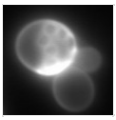

yeast single cell RNA-seq (yscRNA-seq)

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ABSTRACT

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

Please follow all precautions that usually you need to follow RNA and single-cell work. Clean the area prior to starting the protocol. Use dedicated reagents only to single cell work (and label them appropriately). Use pipettes and filter tips throughout the entire protocol

Cell growth

1 Grow the desired pre-inoculum of your desired yeast strain in their corresponding media O/N.
To profile exponentially growing cells, we recommend the initial culture not to grow over OD660=1

2 Next morning. Dilute your cells to OD660= 0.05 in the corresponding media and allow for at least 2 cell divisions prior to sorting.

Single cell isolation by FACS sorting

3 Prepare 96/384 well plates* containing **5 µl Absolute Ethanol** ** in each to fix cells immediately from sorting

* During protocol optimization we recommend using break-away plates

(EK-75118). Check with your facility the compatibility of the plates.

** this is the minimum volume required for our sorting facility/plates. Check with your facility

Note: We have obtained the same results sorting cells directly into 5 ul of cell capturing solution and lysis (see below). If doing so, prepare plates right before sorting and keep them on **4 °C ice**

4 Dilute cells prior to sorting to OD=0.05 in **3 ml** of media and vortex vigurosuly to separate cell clumps.

* At this step propidium idodide (PI) can be added to check for cell viability

5 At the FACS facility, filter cells with Cell Strainer Tubes (check with your facility which tubes they prefer) and put cells in the appropiate sorting tube for live single cell sorting.

6 Check the alignment of the plate with the sorter. For example, this can be done by sorting a drop into a covered plate and look for the droplet would fall inside each well.

7 Sort live single yeast into the each well of the plates and leave one well (we ususally do H12 as empty/not sorted) asnegativecontrol.
* Should you want to do a positive control, sort 100 cells into one well (we usually do A1).

8 Cover plates with aluminim seal if plates will not be processed right away or with temporary plastic lid.

9 Quickly spin plates to collect cells at the bottom

10 Let EtOH avaporate in a sterile environment (stril hood) for **00:45:00 maximum**

11 Once EtOH is completely evaporated, add **5 µl yeast cell lysis solutuin** of cell capturing solution and lysis. Spin down and freeze immediately.

Regardless if cells are sorted into EtOH or cell capturing and lysis solution, frozen plates can be stored at -80ºC for at least 6 months.

Cell lysis solution:

Reagents	Reference	Volume (1 rxn)
1% Triton X-100	X100-1L	0.05
UMI_Oligo dT_T31(100 □M)	IDT	0.2
dNTP 25 mM	R0181	0.68
100 mM DTT	18064014	0.5
Zymolyase 100T (100 mg/ml)	37340-57-1	0.6
RNase Inhibitor (40 U/ml)	2313A	0.12
RNase-Nuclease free H2O	10977035	2.769
ERCC (1:1000,0000) (5000 molecules)	4456740	0.081
Total		5

Lysis reaction

12 Do the follwoing lysis cycle from fresh sorted or frozen plates and **quickly** proceed to add RT reaction for 1st strand cDNA synthesis

Incubate at 30ºC for at least 10 minutes *
00:10:00
3 minutes at 72ºC
00:03:00
1 minutes at 4C

* We have changed the length of cell lysis up to 30 minutes

1st strand cDNA synthesis

13 5 Add **5 µl Reverse transcription mix (RT mix)**

Reagents	Volume (1 rxn)	Reference
5X SuperScript Stand Buffer	2	18064014
MgCl2 (1M)	0.06	AM9530G
Betaine (5M)	1.6	61962
UMI_TSO 100uM	0.2	IDT
200 U/ul SuperScript II	0.25	18064014
RNase inhibitor (40U/ml)	0.125	2313A
RNase-Nuclease free H2O	0.765	10977035
Total	5	

