Biochemical and Biophysical Research Communications 422 (2012) 643–646

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A simple strand-specific RNA-Seq library preparation protocol combining the Illumina TruSeq RNA and the dUTP methods

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ARTICLE INFO

Article history:
Received 2 May 2012
Available online 15 May 2012

Keywords:
Next generation sequencing
RNA-Seq
TruSeq RNA
Strand-specific sequencing
Transcriptome
Illumina
dUTP

ABSTRACT

Preserving the original RNA orientation information in RNA-Sequencing (RNA-Seq) experiment is essential to the analysis and understanding of the complexity of mammalian transcriptomes. We describe herein a simple, robust, and time-effective protocol for generating strand-specific RNA-seq libraries suited for the Illumina sequencing platform. We modified the Illumina TruSeq RNA sample preparation by implementing the strand specificity feature using the dUTP method. This protocol uses low amounts of starting material and allows a fast processing within two days. It can be easily implemented and requires only few additional reagents to the original Illumina kit.

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1. Introduction

RNA-Sequencing is a widely used and powerful method to explore transcriptomes [1,2]. It enables within a single experiment to investigate the expression levels and structure of transcripts without prior knowledge of the transcriptome content [3,4]. To closely reflect the original cellular RNA content of a sample, the library preparation step is crucial. A particularly relevant aspect in transcriptomics is the information of the transcript orientation, which is key for a comprehensive data analysis. It facilitates the detection of overlapping transcripts coded in opposite orientations, and allows an accurate measure of gene activity levels. Among the methods designed for keeping the original RNA strand information, the deoxy-UTP (dUTP) strand-marking protocol [5] has been rated as leading methodology enabling to identify antisense-transcription [5,6] and has been applied in various setups [7–9]. However, the dUTP-based methods published up to now are laborious and difficult to automate given that they include gel purification steps [5,8] or require time-consuming preparation and calibration of several reagents [7,9]. For these reasons, many high-throughput RNAseq production pipelines are currently using commercial kits, such as the Illumina TruSeq RNA protocol optimized for 0.1 to 4 µg total RNA, and allowing the processing in 96 microtiter plates format. However, a major drawback of the Illumina procedure is the loss of the original RNA strand information. In order to circumvent this problem, we combined here the advantages of the Illumina TruSeq protocol with that of the dUTP protocol by introducing the strand specificity feature in the Illumina method. We modified the original protocol to a simple scalable polyA + library preparation method, which is easy to implement in both small and large-scale operations. In brief, we modified a step in the Second Strand Synthesis by incorporation of dUTP instead of dTTP, which is then selectively degraded after the adapter ligation step. All the other steps of the Illumina original protocol were preserved. The library preparation procedure takes only 2 days.

2. Material and methods

We started with 0.5 µg of DNAse-treated human total RNA in the first step of the TruSeq RNA Sample Preparation v2 protocol (Illumina, Part# 15026495 Rev. A). But any amount of total RNA between 0.1 and 4 µg of total RNA, as recommended in the Illumina protocol can be used. Following reagents are required:

- TruSeq RNA sample preparation Kit v2, Set A (Illumina, #RS-122-2001).
- illustraMicroSpin G-50 Columns (GE Healthcare; #27-5330-02).
- 1 mM Tris pH 8.0 (dilution from 1 M Solution, Ambion, #AM9855G).
- Elution Buffer (Qiagen, #19086).
- 10× Reverse Transcription Buffer (Invitrogen, #53032).

