strand cDNA is flanked by the anchor sequence at its 5' end and the reverse complement of the anchor sequence at its 3' end.

- Thaw reagents by resting on their side on top of ice; once thawed, gently vortex, spin briefly and keep on ice.
- Each group will be provided with four single neurons isolated by patch pipette, each in a tube containing 1 µl 10x Reaction Buffer and 4 µl nuclease-free water.
- You will use your 2 pg/µl control RNA to prepare a positive control, and nuclease-free water to prepare a negative control.

1. Prepare 10x Reaction Buffer by combining 10x Lysis Buffer and RNase Inhibitor as shown, vortex briefly, spin down and keep on ice:

	Vol. (µl)
10x Lysis Buffer	19
RNase Inhibitor (40U/µl)	1
Total	20

- Do not put RNase Inhibitor away; you will need it again soon.

2. Prepare negative and positive controls as shown below, and keep on ice:

	Vol. for negative control (µl)	Vol. for positive control (µl)
10x Reaction Buffer	1	1
Nuclease-free water	9	4
Control RNA (2 pg/µl)	-	5
Total	10	10

3. Check the volume of your single cells using a pipette and adjust to 10 µl using nuclease-free water. You do not need to do this for the positive and negative controls.

4. Add 1 μl 3' SMART CDS Primer II A to each sample (single cells and controls), gently vortex, and spin briefly. Keep on ice.

5. Prepare the following mastermix, in the order shown, and keep on ice. **Do not add it to the samples yet!**

	Vol. for 1 sample (μl)	Vol. for 6.6 samples (μl)
5x First-Strand Buffer	4	26.4
DTT (100 mM)	0.5	3.3
dNTP Mix (20 mM)	1	6.6
SMARTer II A Oligonucleotide (12 μM)*	1	6.6
RNase Inhibitor (40U/μl)	0.5	3.3

*Stored at -80°C

6. Incubate samples (without mastermix added) at 72°C for 3 min. During the incubation, complete Step 7.

7. Mix the SMARTScribe Reverse Transcriptase without vortexing. Add 13.2 μl to the mastermix you made in Step 5 (i.e. 2 μl per sample), gently vortex and spin briefly. Add 9 μl mastermix to a PCR tube (one per sample) and keep at room temp.

8. At the end of the 3 min incubation at 72°C, immediately place samples on ice to cool, spin briefly and then transfer all 11 μl to a PCR tube containing mastermix. Mix by pipetting and spin briefly. Place samples in a preheated thermocycler and run the following program:

    42°C for 90 min
    70°C for 10 min
    4°C hold

    - Smart-Seq samples can be stored at 4°C overnight.

SMART-SEQ PART 2: PCR AMPLIFICATION (TUESDAY)

The first strand cDNAs are amplified using PCR. PCR Primer II A has the same sequence as part of the 'anchor'. In the first round of PCR, PCR Primer II A binds to the reverse complement of the anchor sequence at the 3' end of the first strand cDNA, and is extended to create a complementary second strand. Like the first strand, the second strand contains the anchor sequence at its 5' end and the reverse complement of the anchor sequence at its 3' end. Because of this arrangement, in all subsequent rounds of PCR, both cDNA strands are primed by PCR Primer II A and amplified. In other words, PCR Primer II A acts as both the 'forward' and the 'reverse' PCR primer.

1. Make the following mastermix, in the order shown:

	Vol. for 1 sample (µl)	Vol. for 6.6 samples (µl)
2x SeqAmp PCR Buffer	25	165
PCR Primer II A (12 µM)	1	6.6
SeqAmp DNA Polymerase	1	6.6
Nuclease-free water	3	19.8
Total	30	

2. Add 30 µl mastermix to each Smart-Seq sample (stored at 4°C overnight). Mix well by pipetting.

3. Place samples in a preheated thermocycler and run the following PCR program. Your total volume will be 50 µl.

95°C 1 min
20 cycles of:
98°C 10 sec
65°C 30 sec
68°C 3 min
72°C 10 min
4°C hold

- When working with your own cells, adjust the number of PCR cycles to the minimum needed in order to end up with >5 ng cDNA after cleanup.
- Smart-Seq samples can be stored at 4°C overnight.