14 Spin down the plate and perform the following cycles

42°C for 90 mins
70°C for 15 mins
4°C forever

01:45:00

Library amplification

15 Add **15 µl PCR mix** for library amplification

Reagents	Volume (1 rxn)
10X Advantage 2 PCR buffer	2.5
dNTPs (25mm)	0.4
UMI_PCR (10uM)	1.2
50X Advantage 2 polymerase mix	0.5
H20	10.4
Total	15

16 Spin down the plate and do the following cycles

Temperature (ºC)	Time	Cycles
95	1 min	1
95	20 sec	
58	4 min	5
68	6 min	
95	20 sec	
64	30 sec	9
68	6 min	
95	30 sec	
64	30 sec	7
68	7 min	
72	10 min	1

hold at 4ºC overnight (if necessary). This is usually a safe stopping point

qPCR validation

17 Perform a 1:20 dilution of the amplified library to check the percentage of positive libraries.
Use your favorite protocol, and use as a target primers to a housekeeping gene or ERCCs.
This step is specially useful during the protocol set up as it allows to isnpect the efficiency of the protocol before moving forward.

Reagent	Volume (µl)
Sybergreen 2X	2.5
Primer mix*	0.125
H2O	1.375
DNA	1

* Primer mix is a mmix of Fw and Rv primer (10µM each)
1 cycle: 95ºC 10 min
40 cycles: 95ºC 30 sec, 58ºC 30 sec and 72ºC 30 sec
1 cycle: 95ºC 15 sec, 60ºC 15 sec and 95ºC 15 sec (melting curve)

Primer sequence for qPCR:

SOMN17 Fw_TDH3_probe TCGTCAAGTTGGTCTCCTGG
SOMN18 Rv_TDH3_probe GGCAACGTGTTCAACCAAGT SOMN21 Fw_ADH1_probe TGGTGCCAAGTGTTGTTCTG
SOMN22 Rv_ADH1_probe GGCGAAGAAGTCCAAAGCTT
SOMN310 Fw_5_ERCC_00130 CGGAAAAGTACTGACCAGCG
SOMN311 Rv_5_ERCC_00130 TGCCAATGACTTCAGCTGAC

A good plate will have around 70% positive wells, as far as it's around 50% it should be OK. Rearrange positive cells into a new plate to proceed to tagmentation.

dscDNA library clean up

18 Before commencing the purification steps, equilibrate Ampure XP beads at room temperature for 15 min, and then vortex well for several seconds.
Add **15 µl Ampure XP beads** (1:0.6 ratio) to each sample (each tube might have slightly different volumes due to evaporation).
*Do not increase the volume of beads in the purification step above the 1:1 ratio. A less-than-standard amount of beads ensures that primer dimer carryover is kept to a minimum.

19 Mix by pipetting up and down ten times or until the solution appears homogeneous. Transfer solutions to a 96-well plate with compatible magnet stand

20 Incubate the mixture for 10 min at room temperature to let the DNA bind to the beads.
00:10:00

21 Place the 96-well plate on the magnetic stand for 5 min or until the solution is clear and the beads have been collected
00:05:00

22 While samples are on the magnet, carefully remove the liquid without disturbing the beads.

23 Wash the beads with 200 l of 80% (vol/vol) ethanol solution. Incubate the samples for 30 sec and then remove the ethanol.

*It is important that the ethanol solution is freshly prepared every time, as ethanol absorbs moisture from the environment, thus changing the final concentration. Repeat ethanol washing.

24 Repeat ethanol washing one more time.

25 Remove any trace of ethanol and let the beads dry completely, leaving the plate at room temperature for 5 min or until a small crack appears on the surface of the beads.

*Avoid over drying the beads because this will make their resuspension in the designated buffer more difficult.
** As a precaution, cover the plate during this step or protect it from any possible source of contamination or air flows that might disperse the beads around the well, thus leading to cross-contamination between adjacent wells especially.