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.043

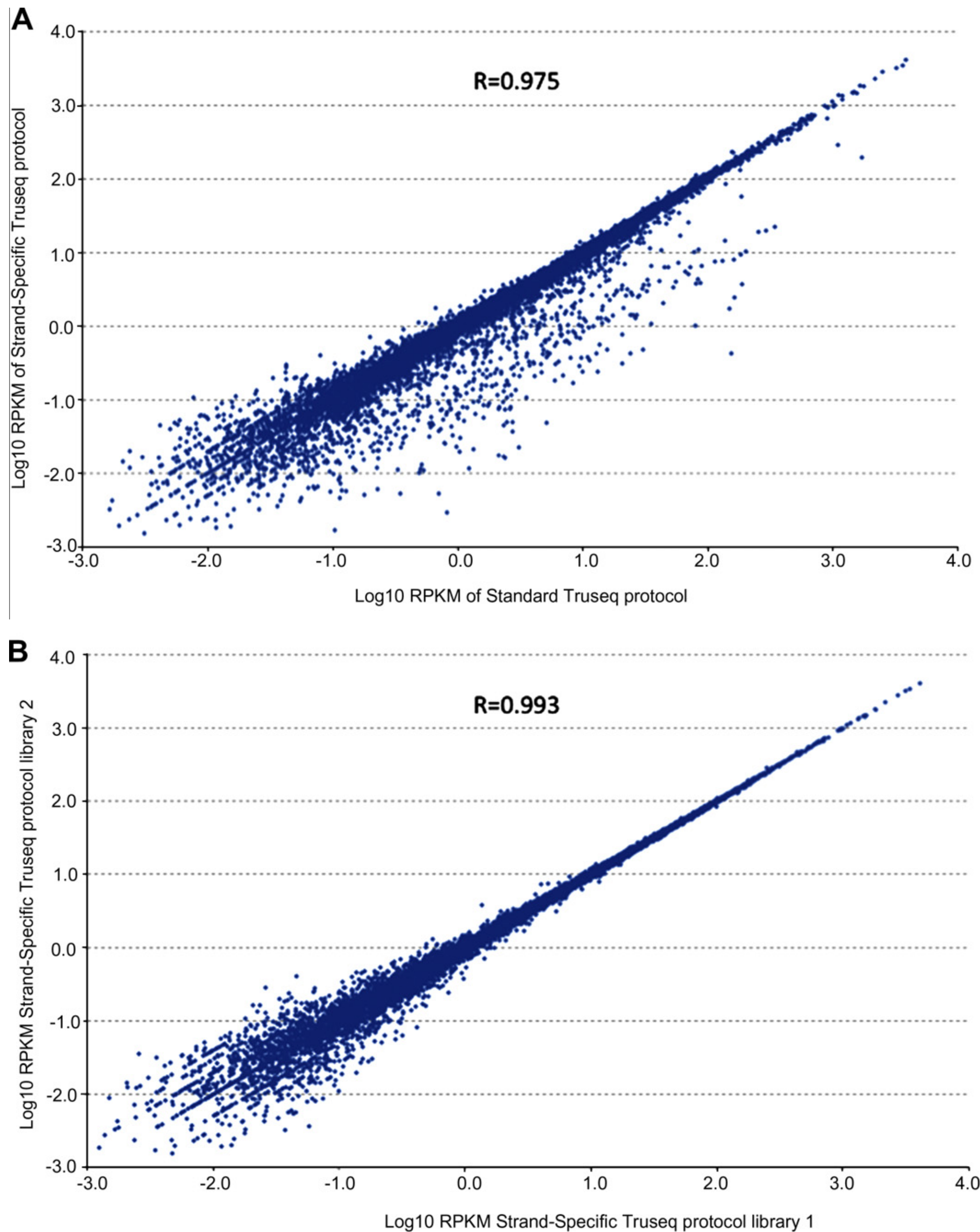

Fig. 1. RPKM scatter plot of 16,470 expressed genes in all three libraries. (A) Scatter plot of the Log10 rpkm values of standard TruSeq protocol (*x*-axis) and Strand specific TruSeq protocol (*y*-axis). (B) Scatter plot of the Log10 rpkm values of two Strand Specific TruSeq libraries. *R* is the correlation coefficient.

- 5× Second Strand Synthesis Buffer (Invitrogen, #11917-010).
- 100 mM DTT (Invitrogen, #11917-010).
- *E.Coli* DNA ligase (10 U/μl) (NEB, #M0205L).
- DNA Polymerase I (10 U/μl) (NEB, #M0209L).
- RNase H (2 U/μl) (Invitrogen, #100004927).
- 100 mM $MgCl_2$ (Dilution from 1 M Solution, Ambion, # AM9530G).
- dUNTP Mix (10 mM each dATP, dCTP, dGTP, dUTP) (Fermentas; #R0146, #R0156,#R0166, #R0133).
- UDGase (1 U/μl) (NEB, #M0280S).
- 10× UDG Buffer (NEB, #B0280S).
- RNase free water

The first steps (1) Purify and Fragment mRNA and (2) First Strand Synthesis were performed as described in the Illumina kit. Step (3) Second Strand cDNA Synthesis was modified as follows:

1. Spin illustra MicroSpin G-50 Columns at 700×*g* for 1 min.
2. Wash Columns three times with 1 mM Tris–HCl pH 8.0:
   i. Add 500 μl Tris–HCl to the column and resuspend the resin by gentle mixing.
   ii. Centrifuge column at 700×*g* for 1 min.
3. Bring the column into a 1.5 ml Low Binding tube.
4. Add 5 μl Elution Buffer to the sample.
5. Add the sample (30 μl) to the G-50 Column and spin the column at 700×*g* for 2 min.
6. Measure the volume of the eluate (should be between 30 and 50 μl).
7. Add RNAse free water to the sample up to a total volume of 52.5 μl.
8. Add Second Strand Mix (22.5 μl) to the sample:

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