SMART-SEQ PART 3: CLEANUP

In this stage, we use AMPure XP beads to purify the amplified cDNA, removing all other components that were in the PCR reaction.

- Thaw 10x Lysis Buffer by resting on its side on top of ice, gently vortex, spin briefly and keep on ice.
- Thaw Elution Buffer at room temp.

First cleanup:

1. Ensure that the AMPure XP beads have been at room temp for at least 30 min.

2. Add 1 µl 10x Lysis Buffer to each sample.

3. Vortex AMPure XP beads thoroughly, then add 50 µl beads to a 1.5 ml tube (one per sample).

4. Add each sample to a tube of beads. Pipette up and down 10 times or vortex to mix. Incubate at room temp for 8 min. *Meanwhile, make 3 ml 80% ethanol.*

5. Briefly spin the samples, then place on magnetic stand with lids open for 5 min.

6. Leave samples on stand. Use a pipette to remove and discard the supernatant.

7. Carefully add 200 µl freshly prepared 80% ethanol to each sample, without disturbing the beads. Wait 30 sec, then carefully remove the 80% ethanol.

8. Repeat Step 7.

9. Remove samples from the magnetic stand, spin briefly and return to the magnetic stand. Wait 30 sec, then remove all remaining ethanol with a small size pipette.

10. Leave samples on the magnetic stand for 2.5 – 3 min, until beads are dry. When wet, they will look 'glossy' or 'shiny', and when dry, they will look 'matte' or 'non-shiny'. **DO NOT OVER-DRY THE BEADS!** If beads are over-dried, cracks will appear. If this happens, increase the length of the rehydration step (Step 12) to 5 min.

11. Add 52.5 µl Elution Buffer to cover the bead pellet. Remove samples from the magnetic stand and mix thoroughly with a pipette to resuspend the beads; try to keep all the beads together so they don't end up smeared all over the inside of the tube (ask for a demonstration if this is unclear).

12. Incubate at room temp for 2 min to allow beads to rehydrate.

13. Briefly spin the samples, then place on the magnetic stand for 1 min.

14. Leave samples on the magnetic stand. Use a pipette to transfer 50 µl supernatant, which contains your cDNA, to a fresh tube.
    - Smart-Seq samples can be stored at 4°C overnight

<u>Second cleanup:</u>

1. Ensure that the AMPure XP beads have been at room temp for at least 30 min.

2. Add 1 µl 10x Lysis Buffer to each sample.

3. Vortex beads thoroughly, then add 30 µl to each sample (note that you are adding fewer beads than in the first cleanup). Pipette up and down 10 times or vortex to mix. Incubate at room temp for 8 min. *Meanwhile, prepare 5 ml 80% ethanol.*

4. Briefly spin the samples, then place on the magnetic stand with lids open for 5 min.

5. Leave samples on magnetic stand. Use a pipette to remove and discard the supernatant.

6. Carefully add 200 µl freshly prepared 80% ethanol to each sample, without disturbing the beads. Wait 30 sec, then carefully remove the 80% ethanol.

7. Repeat Step 6. Do not discard the 80% ethanol; you will use it again later.

8. Remove samples from the magnetic stand, spin briefly and return to the magnetic stand. Wait 30 sec, then remove all remaining ethanol with a small size pipette.

9. Incubate at room temp for 2.5 – 3 min, until the beads are dry. **DO NOT OVER-DRY THE BEADS!**

10. Add 17 µl Elution Buffer to cover the bead pellet. Remove samples from magnetic stand and mix thoroughly with a pipette to resuspend the beads.

11. Incubate at room temp for 2 min to allow beads to rehydrate.

12. Briefly spin the samples, then place on the magnetic stand for 1 min.

13. Leave samples on stand. Use a pipette to transfer the supernatant, which contains purified cDNA, to a fresh tube. Make a 3 µl aliquot of each sample for Bioanalyzer analysis. For more information on Bioanalyzer analysis, see p11.

    - At this point, samples may be stored at -20°C indefinitely.