26 When beads are dried, elute dscDNA libraries with **16.5 µl elution buffer (EB buffer Qiagen)** (19086)

27 Remove plate from the magnet and mix vigurously by pipetting up and down x3 times to resuspend beads out of the magnet

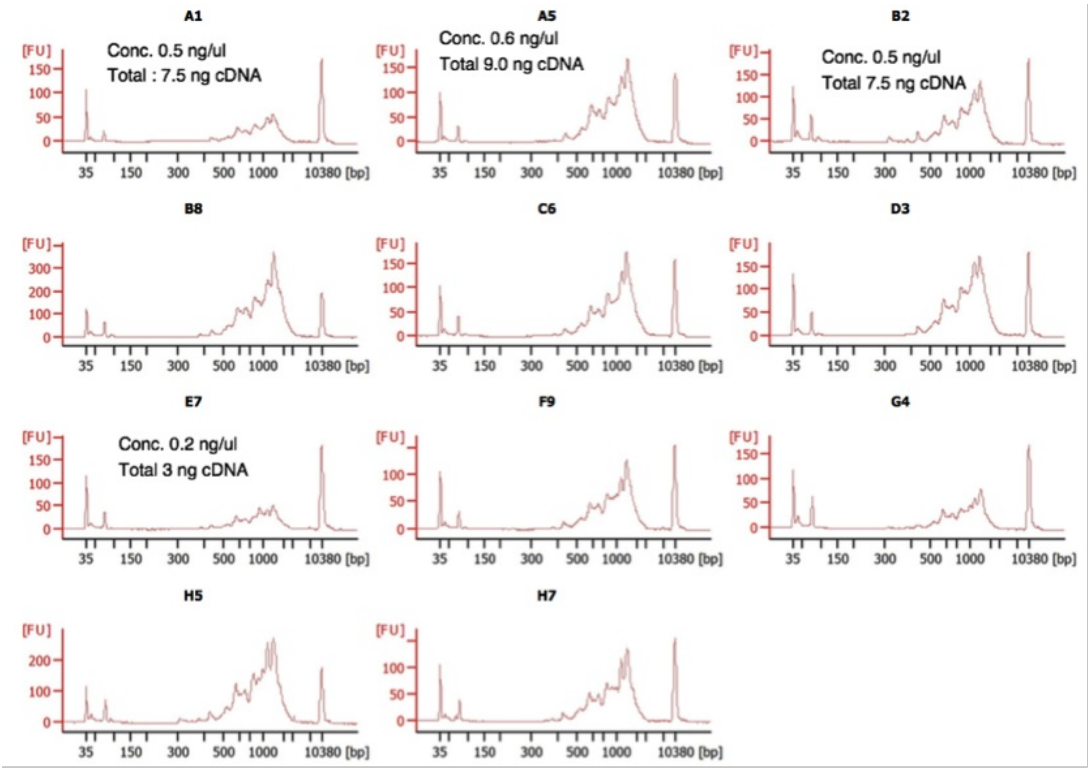
28 Place the plate on the magnetic stand and leave it for 2 min or until the solution appears clear and beads have accumulated in a corner of the well.
00:02:00

29 Recover 15 ml of SPN from each well and transfer to a new plate. Label correctly as this plate will be stored.
SAFE STOPPING POINT: cDNA llibraries can be stored at -20ºC before proceeding to tagmentation.

dscDNA library/concentration size validation

30 Run **1 µl** of several purified dscDNA libraries to check the size distribution and estimate of concentrations using a High Sensitivity DNA ChIP (2100 Bioanalyzer). qPCR validation using a housekeeping gene is valuable to guide well selection. Always run your negative control.

