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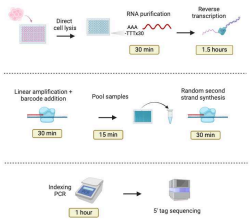
TIREseq - Turbocapture Integrated RNA Expression Sequencing



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We use this protocol and it's working

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Funders Acknowledgements:
WEHI NMAT theme

Abstract

To streamline sample processing we developed a novel protocol integrating RNA purification with library preparation, Turbocapture Integrated RNA Expression Sequencing (TIREseq) This new protocol exhibits a significantly accelerated workflow, higher sequencing efficiency and lower molecular contamination.

Image Attribution

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Guidelines

- All reagents and plastic-ware can be found in the 'Materials' section.
- Use only RNase free supplies and clean all surfaces and tools with RNase Away prior to working
- Make sure all steps involving cell lysate and RNA before reverse transcription are carried out swiftly and on ice.
- Universal primer sequences are listed below:

	A	B	C
	Name	**Sequence**	**Notes**
	TIRE_TSO	/5Biosg/AGAGACAGATTGCGCAATGNNNNNNNNNNWWrGrGrG	5' biotin. 3× 3' RNA 'G'. Order HPLC purified
	TIRE hexamer	CTACACGACGCTCTTCCGATCTGANNNGGNN*N*B	Desalted, 3' phosphorothioate bonds
	TIRE PCR1 Fwd	AGTTCAGACGTGTGCTCTTCCGAT*C*T	Desalted, 3' phosphorothioate bonds
	TIRE PCR1 Rev	CTACACGACGCTCTTCCGAT*C*T	Desalted, 3' phosphorothioate bonds

Universal primer sequences

Well barcoding primer sequences: TIRE_read1_wellBC.csv

SI-TT plate primer sequences: Dual_Index_Kit_TT_Set_A.csv

Combination of plate and well barcoding sequences: plate_and_well_BCs.csv

Materials

- SPRIselect reagent kit **Beckman Coulter Catalog #**B23317
- beta mercaptoethanol
- HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #**KK2601
- Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog ##**EP0741
- DNA Polymerase I Klenow Fragment - 1,000 units **New England Biolabs Catalog #**M0210L
- 10 mM dNTPs **Life Technologies Catalog #**10297-018
- SUPERase• In™ RNase Inhibitor (20 U/µL) **Thermo Fisher Scientific Catalog #**cat# AM2694
- Buffer TCL **Qiagen Catalog #**1031576
- Buffer TCL, 2x **Qiagen Catalog #**1070498
- TurboCapture 96 mRNA Kit **Qiagen Catalog #**72251
- Ultrapure Distilled, Nuclease Free Water
- 0.2 ml PCR Tube strips **Eppendorf Catalog #**0030124359
- Nalgene™ Disposable Polypropylene Robotic Reservoirs, flat **Thermo Fisher Catalog #**1200-1300
- Adhesive PCR Plate Seals **Thermo Fisher Scientific Catalog #**AB0558

Safety warnings

Please follow all Manufacturer safety warnings and recommendations.
Keep a separation of pre and post PCR steps in separate work areas.

Before start

Wipe bench surfaces with PCR clean wipes and keep working environment clean.

Cell lysis

30m

1 Prepare TCL lysis buffer

> Note
>
> The most concentrated lysate I have made is 2,000 cells per uL. Beyond this concentration the lysate becomes too viscous from genomic DNA.

1.1 For adherent cells prepare enough 1x TCL to cover the surface of the culture dish. Add 1% v/v 2-Mercaptoethanol.

1.2 For suspension cells prepare an equal volume of 2x TCL as the cell culture media volume. Add 1% v/v 2-Mercaptoethanol.

2 Prepare cell lysate

2.1 For adherent cells, remove cell culture media by vacuum and add 1 volume of PBS to wash.
After removing PBS, add the relevant volume of 1x TCL buffer based on estimate cell number.
Use a cell scraper and p1000 pipette to homogenize lysate.

2.2 For suspension cells, add the relevant volume of 2x TCL buffer directly to culture media.
Use a p1000 pipette to homogenize lysate.