    - Options for library preparation following Smart-Seq include the Illumina Nextera XT kit, the Illumina Nextera kit with a modified protocol (available on the Clontech website), or Covaris shearing following by library prep with the Clontech Low Input Library Prep Kit.

Index guide

To allow for multiplexing of different libraries in a single flow cell lane, each library has a 6 bp index or 'barcode' incorporated, via the index adapter.

During sequencing of the index, A and C bases are read by excitation with a red laser, while G and T bases are read by excitation with a green laser. Within a set of multiplexed libraries, there should be roughly equal numbers of bases of each color at each base position (1 – 6), otherwise the run will fail. This is referred to as 'balancing' the indexes.

The index adapters you will use are shown below. Each set of four is balanced (2 red and 2 green bases at each position). Any two sets of four combined will also be balanced.

If all 16 libraries are successful, 8 libraries will be run on each of two flow cell lanes. If not all libraries are successful, the arrangement below gives some flexibility in terms of multiplexing libraries.

Group 1:

Index adapter	Sequence
1	A T C A C G
3	T T A G G C
10	T A G C T T
16	C C G T C C

Group 2:

Index adapter	Sequence
2	C G A T G T
7	C A G A T C
9	G A T C A G
11	G G C T A C

Group 3:

Index adapter	Sequence
4	T G A C C A
5	A C A G T G
13	A G T C A A
23	G A G T G G

Group 4:

Index adapter	Sequence
6	G C C A A T
12	C T T G T A
18	G T C C G C
25	A C T G A T

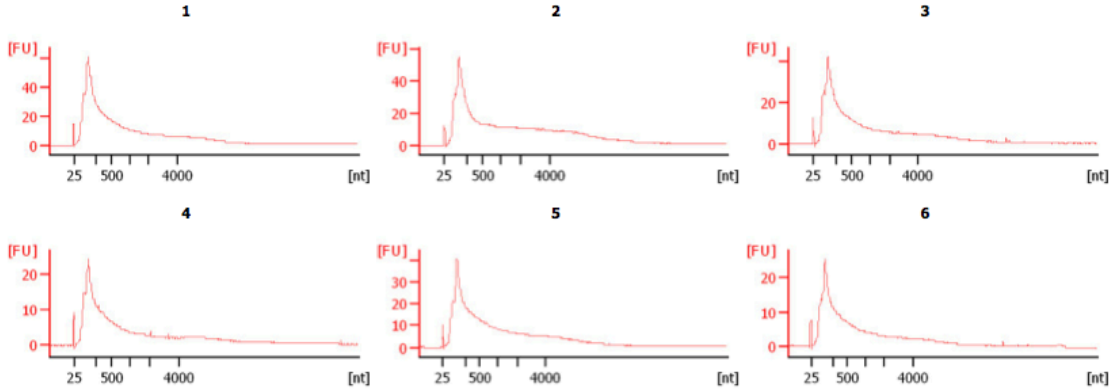
Bioanalyzer and Nanodrop analysis

The Bioanalyzer gives information about nucleic acid concentration and length. The Nanodrop gives information about nucleic acid concentration and the presence of contaminants, such as proteins, ethanol or glycogen.

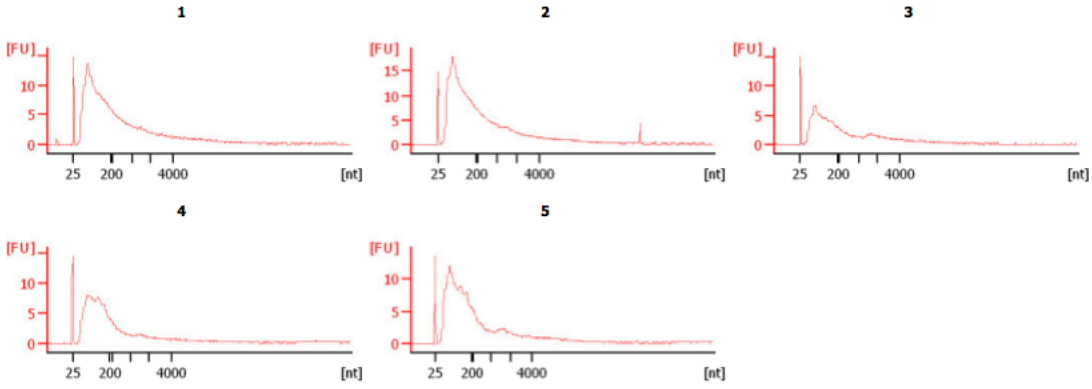
- After two rounds of aRNA amplification, RNA can be detected with the Bioanalyzer RNA 6000 Pico assay.
- After three rounds of aRNA amplification, RNA can be detected with the Nanodrop, as well as the Bioanalyzer (usually RNA 6000 Nano assay).
- After library construction, library cDNAs can be analyzed with the Bioanalyzer DNA 1000 assay.
- After Smart-Seq amplification, cDNA can be analyzed with the Bioanalyzer High Sensitivity DNA assay.

