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Labeling and Purification of Temporally Expressed RNAs During the S-Phase of the Cell Cycle in Living Cells

Matthieu Meryet-Figuere, Mohamad Moustafa Ali, Santhilal Subhash, and Chandrasekhar Kanduri

Abstract

From high-throughput DNA and RNA sequencing technologies, it is evident that more than two-thirds of the mammalian genome is transcribed and nearly 98% of the transcriptional output in humans constitute noncoding RNA, comprising tens of thousands of small and long noncoding RNAs. These observations have put the study of RNA expression levels at the center of molecular biology research. The transcriptional output of cells changes temporally throughout different cell cycle phases, or in response to a large panel of stimuli. In such instances, the measure of induced RNA transcripts might be obscured by the presence of steady-state RNA levels in the total transcriptome. With this protocol, we provide a method for labeling and purification of the nascent RNAs transcribed over short periods of time in cultured cells. The supplementation of cell culture medium with a chemically modified analog of uridine, ethynyl-uridine, allows for the subsequent biotinylation of ethynyl-uridine residues with a click-chemistry reaction. The labeled RNA is then purified on streptavidin beads and eluted. The purified RNA is suitable for use in RT-qPCR assays as well as in deep sequencing applications.

Key words RNA labeling, Nascent RNA, Cell cycle, S-phase, Next generation sequencing, Temporal gene expression, Nascent RNA sequencing

1 Introduction

The recent decade-long advancement in ultradeep sequencing technologies has revolutionized our understanding of RNA biology, allowing unprecedented accuracy in both qualitative and quantitative studies of the global transcription. In a physiological or pathological condition, the total cellular RNA levels undergo a dynamic fluctuation under various stimuli, owing to the functional coordination between RNA biosynthesis and degradation processes [1, 2]. Thus, the steady-state level of a given transcriptome reflects the accumulated total RNA in a cell at a given time and under a particular condition. However, the ultralow expression levels of some regulatory noncoding RNAs, such as enhancer RNAs,

coupled with the intrinsic short half-life and/or the high turnover rate of various RNA species may not allow for precise quantitation of their expression levels in a total steady-state transcriptome [3–5]. Moreover, the differential expression analyses of steady-state RNA levels may not be able to recapitulate the rapid and specific transcriptional events occurring in response to a given stimulus [5, 6].

In order to overcome the challenges of steady-state RNA sequencing, various technologies have been developed to capture nascent RNAs associated with the active transcriptional machinery in real-time. Most of these modern technologies rely on the original Nuclear Run-On (NRO) technique with various modifications [7, 8]. For example, Genomic Run-On coupled with high-throughput sequencing (GRO-seq) utilizes bromouridine analog (Br-UTP) to label the nascent RNAs during transcriptional elongation in detergent-treated nuclei. The labeled RNAs can be captured through immunopurification and subsequently subjected to deep sequencing to map the positions of actively engaged RNA polymerases [9]. Similarly, NRO-seq uses biotinylated uridine analog (Bio-UTP) to label and retrieve nascent RNAs with streptavidin-coated beads [10]. Further techniques have also been optimized to enhance the sequencing resolution, such as PRO-seq and PRO-cap [11]. In addition to the latter, a couple of antibody-based purification methods Mammalian NET-seq [12] and ChRO-seq [3] have been developed to capture the actively transcribing RNA polymerase complex. Despite the high resolution of recent Nuclear Run-On and other antibody-based methods, obtaining suitable nuclei for the run-on reactions is technically challenging, and the limited specificity of antibodies for immunoprecipitation may restrict the success of these technologies. Therefore, the introduction of a technique that labels RNA in living cells in normal cell culture conditions, and eliminates the use of antibody-based purification is of prime importance.

In the present protocol, we devised a method for the metabolic labeling and subsequent purification of nascent RNAs transcribed over short periods of time in cultured cells. The labeling of nascent RNA molecules relies on the incorporation of the modified ethynyl-uridine (EtU) residues into the actively transcribed RNAs. RNA molecules labeled with EtU undergo a copper-catalyzed click-chemistry reaction in the presence of biotin-tagged azide moiety. The labeled RNAs can be retrieved using streptavidin-coated beads for further downstream applications. This method was used successfully to characterize temporal transcription events during different stages (early, mid and late stages) of the S-phase of cell cycle in HeLa cells [13], which led to the identification of several lncRNAs of interest in the context of cancer studies [14]. Our protocol is easily adaptable to explore the other subphases of cell cycle or to monitor the early transcriptional response to any stimuli of interest applied to cultured cells.

