

# Single-cell ScarTrace

Alexander van Oudenaarden (✉ [a.vanoudenaarden@hubrecht.eu](mailto:a.vanoudenaarden@hubrecht.eu))

AvO lab, Hubrecht Institute

Josi Peterson-Maduro

AvO lab, Hubrecht Institute

Maria Florescu

AvO lab, Hubrecht Institute

Chloé S Baron

AvO lab, Hubrecht Institute

Anna Alemany

AvO lab, Hubrecht Institute

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## Method Article

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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 scarring. 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 protocol<sup>1</sup> 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 9 $\mu$ l of the Nanaodrop II dispenser.

1. Cell capture plates preparation - Add 5 $\mu$ l of mineral oil per well using a multichannel pipette and a reagent reservoir. - Dispense 50nl of 7.5 ng/ $\mu$ l 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: 44.4 $\mu$ l (0.02 $\mu$ l per well) • dNTP 10mM: 22.2 $\mu$ l (0.01 $\mu$ l per well) • SUPERase-in: 22.2 $\mu$ l (0.01 $\mu$ l per well) • H2O: 22.2 $\mu$ l (0.01 $\mu$ l per well) • Total: 111 $\mu$ l (0.05 $\mu$ l per well) - Pipette 13.8 $\mu$ l 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: 60.48 $\mu$ l (0.035 $\mu$ l per well) - 0.1M DTT: 30.24 $\mu$ l (0.0175 $\mu$ l per well) - H2O: 8.64 $\mu$ l (0.005 $\mu$ l per well) - RNaseOUT: 15.12 $\mu$ l (0.00875 $\mu$ l per well) - Superscript II: 15.12 $\mu$ l (0.00875 $\mu$ l per well) - Total: 129.6 $\mu$ l (0.075 $\mu$ l per well) - Pipette 15.5 $\mu$ l 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: • H2O: 569.3 $\mu$ l (0.67375 $\mu$ l per well) • 2nd strand buffer 5x: 184.8 $\mu$ l (0.21875 $\mu$ l per well) • dNTP 10mM: 18.5 $\mu$ l (0.021875 $\mu$ l per well) • E.coli ligase: 6.65 $\mu$ l (0.007875 $\mu$ l per well) • E.coli DNA polymerase I: 25.9 $\mu$ l (0.030625 $\mu$ l per well) • RNase H: 6.65 $\mu$ l (0.007875 $\mu$ l per well) • Total: 811.8 $\mu$ l (0.960075 $\mu$ l per well) - Pipette 101 $\mu$ l 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  $\mu$ l of Proteinase K with 90  $\mu$ l H<sub>2</sub>O (1:2,5 dilution) and Pipette 18.6 $\mu$ l 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  $\mu$ M): 20.6 $\mu$ l (0.025 $\mu$ l per well) • Primer eGFP-R1 (10  $\mu$ M): 20.6 $\mu$ l (0.025 $\mu$ l per well) • NEBNext® High-Fidelity 2X PCR Master Mix: 1030 $\mu$ l (1.25 $\mu$ l per well) • Total: 1071.2 $\mu$ l (1.3 $\mu$ l per well) - Pipette 133.8 $\mu$ l 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  $\mu$ M eGFP-F2\_BC\_5 using the Mosquito. - Make a PCR II master mix as following for 2 plates: • eGFP-R2\_3 (10  $\mu$ M): 70.59 $\mu$ l (0.075 $\mu$ l per well) • NEBNext® High-Fidelity 2X PCR Master Mix: 235.29 $\mu$ l (0.25 $\mu$ l per well) • H<sub>2</sub>O: 94.12 $\mu$ l (0.1 $\mu$ l per well) • Total: 400 $\mu$ l (0.425 $\mu$ l per well) - Pipette 49.8 $\mu$ l 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 8 $\mu$ l 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 8 $\mu$ l of supernatant to a new tube.

9. Splitting the sample - Transfer 1.6  $\mu$ l of the sample for SCAR library (20%). - Transfer 6.4  $\mu$ l of the sample for transcriptome library (80%).

SCAR library preparation

1. EXO-SAP treatment - Add 8.4 $\mu$ l of H<sub>2</sub>O to 1.6 $\mu$ l of samples. - Add 2 $\mu$ l 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 12 $\mu$ l to the 12 $\mu$ l 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 200 $\mu$ l freshly prepared 80% Ethanol. - Incubate at least 30 seconds, then remove and discard the supernatant without disturbing beads. - Add 200 $\mu$ l 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 10 $\mu$ l 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 10 $\mu$ l of supernatant to new tube. 3. Library PCR - Add 38 $\mu$ l of the following mix: Ultra Pure Water 11 $\mu$ l NEBNext® High-Fidelity 2X PCR Master Mix 25 $\mu$ l RNA PCR Primer \ (RP1, from Illumina kit) 2 $\mu$ l - To each reaction add 2 $\mu$ l 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 40 $\mu$ l to the 50 $\mu$ l 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 200 $\mu$ l freshly prepared 80% Ethanol. - Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads. - Add 200 $\mu$ l 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 25 $\mu$ l 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 25 $\mu$ l of supernatant to new tube. 5. Bead Clean up of PCR products II - Repeat as above, adding 22.5 $\mu$ l beads and eluting in 11 $\mu$ l water at the end. Transfer 10 $\mu$ l to a new tube. 6. Check library amount and quality Check concentration of DNA by Qubit, 1 $\mu$ l should be enough to measure using the high sensitivity reagent; expected concentration is at least ~1ng/ $\mu$ l. Run 1 $\mu$ l 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 2 $\mu$ l 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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