RT&Tag (Reverse Transcribe & Tagment)

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Oct 05, 2022

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dx.doi.org/10.17504/protocols.io.x54v9jyjqg3e/v1

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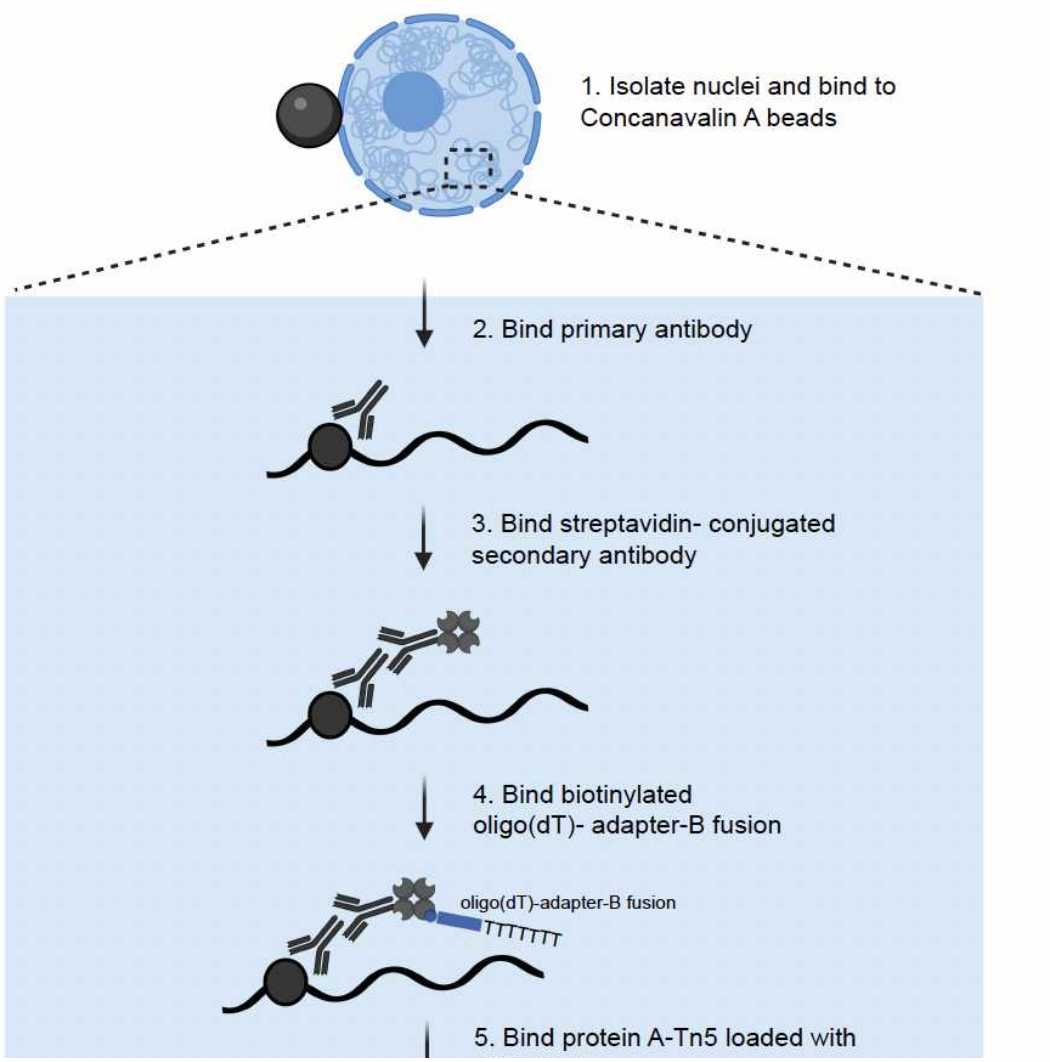
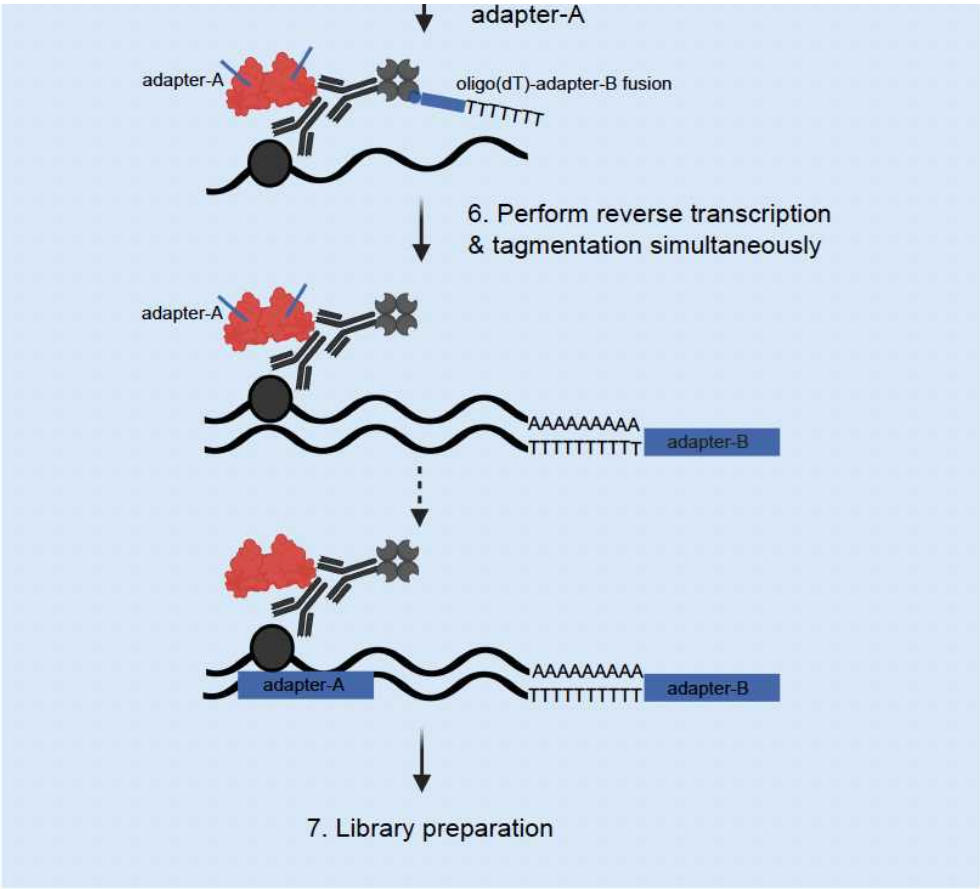
ABSTRACT