3 The lysate may be stored for at least 6 months at -80 °C without any loss in performance.

TIRE-seq preparation

5m

4 Clean all surfaces and pipettes with PCR clean wipes

5 Turn on a lab oven to 50 °C and another to 80 °C

6 Thaw frozen buffers and primers on ice

7 Prepare fresh 80% EtOH

mRNA capture

2h 45m

8 Thaw the cell lysates On ice 1m

9 Transfer 20 µL cell lysate to a TurboCapture plate. 10m
I use a 96w pipetting robot for this step.

> Note
>
> Avoid creating bubbles or foam.
> A maximum of 80uL cell lysate may be used but be aware an equal volume of Kappa HiFi polymerase should be added at a later step driving up costs.

10 Cover the plate with a plastic seal and incubate at Room temperature for 1h
01:00:00 on a laboratory rocker

11 Remove the liquid from the TurboCapture plate by flicking over a sink. Use the ELISA flicking technique. 1m
Dilute out the liquid in the sink by running the tap.

12 Wash the TurboCapture plate using 100 µl Buffer TCW per well. 5m
Flick the TCW into the sink

> Note
>
> Pipetting accuracy not critical here.
> Prepare the reverse transcriptase mix before the final wash so the TurboCapture plate does not dry out.

13 go to step #12 repeat the wash an additional 2 more times for a total of 3 washes 5m

Reverse transcription

2h 45m

14 Prepare reverse transcriptase mixture: 5m

	A	B	C	D
	Reagent	**1 rxn**	**96 rxn + 20%**	**Conc**
	5x Maxima buffer	4	460.8	1x
	100mM dNTPs	0.2	23	1mM
	100uM TIRE_TSO	0.2	23	1uM
	20U/uL SuperaseIN	0.2	23	0.2U/uL
	200U/uL Maxima reverse transcriptase	2	230.4	100U
	Water	13.4	1543.7	
	Total	**20**	**2304**	

15 Remove all buffer TCW from the TurboCapture plate by pulse centrifugation and aspirating with a multi channel pipette. 10m

16 Add 20 µL reverse transcription mix 1h 20m

Incubate in a thermocycler for:

1. 50 °C 1 hour
2. 80 °C 10min
3. 4 °C Hold

cDNA denaturing

25m

17 Add 150 µL [M] 100 millimolar (mM) NaOH to the plate with reverse transcription mixture and immediately flick out the liquid over the sink. 5m

18 Add 100 µL [M] 100 millimolar (mM) NaOH to the plate. Incubate for 00:05:00 room temp 5m

19 Wash with 150 µL [M] 10 millimolar (mM) Tris pH 7.5 5m

Flick out the liquid over the sink.

20 go to step #19 5m

Repeat wash for a total of 2 washes

Note

Have Kappa HiFi and the well barcoding primers thawed and ready so the plate does not dry out

21 Remove all Tris buffer from the TurboCapture plate by pulse centrifugation and aspirating with a multi channel pipette. 5m

Linear amplification 1h 20m

22 Dispense 10 µL 2x Kappa HiFi Polymerase into each well with a repeat dispenser or multichannel pipet. 5m

23 Dispense 10 µL of [M] 1 micromolar (µM) barcoding primer to each well. Mix well. 10m

I use a 96w pipetting robot for this step.

24 Incubate in a thermocycler: 1h

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Inital denaturation	98	2min	1
	Denature	98	20sec	10 cycles
	Anneal	60	30sec	
	Extension	72	30sec	
	Final elongation	72	2min	1

	A	B	C	D
	Hold			

Linear amplification protocol. Sample can be held overnight.

Sample pooling and purification

1h 20m

25 Prepare SPRI beads by diluting SPRIselect commerical beads 1:4 in SPRI buffer.
Prepare 2.5 mL diluted SPRI beads per 96 well plate.

26 Pool all wells together. You can invert the plate into a SBS reservoir and centrifuge.
Or use a multichannel pipette.

27 After pooling, mix the sample well in a single tube then distribute across 4× 1.5mL eppendorf tubes.
Measure the volume of each tube with a p1000 pipette. Use the dialing down method.

28 Perform a 1.2x SPRI bead : sample cleanup.
For example for 450uL of sample add 540 µL diluted SPRI beads.

29 Incubate for 00:05:00 at room temp 5m

30 Incubate on a magnet for 00:05:00 at room temp 5m

31 Keep tubes on magent and remove the supernatant carefully.
Add 500 µL 80% ethanol

32 go to step #31 for a total of 2 ethanol washes.

