Watt lab
Single cell cDNA library protocol

Prepare beforehand

1. 1mM dNTP mix from 100mM dATP, dGTP, dCTP, dTTP (separately from Thermo Scientific)

2. Spike RNA mix from *A. thaliana* mRNA LTP4, LTP6, NAC1, TIM (separately from Stratagene) diluted to 10^{-1}pg/μl, 10^{-2}pg/μl, 10^{-3}pg/μl, 10^{-4}pg/μl, respectively

First-strand synthesis

First-strand synthesis buffer:

5x SuperScript III buffer (Invitrogen)	1μl
5% NP-40 (Pierce)	0.5μl
1mM dNTP mix (Thermo Scientific)	0.25μl
1μM MO_4d(T) primer*	0.075μl
0.1M DTT (Invitrogen)	0.05μl
SuperRNaseIN (Ambion)	0.25μl
RNaseOUT (Invitrogen)	0.25μl
Spike RNA mix (Stratagene)	1.0μl
Nuclease-free water (Ambion)	0.625μl
Total	4μl

*MO_4d(T) primer sequence (Osawa et al., Development, 2005):
AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG GCC GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT T (DNA Technology, HPLC-purified)

1. Aliquot 4μl of first-strand synthesis buffer into thin-wall PCR tubes on ice.
2. Seed single cell in 0.5μl into tube.
3. Freeze immediately on dry ice.
4. Incubate at 65ºC for 5 min to lyse cell.
5. Incubate at 45ºC for 2 min to allow the primer to anneal.
6. Add 0.5μl SuperScript III (Invitrogen).
7. Incubate at 45ºC for 15 min.
8. Incubate at 65ºC for 10 min to inactivate the RT enzyme.

Exonuclease I treatment

Exonuclease I reaction mix:

Exonuclease I (Thermo Scientific)		0.2μl
75mM $MgCl_2$ (Ambion)		0.6μl
Nuclease-free water (Ambion)		0.2μl
	Total	1μl

1. Add 1μl to each tube.
2. Incubate at 37°C for 30 min.
3. Incubate at 80°C for 25 min to inactivate the enzyme.

RNase H treatment and poly-adenylation

Reaction mix:

5x TdT buffer (Promega)		2.6μl
100mM dATP (Thermo Scientific)		0.097μl
RNase H (Invitrogen)		0.5μl
TdT (Promega)		0.5μl
Nuclease-free water (Ambion)		3.303μl
	Total	7μl

1. Add 7μl to each tube.
2. Incubate at 37°C for 15 min.
3. Incubate at 70°C for 10 min to inactivate the enzymes.

PCR amplification 1

PCR reaction mix:

10x ExTaq buffer (Takara)		2μl
100mM dATP (Thermo Scientific)		0.13μl
100mM dGTP (Thermo Scientific)		0.13μl
100mM dCTP (Thermo Scientific)		0.13μl
100mM dTTP (Thermo Scientific)		0.13μl
100μM MO_4d(T) primer		1.65μl
ExTaq polymerase (Takara)		0.2μl
Nuclease-free water (Ambion)		11.63μl
	Total	16μl

1. Aliquot 16μl into fresh tubes; add 4μl poly-adenlyated cDNA in triplicate.

2. Perform PCR:

94°C	1 min	
50°C	2 min	1 cycle
72°C	2 min	
94°C	30 sec	
60°C	30 sec	35 cycles
72°C	2 min	

3. Pool the triplicate PCR products.

PCR amplification 2

PCR reaction mix:

10x ExTaq buffer (Takara)	2μl
2.5mM dNTP mix (Takara)	1.6μl
100μM T7-MO_4 primer*	0.4μl
ExTaq polymerase (Takara)	0.2μl
Nuclease-free water (Ambion)	13.8μl
Total	18μl

*T7-MO4 primer:
GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGAAGCAGTGGTATC
AACGCAGAGTGGCCATTACGGCCGTAC (DNA Technology, HPLC-purified)

1. Aliquot 18μl into fresh tubes; add 2μl pooled PCR 1 product in duplicate.
2. Perform PCR:

94°C	30 sec	
60°C	30 sec	35 cycles
72°C	2 min	

3. Pool the duplicate PCR products.

PCR product purification

Purify PCR product with QIAquick PCR Purification Kit (QIAgen).

Analyse amplified cDNA

Run 5ul PCR product + 1ul 6x DNA loading buffer on a 2% agarose gel. A smear 500-700bp in size should be seen. The amplified cDNA can now be used for the in vitro transcription reaction to generate labelled cRNA for hybridisation to microarrays.