Single-cell ScarTrace

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Abstract

ScarTrace is a protocol that allows the simultaneous amplification of the transcriptome and Cas9-induced genetic scars of thousands of zebrafish single cells. Initially, Tg(H2Af/va-GFP)(kca66) zebrafish one-cell embryos are injected with Cas9 RNA or protein and a gRNA targeting a GFP transgene to allow scaring. At adulthood, various zebrafish organs are dissected, dissociated and single cells are sorted. The following protocol describes the steps to generate transcriptome and scar sequencing libraries from single cells from the WKM, brain, eyes and caudal fin. Briefly, ScarTrace is an adaptation of the SORT-Seq protocol1 where a nested PCR step was integrated to amplify scars after mRNA conversion to cDNA.

Procedure

The protocol depicts the procedure for two 384-well plates, with a dead volume of 9µl of the Nanaodrop II dispenser.

1. Cell capture plates preparation

Add 5µl of mineral oil per well using a multichannel pipette and a reagent reservoir.
Dispense 50nl of 7.5 ng/µl CEL-seq2 primers per well using the Mosquito.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C.
Make a master mix as following for 2 plates:

- Spike in 1:50.000 dilution: 44.4µl (0.02µl per well)
- dNTP 10mM: 22.2µl (0.01µl per well)
- SUPERase-in: 22.2µl (0.01µl per well)
- H20: 22.2µl (0.01µl per well)
- Total: 111µl (0.05µl per well)

Pipette 13.8µl of master mix in each tube of a PCR 8-tube strip and place the strip in the correct location for dispensing in the Nanodrop II.
Dispense 50nl of master mix per well.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C. and keep on ice.

NOTE: Plates containing CEL-seq2 primers, Spike in, dNTP and Superase-in can be stored at -80 until sorting.

2. Sort single cell

Sort single cells into the plates containing primers, Spike in, dNTP and Superase-in.
Cover with silver sealer aluminium and spin down the plates for 1 minute at 2000x g at 4˚C
Snap freeze on dry ice, then store at -80˚C.

3. Lysis and RT

Before lysis, make the following RT master mix as following for 2 plates:
5x First strand buffer: 60.48µl (0.035µl per well)
0.1M DTT: 30.24µl (0.0175µl per well)
H20: 8.64µl (0.005µl per well)
RNaseOUT: 15.12µl (0.00875µl per well)
Superscript II: 15.12µl (0.00875µl per well)
Total: 129.6µl (0.075µl per well)
Pipette 15.5µl of RT master mix in each tube of a PCR 8-tube strip and keep on ice.
Lyse cells at 65˚C for 5 minutes in a 384 well Thermocycler.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C and cool on ice.
Place the strip in the correct location for dispensing in the Nanodrop II and dispense 75nl of the RT mix.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C.
Incubate at 42˚C for 1 h then at 70˚ for 10 min in a 384 well Thermocycler, keeping the lid open.
Cool on ice.

4. 2nd Strand Synthesis

Make a second strand master mix as following for 2 plates:

• H20: 569.3µl (0.67375µl per well)

• 2nd strand buffer 5x: 184.8µl (0.21875µl per well)

• dNTP 10mM: 18.5µl (0.021875µl per well)

• E.coli ligase: 6.65µl (0.007875µl per well)

• E.coli DNA polymerase I: 25.9µl (0.030625µl per well)

• RNAse H: 6.65µl (0.007875µl per well)

• Total: 811.8µl (0.960075µl per well)

Pipette 101µl of second strand master mix in each tube of a PCR 8-tube strip and place the strip in the correct location for dispensing in the Nanodrop II.
Dispense 960nl of the master mix.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C.
Incubate at 16 ˚C for 2 hours in a 384 well Thermocycler, keeping the lid open.

5. Protease treatment

Mix 60 µl of Proteinase K with 90 µl H2O (1:2,5 dilution) and Pipette 18.6µl in each tube of a PCR 8-tube strip.
Place the strip in the correct location for dispensing in the Nanodrop II a dispense 100nl of the diluted Proteinase K.
In a 384 well Thermocycler incubate at 55 ˚C for 1h and heat inactivate at 80°C for 10 min.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C and cool on ice.

6. PCR I

Make a PCR I master mix as following for 2 plates:

• Primer eGFP-F1 (10 µM): 20.6µl (0.025µl per well)

• Primer eGFP-R1 (10 µM): 20.6µl (0.025µl per well)

• NEBNext® High-Fidelity 2X PCR Master Mix: 1030µl (1.25µl per well)

• Total: 1071.2µl (1.3µl per well)

Pipette 133.8µl of master mix in each tube of a PCR 8-tube strip and place the strip in the correct location for dispensing in the Nanodrop II.
Dispense 1300nl of the PCR I master mix.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C.