33 After removing supernatant perform a pulse centrifuge and remove all residual ethanol with a pipet

34 Incubate on the magnet for 00:02:00 2m

35 Elute the sample with 30 µL water

Transfer 28 µL supernatant of each of the 4 aliquots into separate positions of a 0.2mL strip tube.

Sample can be stored for several weeks at -20 °C

Library preparation

1h

36 Keep half the sample at -20 °C as a backup.

Prepare the following random hexamer master mix:

	A	B	C
	Reagent	**1 rxn**	**4 rxn + 10%**
	10x NEB buffer 3.1	2	8.8
	100uM TIRE hexamer primer	2	8.8
	10mM dNTPs	2	8.8
	Linear amp product	13	
	Total	19	83.6

37 Add random hexamer mix to the samples and incubate 95 °C for 2 min in a thermocycler.

38 Remove from thermocycler and allow the sample to cool to room temperature.

Add 1 µL Large (Klenow) Fragment and mix well.

39 Incubate at 25 °C for 30min

30m

40 Incubate at 75 °C for 20min

20m

41 Perform a 1.8x SPRI bead cleanup with undiluted beads with the sample protocol as go to step #29

42 Elute in 20 µL water in a 0.2mL strip tube.

First PCR amplification

2h

43 Prepare a PCR master mix for each of the 4 aliquots from the Klenow reaction:

	A	B	C	D
	Reagent	**1 rxn**	**4 rxn + 10%**	**Conc**
	Purified Klenow reaction	20		
	2x Kappa HiFi polymerase	25	110	
	10uM each TIRE PCR1 primer premix	5	22	400nM
	Total	**50**	**220**	

PCR1 master mix

44 Add 30 µL PCR1 master mix to each purified Klenow reaction.

45 Run the following PCR protocol:

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Inital denaturation	98	2min	1
	Denature	98	20sec	10 cycles*
	Anneal	60	30sec	
	Extension	72	30sec	

	A	B	C	D
	Final elongation	72	2min	1
	Hold			

PCR1 cycling conditions

Note

*I have performed experiments comparing PCR cycle numbers in the PCR1 and PCR2 steps.
10 PCR cycles has worked well for 1 million - 10 million cells across the 96w plate
For 100,000 cells total I use 12 PCR cycles.
I found it better to err on the side of more PCR cycles in the 1st PCR step and less in the 2nd PCR step.

46 Perform a 1x SPRI bead cleanup with undiluted beads following the protocol in go to step #29 PCR cleanup

47 Elute in 15 µL EB and transfer 13 µL to a new tube.

Second PCR amplification

5m

48 Keep the 4 tubes separate for this step but use the same index primer set.

2h

49 Prepare a PCR master mix for each of the 4 aliquots from the PCR 1 reaction:

	A	B	C
	Reagent	**1 rxn**	**4 rxn + 10%**
	Purified PCR1 reaction	5	
	2x Kappa HiFi polymerase	12.5	55
	Index set SI-TT-xx	5	22

	A	B	C
	water	2.5	11
	Total	**25**	**220**

PCR2 master mix

50 Keep Run the following PCR protocol:

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Inital denaturation	98	2min	1
	Denature	98	20sec	8 cycles*
	Anneal	54	30sec	
	Extension	72	20sec	
	Final elongation	72	1min	1
	Hold			

PCR2 cycling conditions

Note

*I have performed experiments comparing PCR cycle numbers in the PCR1 and PCR2 steps.
8 PCR cycles has worked well for 1 million - 10 million cells across the 96w plate
For 100,000 cells total I use 10 PCR cycles.
I found it better to err on the side of more PCR cycles in the 1st PCR step and less in the 2nd PCR step.

51 Perform a 2 sided SPRI bead size selection to obtain compatible fragments for Illumina sequencing.
Combine all 4 aliquots into a single tube ~ 100 µL Crude PCR2 product

51.1 Add 50 µL (0.5x) undiluted SPRI beads to the pooled sample.

51.2 Incubate for 00:05:00 at room temp 5m

51.3 Incubate on a magnet for 00:05:00 at room temp 5m

51.4 **Keep the supernatant!** Transfer cleared supernatant to a new 1.5mL tube.

