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dU-Tn5 stranded RNA-seq experiment

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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-NH2 -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	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC	DSL	Index primers for illumina
N503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC	DSL	Index primers for illumina
N504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC	DSL	Index primers for illumina
N505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC	DSL	Index primers for illumina
N506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC	DSL	Index primers for illumina
N507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC	DSL	Index primers for illumina
N508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC	DSL	Index primers for illumina
N701	CAAGCAGAAGACGGCATACGAGATTAAGGCGAGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N702	CAAGCAGAAGACGGCATACGAGATCGTACTAGGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N703	CAAGCAGAAGACGGCATACGAGATAGGCAGAAGTCTCGTGGGCTCGG	DSL	Index primers for illumina

A	B	C	D
N704	CAAGCAGAAGACGGCATACGAGATTCCTGAG CGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N705	CAAGCAGAAGACGGCATACGAGATGGACTCC TGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N706	CAAGCAGAAGACGGCATACGAGATTAGGCAT GGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N707	CAAGCAGAAGACGGCATACGAGATCTCTCTA CGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N708	CAAGCAGAAGACGGCATACGAGATCAGAGAG GGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N709	CAAGCAGAAGACGGCATACGAGATGCTACGC TGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N710	CAAGCAGAAGACGGCATACGAGATCGAGGCT GGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N711	CAAGCAGAAGACGGCATACGAGATAAGAGGC AGTCTCGTGGGCTCGG	DSL	Index primers for illumina
N712	CAAGCAGAAGACGGCATACGAGATGTAGAGG AGTCTCGTGGGCTCGG	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) $MgCl_2$（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 µl each of 100 mM dATP, dTTP, dGTP and dCTP, and add 60 µl to a volume of 100 µl;

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

(7) Oligo (dT)23VN

(8) Random primers

(9) Hiscript III Reverse Transcriptase（5 × 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/μL, 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/μl) (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 $MgCl_2$, 0.75 mM β-NAD, 0.25 mg/mL BSA
(3) 5x Tn5 Tagmentation Buffer: 50 mM Tris, 25 mM $MgCl_2$

1 Dilute oligos (Primer A, Primer B, and dU-Primer C, refer to Table S1) to 100 μM concentration using the annealing buffer.

dU-Tn5 assembly

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 adapters 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 adapters 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 commerical 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.

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 ddH_2O.

mRNA purification

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

Stranded library preparation

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 tagmented 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 µHieff NGS® Smarter DNA Clean Beadsto the tagmentation products, purified the DNA following the user guide.Dissolvethe resulting DNA in 20 µl ddH_2O.

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.

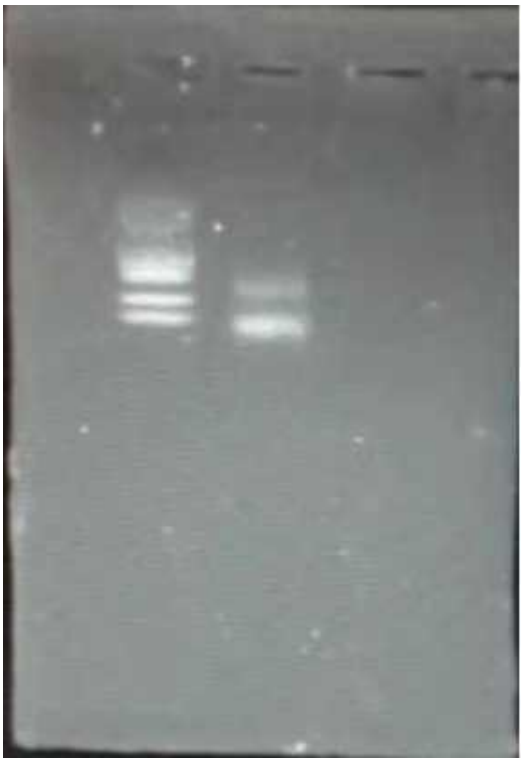
10 Add 1.5 µl Heat-labile UDG (1 U/µl), 1 µl Phanta® Max Super-Fidelity DNA Polymerase, 1 µl primer N50X (20

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 Take3 μl of the PCR productfor 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 μl (1.2 volume) Hieff NGS® Smarter DNA Clean Beads (Yeasen) following the user guide, and the resulting library was dissolved in 30 μl ddH_2O for further quality control (QC) and NGS sequencing.