Single Cell/Low Input cDNA Synthesis & Amplification Module

RK20310

Instruction Manual

Version: N18D02v1.1

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1. Overview

Single Cell/Low Input cDNA Synthesis & Amplification Module (ABclonal, Cat: RK20310) is capable of synthesizing full-length cDNA and enriching it using 1-1000 single cells or 10 pg-10 ng Total RNA as templates. Compared to traditional RNA-Seq methods, this reagent kit is compatible with lower input samples, addressing the library construction issues of samples with low RNA content.

The kit employs optimized Oligo (dT) VN primer as the reverse transcription primer, utilizing the Template-switching activity of Reverse Transcriptase to add adapter sequences at the 3' end of cDNA. Finally, the double-stranded full-length cDNA synthesis and enrichment are completed by PCR amplification using this adapter sequence as a primer. The obtained cDNA exhibits no 3' end bias, and a low residual rRNA rate.

Each reagent in the kit undergoes rigorous quality control, and each batch of reagents is validated for library construction and on-machine sequencing to ensure stable performance of each batch of the kit.

2. Kit Compents

Module Name	Tube Color & Name	24 RXN	96 RXN
Box1	Control Total RNA(1μg/μL)	5 μL	5 μL
	TSO Primer II	48 μL	192 μL
Box2	Cell Lysis Buffer	72 μL	288 μL
	Oligo (dT) RT Primer	24 μL	96 μL
	dNTP Mix	24 μL	96 μL
	5 X RT Buffer	96 μL	384 μL
	Reverse Transcriptase	24 μL	192 μL
	RNase Inhibitor	48 μL	192 μL
	PCR Primer	24 μL	96 μL
	2X PCR Master Mix	600 μL	2400 μL
	Nuclease-free Water	1 mL	2 mL
	Low-EDTA TE	1 mL	2 mL

Note: ○ denotes the color of the tube cap.

3. Storage

Single Cell/Low Input cDNA Synthesis & Amplification Module includes two packages:

Module Name	Storage	Transportation
Box-1	-80℃	dry ice
Box-3	-20℃	dry ice

4. Additional Materials

4.1. cDNA Lib Prep Kits

One-step DNA Lib Prep Kit for Illumina V2 (1 ng Input DNA) (ABclonal，Cat：RK20237)；

One-step DNA Lib Prep Kit for Illumina V2 (5ng Input DNA) (ABclonal，Cat：RK20238)；

4.2. Adapter Kits

Dual DNA Adapter 96 Kit for One-step DNA Lib Prep (ABclonal，Cat：RK20290)。

4.3. Magnetic beads for purification

AFTMag NGS DNA Clean Beads (ABclonal，Cat.NO. RK20257)，or other equivalent magnetic beads for nucleic acid purification.

4.4. RNA sample concentration and quality evaluation

Qubit fluorometers.

Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat. Q32855).

Nanodrop.

Agilent RNA 6000 Pico chip (Agilent #5067-1513).

4.5. Library quality control

Qubit fluorometers.

ABQubit dsDNA Quantitation Kit (ABclonal, Cat. RK30140)；

Agilent high sensitivity DNA Chips (Agilent #5067-4626)；

Agilent DNA 1000 chip (Agilent #5067-1504)；

4.6. Other reagents and consumables

Freshly prepared 80% ethanol.

Magnetic rack.

PCR system.

Thermostatic metal bath or thermostatic water bath.