51.5 Add 25 µL (0.8x) undiluted SPRI beads and incubate off magnet for 00:05:00 5m

51.6 Transfer to a magnet and incubate 00:05:00 5m

51.7 Remove and discard supernatant. Add 500 µL 80% ethanol while the sample is still on the magnet.

51.8 Repeat the ethanol wash go to step #51.7 for a total of 2 washes.

51.9 Pulse centrifuge to collect the liquid to the bottom of the tube.
Incubate on magnet with the cap open for 00:02:00 2m

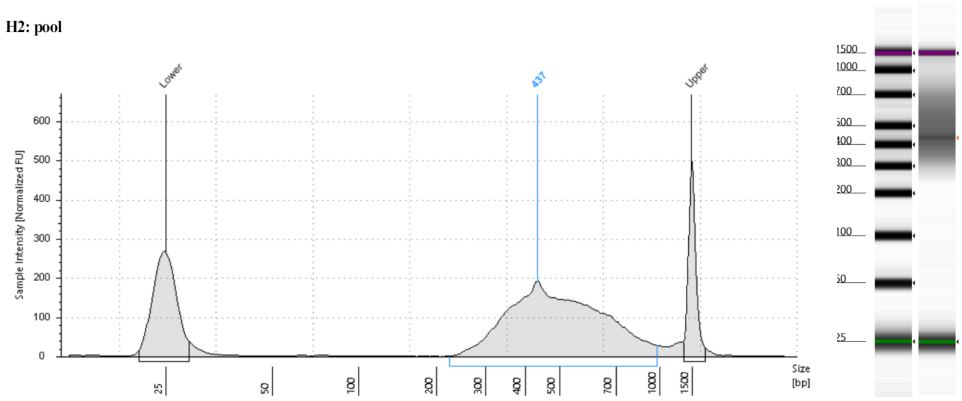
51.10 Elute in 27 µL EB
Transfer 25 µL to a new tube as a finished library.

Quality control 15m

52 Quantify the final library by Qubit DNA high sensitivity kit.
Expect a concentration of between 1 - 20ng/uL.

53

Expected result



Example tapestation trace of finished library on D1000 tape

Sequencing

54 The minimum sequencing requirement is:

1. Read 1: 50nt
2. Index 1: 8nt
3. Index 2: 8nt
4. Read 2: 72nt

Note

Read 1 is the transcript and can be increased if kit capacity allows.
Read 2 contains the well barcode, TSO sequence and UMI.
I typically begin by sequencing 2 million reads per well ~ 200M reads per 96w plate.

Bioinformatic preprocessing

55 The fastqs may be preprocessed with your pipeline of choice. I prefer **zUMIs** where the parameters are:

Command

zUMI config.yaml

```
sequence_files:
  file1:
    name: path_to_read2.fastq.gz
    base_definition:
      - BC(1-10)
      - UMI(28-37)
  file2:
    name: path_to_read1.fastq.gz
    base_definition:
      - cDNA(1-50)
```

55.1 When I have multiple plates in a run I have bclconvert write the index reads to file and concatenate fastqs from all plates into a single file:

Command

concatenate multiplate plate fastqs

```
cat *I1_001.fastq.gz > combined_S1_I1_001.fastq.gz
cat *R1_001.fastq.gz > combined_S1_R1_001.fastq.gz
cat *R2_001.fastq.gz > combined_S1_R2_001.fastq.gz
```

55.2 I then use the index read as the first part of the well barcode:

Command

zUMI multi plate.yaml

```
sequence_files:
  file1:
    name: path_to_index_read1.fastq.gz
    base_definition:
      - BC(1-8)
  file2:
    name: path_to_read2.fastq.gz
    base_definition:
      - BC(1-10)
      - UMI(28-37)
  file3:
    name: path_to_read1.fastq.gz
    base_definition:
      - cDNA(1-50)
```

55.3 You will need to create a well barcode whitelist by concatenating the index reads used to all well barcodes:

Command

concatenate_plate_well_barcodes.R

```
# Read index read 1 barcodes
plateBC <- read.csv(here::here(
  "i7_only_Kit_TT_Set_A.csv"
))

# Read well barcodes
wellBC <- read.csv(here::here(
  "barcodeOrder_v1.csv"
))

# Define the vectors to be concatenated
vector1 <- plateBC$i7_8nt
vector2 <- wellBC$Well_BC

# Get all combinations
combinations <- expand.grid(vector1, vector2)

# Concatenate the combinations
combinations <- as.data.frame(paste(combinations$Var1,
combinations$Var2, sep=""))

# Write the result to a CSV file
write.csv(combinations, file = here::here("all_sample_BCs.csv"),
          row.names = FALSE, quote = F, col.names = FALSE)
```