EXPECTED RESULT



Adaptor annealing for tagmentation

31 In order to load Tn5 with cell-specific adaptors, these need to be annealed as dsDNA cell-specific adaptors.
To anneal the adapters mix in a 96 well plate:

Mix UMI-TN5-U (100 µM) and UMI-TN5_1 (µM) to 96 in TE 1X to final concentration 50 µM (each), a 1:1 dilution.

Primer annealing thermocycler: 95ºC for 3 minutes and gradually cool down to room temperature (0,5 ºC/sec).

This plate can be stored at -20ºC for several months and used to lead several rounds of Tn5.

Tn5 loading with cell-specific adaptors

32 Prepare the following mix and aliquot ,

Prepare the following mix and aliquot **8.75 µl** of the mix (except for adapters) into a new 96 well plate. Then add **1.25 µl** of each adaptor to each well.

Reagent	Volume	Observations
50 µM adapter (96 different)	1.25 µL	add this later individually to each well
80% Glycerol	6.25 µL	
50 µM Tn5 transposase	1.25 µL	Hennig, Bianca P., et al. "Large-Scale Low-Cost NGS Library Preparation Using a Robust Tn5 Purification and Tagmentation Protocol." G3: Genes, Genomes, Genetics (2017): g3-300257.
Nuclease-free water	1.25 µL	
Total volume	10 µL	

33 Incubate 37°C for one hour and freeze -20 °C if not going to be used right away.

01:00:00

* Note, this Tn5-loaded plate can be safely stored for a 1-2 weeks at -20 °C . However leaving the loaded plate on ice 4 °C will significantly reduce Tn5 activity and will result in inefficient tagmentation.

Tagmentation

34 Prepare the following mix

Reagent	Volume (per well)
Harvested DNA	6 µL
Nuclease-free water	8 µL
2x TAPS buffer	2 µL
100% DMF	2 µL
10x Transposome	2.0 µL
Total volume	20 µL

35 Incubate for 55°C for 5 min -> 3 min 85°C to inactivate Tn5 and then cool to 4 °C

00:05:00

* The tagmentation time can vary depending on the Tn5 purification batch.

5' capture through Streaptavidin beads

36 Do a 1:20 dilution of MyOne Streptavidin for the total number of samples (1 µl of beads/sample) .

(Example: 20 µl beads for 20 samples)

2XBWT Buffer:

10 mM Tris-HCl pH7.5
1mM EDTA
2M NaCl
0.02% Tween-20

37 Wash MyOne beads x2 with 2XBWT buffer and resupsend with 20X more volume than the original volume of beads with 2XBWT

(Example: 20 µl beads for 20 samples, will be finally resuspended with 400 µl 2XBWT

38 Add 20 µL beads to each well and incubate at RT for 5 min at room temprature

00:05:00

39 Pool all samples into a single collecting tube (1.5 or 2 ml)

40 Place collecting tube in magnetic rack and allow time enough for the solution to be completely clear

41 Wash beads once with TNT buffer 100 µl

TNT Buffer:
20 mM Tris pH 7.5
50 mM NaCl,
0.02% Tween

42 Wash the beads once in Qiaquick PB 100 µl discard SPN

43 Wash beads 3x with TNT buffer 100 µl again, discard SPN

Removing 3' end fragments

44 Resuspend the beads in the following mix:

Reagent	Volume
CutSmart buffer	10 µL
PvuI-HF enzyme (20 U/µL)	2 µL
Nuclease-free water	88 µL
Total volume	100 µL

45 Incubate at 37°C for one hour with interval mix during the incubation (to avoid beads precipitation); 2 min without mix, 30 sec 1000 rpm mix.
01:00:00