2 Materials

2.1 Cell Culture

1. Growth medium: MEM medium supplemented with 10% FBS (heat-inactivated, 55 °C 5 min), 4 mM glutamine, antibiotics (penicillin + streptomycin).
2. Humidified incubator with 5% CO₂ at 37 °C.
3. 1X PBS.
4. Thymidine medium: growth media supplemented with 2 mM thymidine, prepare just before use.
5. Hydroxyurea medium: growth media supplemented with 1 mM hydroxyurea, prepare just before use.

2.2 Flow Cytometry

1. 1X PBS.
2. Trypsin.
3. Growth media.
4. 100% ethanol.
5. Vortex.
6. DNA staining solution: 1X PBS supplemented with 200 µg/ml RNaseA and 50 µg/ml propidium iodide.
7. Flow cytometer to assess DNA content of cells and characterize the cell cycle profiles of cells.

2.3 Nascent RNAs Labeling with 5-Ethynyl-Uridine

1. Growth medium.
2. DMSO.
3. EtU medium: growth medium supplemented with 1 mM ethynyl-uridine (EtU) (Invitrogen). EtU is supplied as a powder and solubilized in DMSO; appropriate volume of EtU in DMSO is added to growth medium to reach the indicated final 1 mM EtU concentration. Prepare just before use.
4. DMSO medium: growth media supplemented with DMSO, in the same proportion as in EtU medium. Prepare just before use.
5. 1X PBS.

2.4 EtU-Labeled RNA Biotinylation and Staining on Fixed Cells

1. Cytospin device to transfer cells in suspension onto a microscope slide.
2. 1X PBS.
3. 3.7% formaldehyde in PBS.
4. 0.1 M glycine in PBS.
5. Permeabilizing solution: 0.1% Triton X-100 in PBS (PBS-T). Prepare just before use.

6. Blocking solution: 0.1% Triton X-100, 1% BSA, in PBS. Prepare just before use.
7. RNaseA solution: 400 µg/ml RNaseA in PBS.
8. Click-iT cell reaction buffer kit (Invitrogen).
9. Biotin-azide.
10. Avidin staining solution: avidin coupled with Texas-Red (Vector Labs) at 1:500 dilution in blocking solution. Prepare just before use.
11. DAPI solution: DAPI 100 µg/ml in PBS.
12. Vectashield Hard Set Mounting Medium (Vector labs).

2.5 EtU-Labeled RNA Biotinylation for RT-qPCR and NGS Applications

1. Tri-reagent, 100% isopropanol, and chloroform.
2. RNase-free DEPC-treated Milli-Q water.
3. 70% ethanol (100% ethanol diluted to 70% with RNase-free water).
4. RQ1 DNase (Promega, supplied with 10X DNase buffer).
5. NanoDrop spectrophotometer.
6. RiboMinus Eukaryote rRNA depletion kit (Thermo Fisher Scientific).
7. 3 M sodium acetate, pH 5.5.
8. Biotin-azide.
9. Click-iT nascent RNA Capture Kit (Invitrogen).
10. Vortex mixer.
11. Elution buffer: 2 mM Biotin, 1 M NaCl, 50 mM MOPS, 5 mM EDTA, 2 M 2-mercaptoethanol, pH 7.4, in RNase-free water. Prepare just before use.
12. GlycoBlue (Invitrogen).
13. 100% ethanol.
14. Qubit fluorometer.
15. Bioanalyzer 2100 with RNA 6000 pico kit.

3 Methods

3.1 Cell Synchronization

1. For each time point as well as controls, prepare an individual flask by seeding HeLa cells in T75 flasks to reach a confluency of 25–35%. Make sure that cells are individualized to avoid contact inhibition and ensure correct synchronization throughout the whole experiment (*see Note 1*).
2. Next day, replace the growth medium with a fresh thymidine medium and incubate for 10 h. Discard the medium, wash once

with 37 °C pre-heated 1X PBS, and add a fresh hydroxyurea medium for 12 h.