Reverse Transcribe & Tagment (RT&Tag) is a method that capitalizes on the ability of the Tn5 transposase to tagment RNA-cDNA hybrids. RT&Tag bypasses immunoprecipitation and instead uses antibodies to tether a protein A-Tn5 transposase fusion protein *in situ.* By performing localized reverse transcription and tagmentation, one can capture RNAs associated with an epitope of interest within intact nuclei. With RT&Tag, one can generate sequencing libraries using only 100,000 Drosophila cells and require only 8 million sequencing reads per sample.





DOI

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PROTOCOL CITATION

Nadiya Khyzha 2022. RT&Tag (Reverse Transcribe & Tagment). **protocols.io** https://dx.doi.org/10.17504/protocols.io.x54v9jyjqg3e/v1

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

https://www.nature.com/articles/s41592-022-01618-9

KEYWORDS

RT&Tag, RNA, RNA-chromatin

CREATED

Oct 27, 2020

LAST MODIFIED

Oct 05, 2022

PROTOCOL INTEGER ID

43870

MATERIALS TEXT

Solutions

- 1 M Hydroxyethyl piperazineethanesulfonic acid (**HEPES**) pH 7.9 (Sigma-Aldrich Cat# H3375)
- 1 M Potassium Chloride (**KCl**) (Sigma-Aldrich Cat# P3911)
- 1 M Manganese Chloride (**MnCl2**) (Sigma-Aldrich Cat# M5005)
- 1 M Calcium Chloride (**CaCl2**) (Fisher Cat# BP510)
- 10% **Triton X-100** (Sigma-Aldrich Cat# X100)
- 2 M **Spermidine** (Sigma-Aldrich Cat# S0266)
- **Glycerol** (Sigma-Aldrich Cat# G5516)
- 1 M Hydroxyethyl piperazineethanesulfonic acid (**HEPES**) pH 7.5 (Sigma-Aldrich Cat# H3375)
- 5 M Sodium chloride (**NaCl**) (Sigma-Aldrich Cat# S3014)
- 0.5 M Ethylenediaminetetraacetic acid (**EDTA**) (Research Organics Cat# 3002E)
- 30% Bovine Serum Albumen (**BSA**) (Sigma-Aldrich Cat# A8577)
- 1 M N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (**TAPS**) pH 8.5 (Millipore Sigma Cat# T5130)
- 10% Sodium dodecyl sulfate (**SDS**) (Sigma-Aldrich Cat# L4509)
- 1 M tris(hydroxymethyl)aminomethane (**Tris-HCl**) pH 8.0 (Research Organics Inc Cat# 30960T)
- 80% **Ethanol** (Fisher Scientific 04-355-223)