46 Wash the beads three times in TNT. 100 µl

Eluting single stranded cDNA library

47 Resuspend in 30 µL Nuclease-free water.
30 µl

48 Incubate 10 min at 70°C, 850 rpm mix.
00:10:00

49 Bind the beads to the magnet immediately and collect the supernatant.

sscDNA cleanup

50 Add 54µl of room temperature Ampure XP beads to 30µ sscDNA library

54 µl Ampure XP beads

51 Incubate 10 min at RT. 00:10:00

52 Bind the beads to the magnet for 1min and discard supernatant or until solution is completely clear.
00:01:00

53 Wash once with 200 µL fresh 80% ethanol for 20-30 sec. Let the beads be bound to the magnet the entire time.

54 Dry the beads for up to 2 min.
00:02:00

55 Resuspend in 30 µL Qiagen EB buffer and incubate 5 min at RT.
00:05:00 30 µl EB

56 Bind the beads 1 min and transfer SPN to a new tube.

Library Concentration

57 To quantify library concentration, set up a KAPA quantification reaction with a 1:100 and 1:1000 dilutions of the eluted cDNA library.

Reagent	Volume (1 rxn)
KAPA SYBR® FAST qPCR Master Mix containing Primer Premix	12 µl
PCR-grade water	4 µ
Diluted library DNA or DNA Standard	4 µl
TOTAL	20 µl

This kit can be subsituted by your favorite quantification method or by a qPCR using P5-P7 primer pairs with known standards (PhiX is strongly recommended) sybergreen 2X mastermix.

58 qPCR cycling conditions for KAPA and homemade Sybergreen

Temperature	Time	
95ºC	5 min	
95ºC	30 sec	repeat this for 30 cycles for qPCR quantification
60ºC	45 sec	
4ºC	hold	

59 Use the qPCR to calculate library quantification using the template provided by KAPA biosystems or the instructions provided from your manufacturer.
We have used KAPA, NEB and homemade systems with similar results.

Library Size

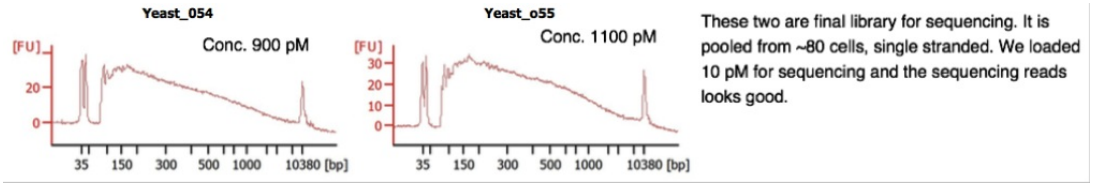
60 Sett up a separate PCR to run a bioanalyzer to determine the final size distribution. Prepare the following mix:

KAPA SYBR Q-PCR Mastermix ABI Prism	10 µL
Primers mix (10 X)	2 □l
Water	6 µL
DNA without dilution	2 µl
Total volume	**20 µl**

61 Run the same PCR as in step 58 but for 11 cycles

62 Run 1 µL into a High sensitivity DNA CHIP to obtain an average library size based on the Bioanalyzer profile.

EXPECTED RESULT



These two are final library for sequencing. It is pooled from ~80 cells, single stranded. We loaded 10 pM for sequencing and the sequencing reads looks good.

Sequencing

63 Sequence the library on the HiSeq 2000 High output using C1-P1-PCR-2 as the *Read 1* primer and UMI-TN5-U as the *Index read* primer.

64 To run the libraries on the HiSeq rapid run, us LNA primers. Spike in at primer at 0.5 uM.
Index 1 primer into HP8 (position 17) in the HiSeq
Read 1 primer into HP9 (position 16) in the Hiseq
(double check this information with your sequencing kit/instrument)

UMI_PCR_read1: +GAATGA+TACGGCG+ACCA +CCGA+T - custom 250 nmole. DNA oligo, HPLC Purification
Index1: CTGT+CT+CTT+ATA+CA +CA+TCTGA+CG+C - custom 250 nmole DNA oligo, HPLC Purification

*Note for High Output run custom primers are needed as well but without LNA

65 Load around 8-14 pmol of each library per lane. Libraries are single stranded DNA, so no denaturing is required.