3. At this stage, cells are synchronized at the early boundary of the S-phase. Observation with a microscope at 20X magnification should reveal individualized cells with a slightly bigger size than control unsynchronized cells. One flask of each synchronized and unsynchronized cells will advance to next step to analyze cell cycle distribution and check the homogeneity of synchronized cells versus the unsynchronized control. The rest of the flasks will proceed directly to Subheading 3.2.
4. Remove growth media from each flask of synchronized and unsynchronized cells set aside for synchronization control, and wash once with 1X PBS. Incubate in trypsin at 37 °C for 5 min and add fresh growth media to stop trypsin action, harvest cells, centrifuge at $2000 \times g$ for 5 min. Remove supernatant and resuspend cell pellet in PBS. Add 100% ethanol volume to reach a final 70% ethanol concentration. Vortex cells well to ensure single cell suspension.
5. Store fixed cells in 70% ethanol at -20 °C where they can stay up to several weeks. These samples will be analyzed for their cell cycle phase together with the samples from EtU RNA-labeled and RNA-unlabeled samples harvested from different stages of the S-phase, at **step 7** in Subheading 3.2.

3.2 Nascent RNAs Labeling

1. At the end of synchronization, cells are arrested at the very beginning of S-phase. Discard hydroxyurea medium, wash once with 1X PBS and add fresh growth medium to the cells to release the block and allow them to progress into the S-phase of the cell cycle.
2. To cover the transcriptional events at the early, mid and late stages of the S-phase of the cell cycle, we labeled nascent RNA for overlapping periods of 2 h covering S-phase. The starting point is demarcated as T0 at the release from the block. The overlapping time points represent 0 h–2 h, 1.5 h–3.5 h, and 3 h–5 h (Fig. 1); with 5 h being the end of S-phase for HeLa cells. For any labeling period, the process is similar. At the beginning of the labeling period, replace growth medium with EtU medium. In control flasks, replace growth media with DMSO containing media and harvested RNA from this constitute unlabeled RNA.
3. At the end of the labeling period, harvest both labeled and unlabeled cells separately. Resuspend each cell pellet in PBS and divide the cells into three fractions; 20,000 cells to check the cells' position in cell cycle at the time of harvesting (**step 4** Subheading 3.2), 5000 cells to confirm the proper RNA labeling using immunostaining (**step 5** Subheading 3.2), rest of the

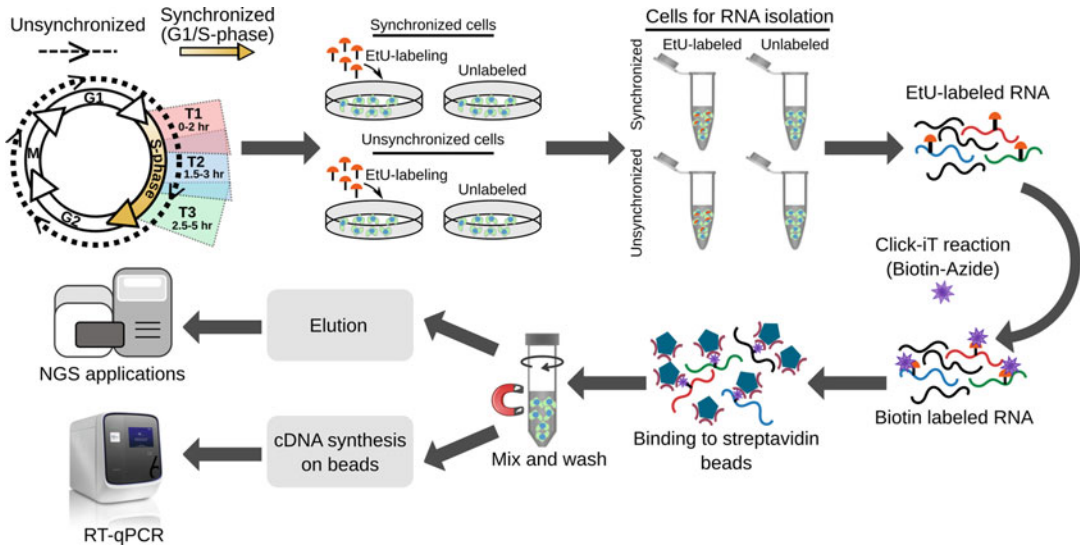


Fig. 1 Schematic representation of time-period labeling over S-phase. EtU-labeled and Unlabeled samples are prepared. Following RNA extraction, EtU-labeled RNA is biotin-labeled with click-chemistry reaction and purified on streptavidin beads for indicated downstream analysis