5. Workflow

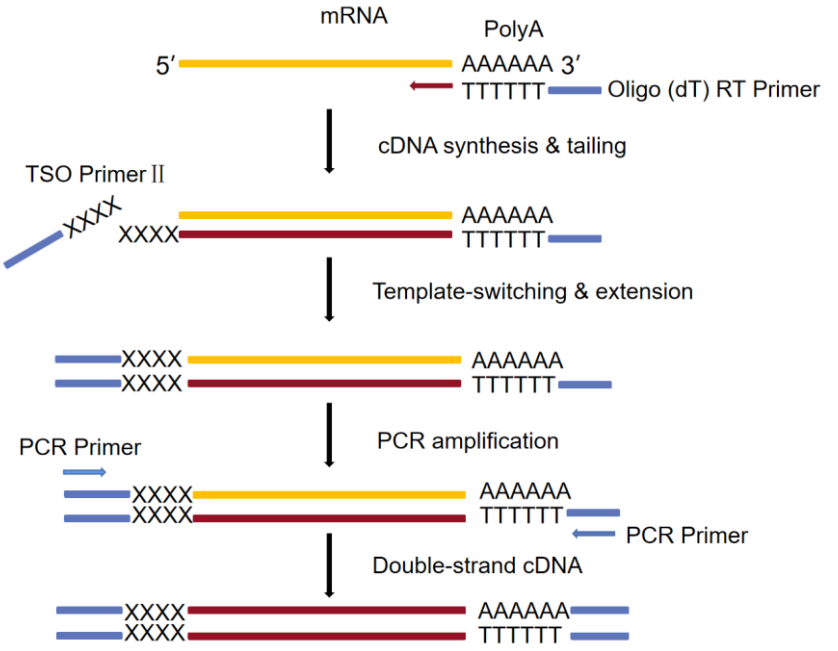

Figure 1. Procedure Diagram

6. Precautions

6.1. Quality Control of RNA Samples

6.1.1. Sample Input Requirements: The initial amount should be 1-1000 cells or 10 pg~10 ng Total RNA.

6.1.2. Sample Type Requirements: Eukaryotic cells containing polyA tails (without cell wall structures) or extracted Total RNA samples are suitable. This method is not applicable to prokaryotic cells or fixed cells.

Cell Samples: Due to factors such as cell viability and the cell cycle, which may affect cDNA output, it is recommended to conduct cell viability assessment before cell sorting. Dead cells may undergo RNA degradation, affecting experimental results. After passing viability assessment, proceed with cell sorting immediately. If sorted cells are not used for experiments immediately, it is advisable to follow step 7.1.3 for processing and immediately store them in a -80°C freezer. Upon retrieval, proceed with the reaction as soon as possible. Components such as cell culture medium and PBS have inhibitory effects on the reaction, so please minimize the volume of these components as much as possible.

RNA Samples: Before starting the experiment, it is recommended to use the Agilent RNA 6000 Pico Kit to assess sample integrity. Degraded samples may affect cDNA output and length analysis, potentially leading to experimental failure.

6.1.3. Different samples contain different mRNA levels. For samples with low mRNA content, it is recommended to increase the number of cycles appropriately to prevent low cDNA synthesis concentration, which may result in experimental failure.

6.2. Use of Magnetic Beads

6.2.1. The magnetic beads should be taken out half an hour in advance and brought to room temperature.

6.2.2. During purification with AFTMag NGS DNA Clean Beads, the Low EDTA TE must be added for elution after ethanol has fully evaporated, namely when the bead color changes from bright brown to frosted brown. Residual ethanol or over-drying (leading to cracks) of beads may reduce the library yield.

6.2.3. During the magnetic bead purification process, handle with care when aspirating the supernatant to avoid aspirating the magnetic beads, which could affect the size and yield of library fragments.

6.3. Library Quality Control

6.3.1. The actual yield of cDNA depends on the quality, quantity, and mRNA content of the cells. Please conduct experiments based on the PCR cycle recommendations provided in step 7.2.1. Typically, the cDNA yield ranges from 4-20 ng.

6.3.2. Evaluate the length of prepared cDNA using Agilent high sensitivity DNA Chips (Agilent #5067-4626). The average length of full-length cDNA peaks should be around 1500-2000 bp, with a peak range of approximately 400-10000 bp. The absence of obvious dimer residues indicates qualified cDNA synthesis.

6.4. Operating Procedures

6.4.1. All buffers and enzymes should be kept on ice at all times.

6.4.2. Handle all reagents with care to avoid cross-contamination between reagents and samples.

6.4.3. Wear a mask and gloves during the experiment. After diluting RNA samples, store them on ice and proceed to the next step as soon as possible to prevent RNA degradation.