Representative Bioanalyzer results

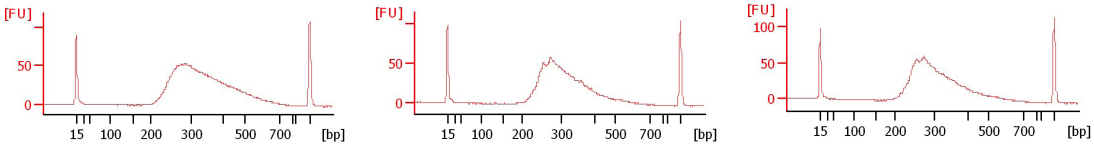
aRNA second round:



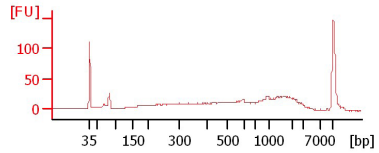
aRNA third round:



TruSeq libraries:



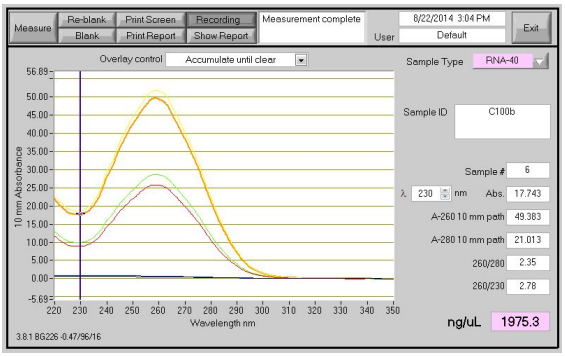
Smart-Seq:

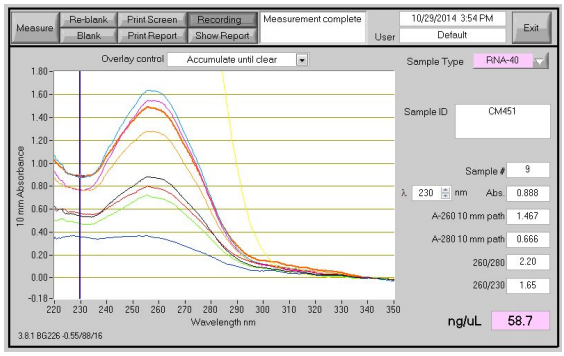


X axis represents length in nucleotides, Y axis represents concentration.

Guide to expected Nanodrop results

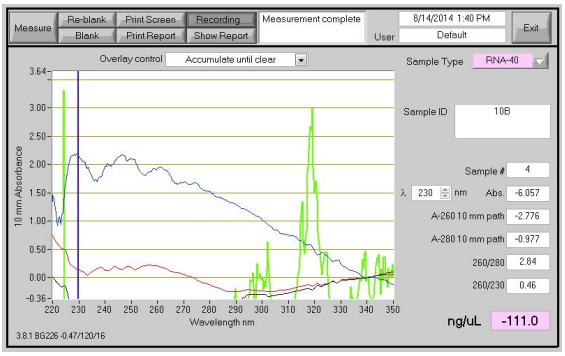
Nanodrop traces from good RNA preparations:

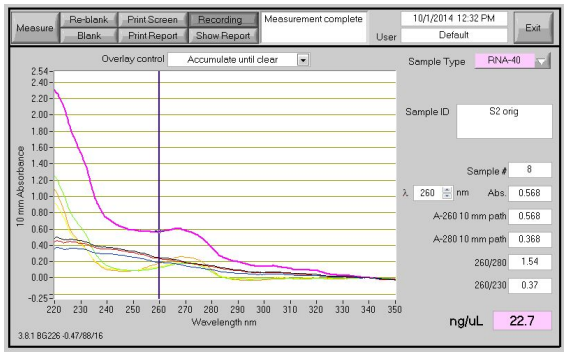




Peak at 260 nm
Trough at 230 nm
260/280 ratio > 2
260/230 ratio ideally > 2

Nanodrop traces from bad RNA preparations:





No peak at 260 nm, indicating low RNA concentration or absence of RNA.
Higher absorbance at 220-230 nm and 270-280 nm, indicating contamination.
Note that peaks caused by contaminants may obscure low levels of RNA present.

X axis represents wavelength, Y axis represents absorbance.

Reagents and supplies used

aRNA amplification and library construction, Smart-Seq:

Product	Vendor	Catalogue #	Quantity / Volume
ERCC RNA Spike-In Mix	Life Technologies	4456740	10 µl
SuperScript III (supplied with First Strand Buffer & DTT)	Life Technologies	AM9937	50 µl
Nuclease-free water	Life Technologies	18080044	10 x 50 ml
dNTP mix (10 mM each)	Life Technologies	18427088	1000 µl
T7-oligo(dT), RP-LC purified*	Midland	Custom order	
RNasin	Promega	N2511	125 µl
Second Strand buffer	Life Technologies	10812014	500 µl
DNA polymerase I	Life Technologies	18010025	100 µl
RNase H	Life Technologies	18021014	15 µl
T4 DNA polymerase	Life Technologies	18005025	50 µl
T7 MegaScript kit	Life Technologies	AMB1334-5	200 samples
SUPERaseIn	Life Technologies	AM2696	500 µl
MegaClear kit	Life Technologies	AM1908	20 samples
Glycogen (20 mg/ml)	Roche	10901393001	1 ml
Random primers	Promega	C1181	40 µl
MinElute kit	Qiagen	28206	250 samples
TruSeq Stranded mRNA kit A	Illumina	RS-122-2101	48 samples
Agencourt AMPure XP beads	Beckman Coulter	A63881	60 mL
DynaMag-2 magnetic stand	Life Technologies	12321D	16 samples
SMARTer Ultra Low Input RNA Kit for Sequencing – v3	Clontech	634848	12 rxns (larger sizes available)
Agilent 2100 Bioanalyzer	Agilent Technologies	G2940CA	1
RNA 6000 Pico kit	Agilent Technologies	5067-1513	275 samples
RNA 6000 Nano kit	Agilent Technologies	5067-1511	300 samples
DNA 1000 kit	Agilent Technologies	5067-1504	300 samples
High Sensitivity DNA kit	Agilent Technologies	5067-4626	110 samples

*T7-oligo(dT) sequence (V = any nucleotide except T):
GGAGGCCGGAGAATTGTAATACGACTCACTATAGGGAGACGCGTGTTTTTTTTTTTTTTTTTTTTTTTV

Single cell pipette isolation:

Product	Vendor	Catalogue #	Quantity / Volume
Capillaries for micropipettes	Kimble Chase	34500-99	2000/case
MP Series pressure type electrode holder	Warner Instruments	64-1263 custom	1
1/16 inch inner diameter PVC tubing	Thermo Scientific	8000-0004	50 feet
Nylon 3-way stopcock, 2 female to male luers	Kimble Chase	420163-4503	10/case
Luer-lock tip 3 ml syringes	BD	309657	800/case
Nunc 35 mm tissue culture dishes	Fisher Scientific	12-565-90	500/case

Isoraft isolation:

Contact Nick Dobes, Ph.D. at Cell Microsystems for more information.
http://www.cellmicrosystems.com/

Further Resources

Information and resources relating to single cell biology techniques can be found on the PPSCB website:
www.med.upenn.edu/ppscb

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