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dU-Tn5 stranded RNA-seq experiment

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We use this protocol and it's working

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Abstract

In this protocol, we describe a novel Tn5-based stranded RNA-seq procedure, in which deoxy-UTP-labeled Tn5 (dU-Tn5) is applied in library construction to preserve the strand-specificity of transcripts. The stranded library preparation section only contains 8 steps, which is straightforward and easy-to-do for library preparation.

Materials

1. Oligos and primers (Table S1)

A	B	C	D
Name	Sequences (5'-3')	Purification Method	Usage
Primer A	5'-phos-CTGTCTCTTATACACATCT-NH ₂ -3' (5'-Phosphate, 3'-AminolinkerC7)	HPLC	Tn5 assembly
Primer B	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	DSL	Tn5 assembly
dU-Primer C	G/ideoxyU/C/ideoxyU/CG/ideoxyU/GGGC/ideoxyU/CGGAGATGTGTATAAGAGACAG	HPLC	Tn5 assembly
N501	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC	DSL	Index primers for illumina
N502	AATGATACGGCGACCACCGAGATCTACACTCTCTATTCGTCTCGGCAGCGTC	DSL	Index primers for illumina
N503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCTCGGCAGCGTC	DSL	Index primers for illumina
N504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC	DSL	Index primers for illumina
N505	AATGATACGGCGACCACCGAGATCTACACTAAGGAGTCGTCTCGGCAGCGTC	DSL	Index primers for illumina
N506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC	DSL	Index primers for illumina
N507	AATGATACGGCGACCACCGAGATCTACACAGGAGTATCGTCGGCAGCGTC	DSL	Index primers for illumina
N508	AATGATACGGCGACCACCGAGATCTACACTAAGCCTTCGTCTCGGCAGCGTC	DSL	Index primers for illumina
N701	CAAGCAGAAGACGGCATACGAGATTAAGGCAGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N702	CAAGCAGAAGACGGCATACGAGATCGTACTAGGTCTCGTGGGCTCGG	DSL	Index primers for illumina

	A	B	C	D
	N703	CAAGCAGAAGACGGGCATACGAGATAGGCAG AAGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N704	CAAGCAGAAGACGGGCATACGAGATTCCTGA GCGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N705	CAAGCAGAAGACGGGCATACGAGATGGACTC CTGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N706	CAAGCAGAAGACGGGCATACGAGATTAGGCA TGGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N707	CAAGCAGAAGACGGGCATACGAGATCTCTCT ACGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N708	CAAGCAGAAGACGGGCATACGAGATCAGAGA GGGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N709	CAAGCAGAAGACGGGCATACGAGATGCTACG CTGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N710	CAAGCAGAAGACGGGCATACGAGATCGAGGC TGGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N711	CAAGCAGAAGACGGGCATACGAGATAAGAGG CAGTCTCGTGGGCTCGG	DSL	Index primers for illumina
	N712	CAAGCAGAAGACGGGCATACGAGATGTAGAG GAGTCTCGTGGGCTCGG	DSL	Index primers for illumina

2. Chemicals

- (1) PEG 8000 (sangon.com, Cat#A100159-0500)
- (2) actinomycin D (J&K, Cat#338112)
- (3) SDS (sangon.com, Cat#A100227-0100)
- (4) Tris hydrochloride (sangon.com, Cat#A610103-0250)
- (5) MgCl₂ (sangon.com, Cat#A601336-0500)
- (6) KCl (sangon.com, Cat#A100395-0500)
- (7) (NH₄)₂SO₄ (sangon.com, Cat#A100191-0005)
- (8) β-NAD (sangon.com, Cat#A600641-0001)
- (9) Bovine Serum Albumin,BSA (solarbio.com, Cat#A8010)
- (10) NaCl (sangon.com, Cat# A100241-0500)

(11) Ethylenediaminetetraacetic acid, EDTA (sangon.com, Cat#A600107-0500)

3. Reagents and kits

(1) oligo(dT)-attached mRNA capture magnetic beads (Vazyme, Cat#401)

(2) dUTP, 100 mM (Yeasen, Cat#10128ES74)

(3) dTTP, 100 mM (Yeasen, Cat#10120ES74)

(4) dATP, 100 mM (Yeasen, Cat#10118ES74)

(5) dGTP, 100 mM (Yeasen, Cat#10121ES74)

(6) dCTP, 100 mM (Yeasen, Cat#10119ES74)

dNTP Mix (10 mM each dATP, dTTP, dGTP, and dCTP) by mixing 10 μ l each of 100 mM dATP, dTTP, dGTP and dCTP, and add 60 μ l to a volume of 100 μ l;

dUTP-containing dNTP Mix (20 mM dUTP, 10 mM each dATP, dGTP, and dCTP) by mixing 20 μ l dUTP, 10 μ l each of 100 mM dATP, dTTP, dGTP and dCTP, and add 60 μ l to a volume of 100 μ l.