6.4.4. The components and procedures of this product have been optimized. Please do not make changes.

6.4.5. Due to potential changes in RNA sample concentration during storage and transportation, it is recommended to perform Qubit quantification after a 10-fold dilution, and conduct experiments according to the measured concentration.

7. Protocols

7.1. First Strand cDNA Systhesis

7.1.1. Thaw the Cell Lysis Buffer, Oligo (dT) RT Primer, dNTP Mix, 5X RT Buffer, Nuclease-free Water on ice and mix well. Centrifuge them briefly to collect residual liquid from the tube wall, and keep on ice. All the following operations are performed on ice.

7.1.2. Prepare the Lysis Mix according to the table below.

Reagent	Volume
Cell Lysis Buffer	3 μL
RNase Inhibitor	1 μL
Total volum	**4 μL**

Note: The Lysis Mix should be prepared immediately before use. Any unused portion of the prepared mix can be stored at -20°C and used within 24 hours for subsequent reactions.

7.1.3. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube on ice.

7.1.4. Sample preparation:

Set up controls and test samples according to the table below, and prepare the reaction system as follows.

Reagent	Negative Control	Positive Control	Test Group
Lysis Mix	4 μL	4 μL	4 μL
Control Total RNA	-	1~6 μL	-
Total RNA/cell	-	-	1~6 μL
Nuclease-free Water	To 10 μL	To 10 μL	To 10 μL

Note 1: The concentration of Control RNA is 1 μg/μL. When in use, it needs to be diluted to the desired concentration using Nuclease-free Water.

Note 2: For RNA samples, take 10 pg-10 ng and add them to the Lysis Mix tube. If the volume of the RNA sample is less than 6 μL, use Nuclease-free Water to bring the total volume to 10 μL.

Note 3: For cell samples, take 1-1000 isolated cells and add them to the Lysis Mix tube. If the volume of the cell sample is less than 6 μL, use Nuclease-free Water to bring the total volume to 10 μL. Mix gently, incubate at room temperature for 5 minutes.

Note 4: Components such as cell culture medium and PBS in the isolated cells may inhibit the reaction. Please minimize their usage.

Prepared cell samples can be stored at -80℃ before use and transported using dry ice.

7.1.5. Place the samples on ice and set up the annealing system according to the table below:

Reagent	Volume
Products from step 7.1.4	10 μL
Oligo (dT) RT Primer	1 μL
dNTP Mix	1 μL
Total volum	**12 μL**

7.1.6. Gently mix, briefly centrifuge to collect residual liquid from the tube wall, and place on ice.

7.1.7. Place the tube in a thermocycler，and run the following program（Lid preheated to 105℃）：

Temperature	Time
72℃	5 min
Transfer the tube to ice *	2 min

Note: After the reaction is complete, immediately place the samples on ice for at least 2 minutes.

7.1.8. Prepare the First Strand cDNA Synthesis reaction according to the table below.

Reagent	Volume
Products from step 7.1.7	12 μL
5X RT Buffer	4 μL
TSO Primer II	2 μL
RNase Inhibitor	1 μL
Reverse Transcriptase	1 μL
Total volum	**20 μL**

7.1.9. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube in a thermocycler，and run the following program (Lid preheated to 105℃).

Temperature	Time
42℃	90 min
70℃	10 min
12℃	Hold

7.2. Amplification of full-length cDNA

7.2.1. Thaw the 2X PCR Master Mix, PCR Primer on ice and mix well. Centrifuge them briefly to collect residual liquid from the tube wall, and keep on ice. All the following operations are performed on ice. Prepare the PCR Enrichment reaction according to the table below.

Reagent	Volume

Products from step 7.1.9	20 μL
2X PCR Master Mix	25 μL
PCR Primer	1 μL
Nuclease-free Water	4 μL
Total volum	**50 μL**

7.2.2. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube in a thermocycler，and run the following program (Lid preheated to 105℃).