Buffer Recipes

- **Binding Buffer** (Can store @4C for 6 months)

Reagent	Volume
1M HEPES-KPH pH7.9	200ul
1M KCl	100ul
1M CaCl2	10ul
1M MnCl2	10ul
Water	Bring up to 10ml

- **NE1 Buffer** (Can store @4C for 1 week after adding spermidine and Protease Inhibitor Tablet)

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Reagent	Volume
1M HEPES-KOH pH7.9	1ml
1M KCl	500ul
10% Triton X-100	500ul
100% glycerol	10ml
2M spermidine	12.5ul
Roche Complete Protease Inhibitor EDTA-Free Tablet	1 tablet
Water	Bring up to 50ml

- **Wash Buffer** (Can store @4C for 1 week after adding spermidine and Protease Inhibitor Tablet)

Reagent	Volume
1M HEPES-KOH pH7.5	1ml
5M NaCl	1.5ml
2M spermidine	12.5ul
Roche Complete Protease Inhibitor EDTA-Free Tablet	1 tablet
Water	Bring up to 50ml

- **Antibody Buffer** (Can store @4C for 1 week)

Reagent	Volume
Wash Buffer	1.985ml
0.5M EDTA	8ul
30% BSA	6.7ul

- **300Wash Buffer** (Can store @4C for 1 week after adding spermidine and Protease Inhibitor Tablet)

Reagent	Volume
1M HEPES-KOH pH7.5	1ml
5M NaCl	3ml
2M spermidine	12.5ul
Roche Complete Protease Inhibitor EDTA-Free Tablet	1 tablet
Water	Bring up to 50ml

- **Post-tagmentation Wash Buffer**

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Reagent	Volume
1M TAPS pH8.5	10ul
Water	990ul

- **SDS Release Buffer** (Make fresh each time)

Reagent	Volume
1M TAPS pH8.5	10ul
10% SDS	10ul
Water	980ul

- **Triton X-100 Neutralization Buffer** (Make fresh each time)

Reagent	Volume
10% Triton X-100	67ul
Water	933ul

Reagents

- **Rnasin Rnase Inhibitor** (Promega N2515)
- **Roche Complete Protease Inhibitor EDTA-Free tablets** (Sigma-Aldrich Cat# 5056489001)
- **Concanavalin A (ConA)-coated magnetic beads** (Bangs Laboratories Cat # BP531)
- **Antibody to an epitope of interest.** Given that *in situ* binding conditions mimic that of immunofluorescence (IF), we suggest using IF-tested antibodies.
- **Streptavidin Conjugation Kit- Lightning-Link** (Abcam Cat# ab102921)
- **Guinea pig α-rabbit secondary antibody** (Antibodies online Cat# ABIN101961)
- **Biotinylated- Oligo d(T)-ME B** (Manufactured by IDT. Resuspend in water to make a 12.5uM working solution.)

(/5Biosg/GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN)

- **Protein A–Tn5 (pA-Tn5) fusion protein** (Can be generated using the following protocol: https://www.protocols.io/view/3xflag-patn5-protein-purification-and-meds-loading-j8nlke4e5l5r/v1)
- **Mosaic end_reverse** [PHO]CTGTCTCTTATACACATCT
- **Mosaic end_Adapter A** TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
- **Thermo Scientific Maxima H Minus Reverse Transcriptase** (Thermo Scientific Cat# EP0751)
- **NEBNext® High-Fidelity 2X PCR Master Mix** (NEB Cat# M0541S)
- **PCR primers** (10 µM stock solutions of a universal i5 primer and i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486 (2015)] in 10 mM Tris pH 8)
- **AMPure XP reagent** (Beckman Coulter Cat# A63880)

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Reagent Preparation

- **Tn5-ME A adapter complex**

Dilute the mosaic end_Adapter A and mosaic end_reverse oligonucleotides to 200µM in annealing buffer (10mM Tris pH8, 50mM NaCl, 1mM EDTA). Then take 8µl of each re-suspended oligonucleotide and mix together in a tube. Incubate at 95C for 5min on a heatblock. Then remove heatblock from the heat source and allow to cool to room temperature for ~45min. Once cool, combine with 100µl of 5.5µM protein A-Tn5 fusion protein and place on a rotating platform for 1hr at room temperature. Store at -20C until use.