(7) Oligo (dT)23VN

(8) Random primers

(9) Hiscript III Reverse Transcriptase (5 $\times$ Hiscript III Buffer included, Vazyme, Cat#R302-01)

(10) Recombinant RNasin® Ribonuclease Inhibitor

Components of (7)-(10) were included in reverse transcription kits, e.g. Hiscript® III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, R312-01)

(11) E. coli DNA ligase (NEB, Cat#M0205S)

(12) DNA polymerase I (Yeasen, Cat#12903ES76)

(13) RNase H (5U/ μ L, Yeasen, Cat#12906-A)

(14) Hieff NGS® Smarter DNA Clean Beads (Yeasen, Cat#12600ES03)

(15) Bst 2.0 (NEB, Cat#M0537S)

(16) Heat-labile UDG (1 U/ μ l) (Vazyme, Cat#P051-01)

(17) Phanta® Max Super-Fidelity DNA Polymerase (2x Phanta® Max buffer was included, Vazyme, Cat#P505-d1)

(18) Commerical Tn5 transposase (e.g. Vazyme, Novoprotein)

4. Recipes for buffers

(1) annealing buffer: 10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA

(2) 5 x second strand buffer: 100 mM Tris-Cl, pH 7.5, 500 mM KCl, 50 mM (NH₄)₂SO₄, 25 mM MgCl₂, 0.75 mM β -NAD, 0.25 mg/mL BSA

(3) 5x Tn5 Tagmentation Buffer: 50 mM Tris, 25 mM MgCl₂

dU-Tn5 assembly

- 1 Dilute oligos (Primer A, Primer B, and dU-Primer C, refer to Table S1) to 100 μM concentration using the annealing buffer.
- 2 Set up the following two reactions in PCR tubes: Reaction 1 (adaptor AB), 10 μL of 100 μM primer A, 10 μL of 100 μM primer B; Reaction 2 (adaptor AC), 10 μL of 100 μM primer A, 10 of μL 100 μM primer C. Anneal the adaptors in the PCR machine using the following program: heat lid (102°C), 75°C for 15 min, 60°C for 10 min, 50°C for 10 min, 40°C for 10 min, 25°C for 30 min. Combine adaptor AB and adaptor AC at 1:1 ratio, designated as "adaptor mix".

Note

The adaptors are partially double-stranded DNA molecules (concentration = 50 pmol/ μL)

- 3 Set up the following reaction in a 1.5 mL centrifuge tube: 5 μL of Tn5 transposase (10 pmol/ μL), 1.2 μL of adaptor mix (50 pmol/ μL), 6.3 μL of coupling buffer (included in the commercial Tn5 products). Pipette 20 times gently to mix well and incubate at 30°C in a water bath for 1 h. The final concentration of transposase = 4 pmol/ μL . Store at -20°C until use.

Note

Molar ratio of adaptor mix: transposase = 1.2:1.

mRNA purification

- 4 mRNA was purified from 1 μg of total RNA using oligo(dT)-attached mRNA capture magnetic beads (Vazyme, cat#401) following the user manual. The final purified mRNA was dissolved in 10.5 μL ddH₂O.

Stranded library preparation

- 5 For first-strand cDNA synthesis, mRNA was reverse transcribed in a PCR tube with the following setup: 10 μL purified mRNA, 1 μL 50 μM Oligo (dT)23VN, 1 μL 50 μM random primers, 4 μL 5 × HiScript III Buffer, 0.5 μL dNTP Mix (10 mM each dATP, dTTP, dGTP, and

dCTP), 1 µl 40 U/µl Recombinant RNasin® Ribonuclease Inhibitor, 1 µl 200 U/µl Hiscript III Reverse Transcriptase, 1 µl 120 ng/µl actinomycin D, and ddH₂O as needed to adjust the reaction volume to 20 µl. The reverse transcription was performed under the following program: using a heated lid, 25°C for 5 min, 55°C for 45 min, and finally 85°C for 5 min for deactivation.

- 6 Second-strand cDNA was synthesized by adding 10 µl 5 x second strand buffer, 1 µl dUTP-containing dNTP mix (20 mM dUTP, 10 mM each dATP, dGTP, and dCTP), 1 µl E. coli DNA ligase, 2 µl DNA polymerase I, 0.06 µl RNase H, and 15.94 µl ddH₂O to adjust the reaction volume to 50 µl. The solution was incubated at 16 °C for 1 h.
- 7 The resulting double-stranded cDNA from previous step was tagged by adding 20 µl 5x Tn5 Tagmentation Buffer, 16 µl 50% PEG 8000, 2 pmol (0.5 µl) Tn5 transposase, and 14 µl ddH₂O to adjust the reaction volume to 100 µl. The tagmentation reaction was performed at 55°C for 10 min, after which 10 µl 0.2% SDS was added and the enzyme deactivated by heating to 85°C for 5 min.
- 8 Add 100 µl HiFF NGS® Smarter DNA Clean Beads to the tagmentation products, purified the DNA following the user guide. Dissolve the resulting DNA in 20 µl ddH₂O.
- 9 Preparation for PCR amplification was then carried out by mixing the 20 µl of eluted DNA with 25 µl 2x Phanta® Max buffer, 2 µl dNTP Mix (10 mM each dATP, dTTP, dGTP, and dCTP), and 1 µl Bst 2.0, and extension was performed at 72°C for 20 min followed by deactivation at 85°C for 20 min.
- 10 Add 1.5 µl Heat-labile UDG (1 U/µl), 1 µl Phanta® Max Super-Fidelity DNA Polymerase, 1 µl primer N50X (20 µM), and 1 µl primer N70X (20 µM) to the PCR tube, and PCR was performed according to the following program: 25 °C for 20 min; 95 °C for 3 min; 14 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min; 4 °C for 1 min.
- 11 Take 3 µl of the PCR product for agarose gel electrophoresis to determine the concentration and size distribution of bulk DNA products.

Note

An additional 1-2 PCR cycles were carried out if necessary until the DNA bands were visible on agarose gel.



- 12 The PCR products were purified using 60 μ l (1.2 volume) Hieff NGS® Smarter DNA Clean Beads (Yeasten) following the user guide, and the resulting library was dissolved in 30 μ l ddH₂O for further quality control (QC) and NGS sequencing.