Temperature	Time	Cycles
98℃	1 min	1 cycle
98℃	10 sec	
67℃	15 sec	8~18 cycles
72℃	3 min	
72℃	5 min	1 cycle
12℃	Hold	

Recommended PCR cycle numbers are as follows:：

Input Total RNA	Input cells	Cycles
10 ng	1000 cells	8-9
1 ng	100 cells	11-12
100 pg	10 cells	14-15
10 pg	1 cell	17-18

Note: The recommended cycle numbers in the table are based on reference data obtained using 293T cells/HeLa cells as templates. In amplification reactions with single cells as starting templates, there is a significant difference in RNA content between different cells, so PCR cycle numbers may vary greatly. Please adjust the amplification cycle numbers according to the samples. When testing a new cell type for the first time, we recommend titrating the PCR cycle numbers.

7.2.3. After the reaction is complete, remove the above products from the PCR and place them on ice immediately. Proceed directly to step 7.3 for purification of the full-length cDNA amplification products. If storage at 4℃ overnight is necessary, do not exceed 12 hours.

7.3. PCR Product Purification

7.3.1. Take out the AFTMag NGS DNA Clean Beads from 2-8℃ in advance and allow them to equilibrate to room temperature. Before use, vortex or mix gently.

7.3.2. Add 30 μL of AFTMag NGS DNA Clean Beads (0.6X) to the reaction tubes from step 7.2.3 and mix thoroughly by pipetting.

7.3.3. Incubate at room temperature for 8 minutes to allow the cDNA to fully bind to the magnetic beads.

7.3.4. Briefly centrifuge the PCR tubes and transfer them to a magnetic rack for ~5 minutes until the solution clears. Carefully discard all of the supernatant.

7.3.5. Keep the centrifuge tubes on the magnetic rack and add 200 µL of 80% ethanol. Incubate for 30 seconds and carefully discard all of the supernatant.

7.3.6. Repeat step 7.3.5, washing the magnetic beads once more with 200 µL of 80% ethanol.

7.3.7. Keep the PCR tubes on the magnetic rack and use a 10 µL pipette to remove all residual liquid.

7.3.8. Dry the beads for 1-2 minutes until the ethanol evaporates completely (the color of the beads changes from shiny brown to matte brown). Remove the PCR tubes from the magnetic rack and add 17 µL of Nuclease-free Water. Mix thoroughly by pipetting.

7.3.9. Incubate at room temperature for 2 minutes. If the beads dry and crack, extend the incubation time appropriately.

7.3.10. Briefly centrifuge the PCR tubes and place them on the magnetic rack for 1 minute until the solution clears. Carefully transfer 16 µL of the supernatant to a new centrifuge tube and store at -20°C for future use.

7.4. Quality control

In general, successful cDNA production should yield between 4 to 20 ng depending on the initial template quantity. The size distribution of full-length cDNA peaks should range from 400-10000 bp, with the main peak averaging around 1500-2000 bp. A negative control without template should show no product (as shown in Figure 2). It is recommended to use the Agilent 2100 Bioanalyzer for detection. Refer to the High Sensitivity DNA Chip operation manual, and take 1 µL of purified cDNA product for analysis on the DNA High Sensitivity Chip.

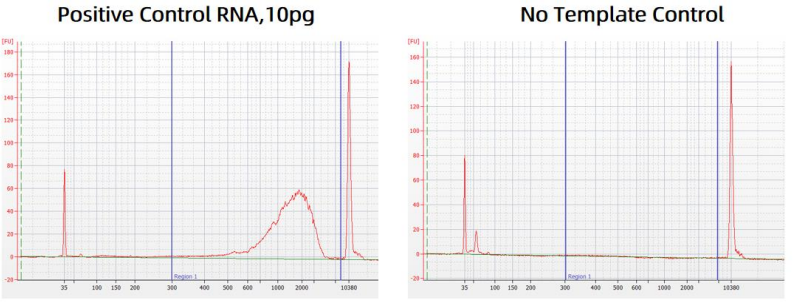

Figure 2. Analysis of full-length cDNA fragment profile

7.5. Library Preparation for Illumina

It is recommended to use 1 ng of full-length cDNA amplification product for cDNA sequencing library preparation using the One-step DNA Lib Prep Kit for Illumina V2 (1 ng Input DNA) (ABclonal, Cat. RK20237).

Experimental procedures can be found in the corresponding instruction manual.

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