- **Streptavidin conjugated secondary antibody**

Use the guinea pig anti-rabbit secondary antibody and the streptavidin conjugation kit- lightning link. Dilute secondary antibody to 1ug/ul in PBS and then add 10ul of Modifier Reagent. Mix. Then add the secondary antibody to the streptavidin mix. Mix. Incubate for 3hrs in the dark. Then add 10ul of Quencher Reagent. Mix. Incubate for 30min in the dark. The secondary antibody is now streptavidin conjugated and ready for use. Store @4C in the dark until use.

Equipment

- 1.5ml Eppendorf tubes
- PCR Rigid 8-tube strips with individually attached flat caps, clear, 0.2ml (BrandTech Cat# P1200)
- 0.2 ml PCR 8 Strip Magnetic Separator (Permagen Labware MSRLV08)
- Magnet that fits 1.5ml Eppendorf tubes
- PCR machine
- Centrifuge
- Vortex

Clean desk and prepare reagents 10m

1

1. Prepare all buffers and reagents.

Prepare Concanavalin A beads 10m

2 1. Mix the Concanavalin A (conA) bead slurry. Transfer 5µl of beads per sample into a 1.5ml eppendorf tube.
2. Wash with 1ml of Binding Buffer.

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3. Place on magnet and remove the Binding Buffer.
4. Perform a second wash with 1ml of Binding Buffer.
5. Resupend in 5µl of Binding Buffer per sample.
6. Aliquot 5µl of ConA beads into 8-tube PCR strips.

Prepare nuclei 1h

3 1. Harvest 4 million S2 cells.
2. Centrifuge for 3min at 600g. Remove media.
3. Re-suspend in 1ml of Wash Buffer.
4. Centrifuge for 3min at 600g. Remove Wash Buffer.
5. Re-suspend in 500µl of NE1 buffer supplemented with Rnasin Rnase Inhibitor (1U/µl, 1:40 dilution). Immediately place on ice and incubate for 10min.
6. Centrifuge for 5min at 2500g. Remove NE1 Buffer.
7. Re-suspend in 500µl of Wash Buffer supplemented with Rnasin Rnase Inhibitor (1U/µl, 1:40 dilution).
8. Add 100k nuclei into each of 8-tube PCR strips. Incubate for 10min at room temperature to allow nuclei to bind to beads.

Primary antibody binding 1h 15m

4 1. Prepare Primary Antibody Master Mixes. Use 50µl of Antibody Buffer per reaction. Dilute antibody of interest 1:100 in Antibody Buffer. Supplement with Rnasin Rnase Inhibitor (1U/µl, 1:40 dilution).
2. Place the PCR strips on a magnet and remove liquid from the nuclei binding step.
3. Re-suspend beads in 50µl of Primary Antibody Master Mix while gently vortexing.
4. Place on a nutator and incubate for 2h at room temperature or overnight at 4°C.

Streptavidin conjugated secondary antibody binding 1h

5 1. Prepare Secondary Antibody Master Mixes. Use 50µl of Wash Buffer per reaction. Dilute streptavidin conjugated secondary antibody 1:100 in Wash Buffer. Supplement with Rnasin Rnase Inhibitor (1U/µl, 1:40 dilution).
2. Place the PCR strips on a magnet and remove the liquid.
3. Re-suspend beads in 50µl of Secondary Antibody Master Mix while gently vortexing.

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4. Place on a nutator and incubate for 45min at room temperature or overnight at 4°C.

Biotinylated Oligo(dT)-ME-B binding 35m

6 1. Prepare Biotinylated Oligo(dT)- ME-B Master Mixes. Use 50µl of Wash Buffer per reaction. Dilute biotinylated Oligo(dT)- ME-B (12.5uM) 1:50 in Wash Buffer. Supplement with Rnasin Rnase Inhibitor (1U/µl, 1:40 dilution).
2. Place the PCR strips on a magnet and remove the liquid.
3. Perform 2 sets of washes using 200µl of Wash Buffer.
4. Re-suspend beads in 50µl of Biotinylated Oligo(dT)- ME-B Master while gently vortexing.
5. Place on a nutator and incubate for 20min at room temperature.

Protein A-Tn5 binding 1h 15m

7 1. Prepare Protein A-Tn5 Master Mixes. Use 50µl of 300Wash Buffer per reaction. Dilute protein A-Tn5 loaded with ME-A 1:200 in 300Wash Buffer. Supplement with Rnasin Rnase Inhibitor (1U/µl, 1:40 dilution). The protein A-Tn5 dilution may need to be established empirically based on the activity level of purified protein A-Tn5.
2. Place the PCR strips on a magnet and remove the liquid.
3. Perform 2 sets of washes using 200µl of Wash Buffer.
4. Re-suspend beads in 50µl of Protein A-Tn5 Master Mix while gently vortexing.
5. Place on a nutator and incubate for 1h at room temperature.