PCR program:

98 ˚C, 1 min

8-10 cycles of:

98 ˚C, 10 sec

58 ˚C, 30 sec

72 ˚C, 30 sec

72 ˚C, 10 min

Hold at 4°C

7. PCR II

Dispense 75nl of 10 µM eGFP-F2_BC_5 using the Mosquito.
Make a PCR II master mix as following for 2 plates:

• eGFP-R2_3 (10 µM): 70.59µl (0.075µl per well)

• NEBNext® High-Fidelity 2X PCR Master Mix: 235.29µl (0.25µl per well)

• H20: 94.12µl (0.1µl per well)

• Total: 400µl (0.425µl per well)

Pipette 49.8µl of master mix in each tube of a PCR 8-tube strip and place the strip in the correct location for dispensing in the Nanodrop II.
Dispense 425nl of PCR II mix.
Spin down the dispensed plate for 1 minute at 2000x g at 4˚C

PCR program:

98 ˚C, 1 min

8 to 10 cycles of:

98 ˚C, 10 sec

64 ˚C, 30 sec

72 ˚C, 30 sec

72 ˚C, 10 min

Hold at 4°C

8. Pool & Clean up

Warm AMPure XP beads to room temperature.
Pool all the wells from 1 plate by centrifuging each plate upside down into a separate container at 200g for 1 minute.
Collect the aqueous phase from the container and transfer into a 2 ml tube.
Wash the container with 1 ml mineral oil, collect and transfer into the same 2ml tube.
Spin down the 2ml tubes at maximum speed for 1 minute to separate the aqueous phase from the mineral oil.
Collect the aqueous phase without collecting any mineral oil and transfer to a clean 1.5ml tube.
Measure how much you recovered in the 1.5ml tube with a pipette.
Add 1x volume of mixed diluted AMPure XP beads (1:8 diluted with bead binding buffer).
Incubate 15 min at room temperature
Incubate on magnet stand for 5 min or until liquid is clear.
Remove supernatant without disturbing the beads.
Wash pellet carefully with 1ml 80% Ethanol. Incubate at least 30 seconds.
Repeat above step.
Remove as much Ethanol as possible.
Dry at room temperature for approximately 10 minutes or until dry.
Resuspend with 8µl water. Pipette entire volume up and down ten times to mix thoroughly Incubate at room temperature for 2 min.
Place on magnetic stand for 5 min, until liquid appears clear.
Transfer 8µl of supernatant to a new tube.

9. Splitting the sample

Transfer 1.6 µl of the sample for SCAR library (20%).
Transfer 6.4 µl of the sample for transcriptome library (80%).

SCAR library preparation

1. EXO-SAP treatment

Add 8.4µl of H20 to 1.6µl of samples.
Add 2µl EXOSAP-IT.
Incubate at 37 °C for 15 minutes then at 80 °C for 15 minutes.

2. Bead Clean up

Prewarm beads to room temperature.
Vortex AMPure XP Beads until well dispersed, then add 12µl to the 12µl sample (1:1 ratio). Mix entire volume up ten times to mix thoroughly.
Incubate at room temperature for 15 min.

Place on magnetic stand for at least 5 min, until liquid appears clear.
Remove and discard of the supernatant.
Add 200µl freshly prepared 80% Ethanol.
Incubate at least 30 seconds, then remove and discard the supernatant without disturbing beads.
Add 200µl freshly prepared 80% Ethanol.
Incubate at least 30 seconds, then remove and discard the supernatant without disturbing beads.
Air dry beads for 15 min, or until completely dry.
Resuspend with 10µl water. Pipette entire volume up and down ten times to mix thoroughly.
Incubate at room temperature for 2 min.
Place on magnetic stand for 5 min, until liquid appears clear.
Transfer 10µl of supernatant to new tube.

3. Library PCR

Add 38µl of the following mix:

Ultra Pure Water 11µl

NEBNext® High-Fidelity 2X PCR Master Mix 25µl

RNA PCR Primer (RP1, from Illumina kit) 2µl

To each reaction add 2µl of a uniquely indexed RNA PCR Primer (RPIX, sequences from Illumina kit)
Amplify the tube in the thermal cycler using the following PCR cycling conditions:

30 seconds at 98°C

10-14 cycles of:

10 seconds at 98°C

30 seconds at 60°C

30 seconds at 72°C

10 minutes at 72°C

Hold at 4°C

4. Bead Clean up of PCR products I

Prewarm beads to room temperature.
Vortex AMPure XP Beads until well dispersed, then add 40µl to the 50µl PCR reaction. Mix entire volume up ten times to mix thoroughly.
Incubate at room temperature for 15 min.
Place on magnetic stand for at least 5 min, until liquid appears clear.
Remove and discard the supernatant.
Add 200µl freshly prepared 80% Ethanol.
Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
Add 200µl freshly prepared 80% Ethanol.
Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
Air dry beads for 15 min, or until completely dry.
Resuspend with 25µl water. Pipette entire volume up and down ten times to mix thoroughly.

Incubate at room temperature for 2 min.
Place on magnetic stand for 5 min, until liquid appears clear.
Transfer 25µl of supernatant to new tube.

5. Bead Clean up of PCR products II

Repeat as above, adding 22.5µl beads and eluting in 11µl water at the end. Transfer 10µl to a new tube.

6. Check library amount and quality

Check concentration of DNA by Qubit, 1µl should be enough to measure using the high sensitivity reagent; expected concentration is at least ~1ng/µl.

Run 1µl of each sample on Bioanalyzer using a high sensitivity DNA chip to see size distribution.

Transcriptome library preparation

The SORT-Seq protocol (Muraro, M. J. et al, 2016) is used for the transcriptome library preparation with the addition of 2µl TURBO DNase (Thermo Fischer Scientific AM2238) after the In Vitro Reaction.

Timing

3 days

References

1. Muraro, M. J. et al. A Single-Cell Transcriptome Atlas of the Human Pancreas. Cell Syst 3, 385-394 e383, doi:10.1016/j.cels.2016.09.002 (2016).

Acknowledgements

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