Reverse Transcription & Tagmentation 2h 15m

8 1. Prepare reverse transcription master mix by combining per reaction:

Reagent	Volume
5x Reverse Transcription Buffer	4 µL
dNTP Mix (10mM)	1 µL
Maxima Reverse Transcriptase	1 µL
Rnasin RNAse Inhibitor (40U/µL)	0.5 µL
Water	13.5 µ

2. Place the PCR strips on a magnet and remove the liquid.
3. Perform 2 sets of washes using 200µl of 300Wash Buffer.
4. Re-suspend beads in 20µl of Reverse Transcription Master Mix while gently vortexing.
5. Incubate for 2h at 37C in a PCR machine.

Tn5 release 1h 15m

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9 1. Place the PCR strips on a magnet and remove the liquid.
2. Perform a wash using 50µl of Post-tagmentation Wash Buffer.
3. Add 5µl of SDS Release Buffer to each PCR tube. Don't mix or resuspend by vortexing as the bead solution will be quite sticky at this point.
4. Incubate for 1h at 58C in a PCR machine.

Amplifying libraries 45m

10 1. Set-up PCR reaction master mix by combining per reaction:

Reagent	Volume
2x NEBNext PCR Master Mix	25 µl
Triton X-100 Neutralization Buffer	15 µl
10 µM barcoded i5 primer	2 µl

2. Add 42 µl of PCR reaction master mix to each sample.
3. Add 2 µl of corresponding 10 µM barcoded i7 primer to each sample.
4. Perform PCR using the following conditions:

A	B	C	D	E
Cycle number	Denature	Anneal	Extend	Final
1			58° C, 5 min	
			72° C, 5 min	
2	98° C, 30 s			
3–14	98° C, 10 s	60° C, 15 s	72° C, 30s	
15			72° C, 1 min	
16				4° C, hold

Library clean-up 30m

11 1. Perform library clean-up using x0.8 AMPure XP beads. To do so mix the 50µl PCR reaction with 40µl of AMPure XP beads in PCR tubes. Vortex at full speed and centrifuge.
2. Allow to incubate for 5-10min.
3. Place the PCR tubes on a magnet and allow a few minutes for beads to gather.
4. Remove the liquid and perform 2 rounds of washes with 200 µl of 80% ethanol while still on magnet.
5. Perform a quick centrifuge to gather the remaining ethanol. Place back on magnet and use a 20 µl pipette to remove the residual ethanol.
6. Re-suspend the beads in 22 µl of 10mM Tris-HCl. Vortex to mix, centrifuge, and allow to incubate for 5-10min.
7. Place on magnet and transfer the final sample to a clean tube.

Tapestation 20m

12 1. Combine 2 µl of sample with 2µl of sample dye and run on D5000 Tapestation to get library size distribution.

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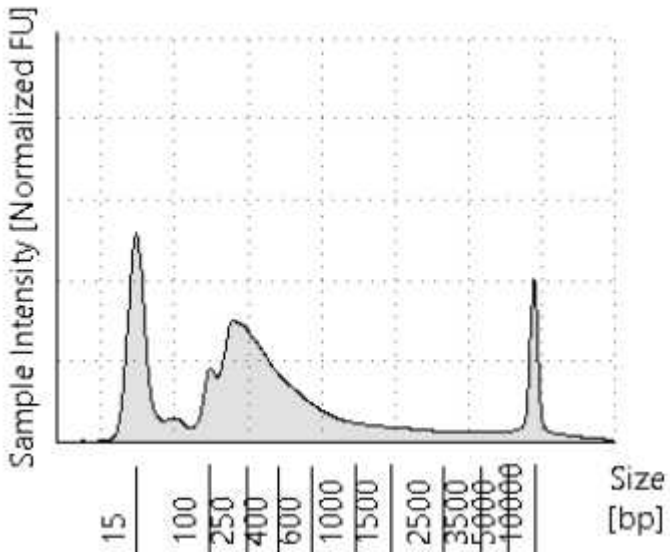
2. Expected library size should be between 200-700bp.



Example of a successful RT&Tag library.

3. If large peak around 150bp is observed, perform an additional round of x0.8 AMPure XP bead clean-up.

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