cells for labeled nascent RNA purification (**step 6** Subheading 3.2), *see Note 2*.

4. To the 20,000 cells fraction, add 100% ethanol volume to a final 70% ethanol concentration. Vortex cells well and store at -20°C for flow cytometry analysis. Samples in 70% ethanol can be stored at -20°C for several weeks.
5. To confirm EtU incorporation into RNA, transfer 5000 cells on to a microscopic slide using a CytoSpin device by spin down for 5 min at 800 rpm. Fix cells in 3.7% formaldehyde in PBS for 15 min. Wash twice in 1X PBS and quench formaldehyde-induced auto-fluorescence by incubating in 0.1 M glycine in PBS for 30 min at RT followed by one time wash with 1X PBS. Slides can be kept for 24–48 h at 4°C in 1X PBS.
6. Spin down the cell fraction designated for nascent RNA purification at $2000 \times g$ for 5 min and discard supernatant. Cell pellet can be stored at -80°C for several weeks.
7. To confirm an accurate stage of S phase in which cells are positioned, spin down samples from **step 5** Subheading 3.1 and **step 4** Subheading 3.2 at $2000 \times g$ for 5 min and discard supernatant. Resuspend cell pellets in 3 ml 1X PBS and incubate at 37°C for 30 min. Centrifuge cells at $2000 \times g$ for 5 min and remove supernatant. Resuspend cell pellet in DNA staining solution (PBS supplemented with 200 $\mu\text{g}/\text{ml}$ RNaseA and 50 $\mu\text{g}/\text{ml}$ propidium iodide) and incubate for 2 h at 4°C in the dark. Analyze the DNA content of cells on a flow cytometer to assess their position in the S-phase of cell cycle. The results

should show desired cell synchronization at all analyzed time-points with identical profiles for labeled and unlabeled controls.

3.3 EtU-Labeled RNA Biotinylation and Staining on Fixed Cells

1. Permeabilize the cells fixed on slides from **step 5** Subheading **3.2** by incubating in permeabilizing solution for 10 min at RT. Incubate the slides in blocking solution for 1 h at RT.
2. To check the specificity of EtU incorporation into RNA, treat one of the fixed slides with RNaseA solution for 30 min at 37 °C prior to incubation in blocking solution. Wash the RNaseA treated slide twice in 1X PBS at RT. Make sure not to contaminate the other slides and reagents with RNase A during the remaining steps of the experiment.
3. Perform a Click-iT reaction on slides according to “Click-iT Cell Reaction Buffer Kit” protocol. Use 5 μM final concentration of biotin-azide. Prepare 300 μl of reaction cocktail per slide, incubate for 30 min at RT in a humidified chamber. During the process of Click-iT reaction, the biotin-azide molecules are added to EtU residues incorporated in RNA (Fig. 1).
4. Terminate the Click-IT reaction by aspirating the reaction cocktail and wash slides three times in the dark in blocking solution. Incubate in avidin staining solution for 30 min in a humidified chamber at RT. Wash three times in blocking solution for 10 min and with 1X PBS for 15 min at RT. Incubate cells in DAPI solution in 1X PBS for 15 min at RT to stain nuclei. Wash slides three times in 1X PBS for 10 min and mount them with Vectashield hardset mounting medium and keep them overnight at 4 °C in the dark to dry. Observe these slides under fluorescence microscope for nascent RNAs staining, *see Note 3*.

3.4 EtU-Labeled RNA Biotinylation for RT-qPCR and NGS Applications

1. Lyse cell pellets from **step 6** Subheading **3.2** by adding 1 ml of Tri-reagent. Proceed to RNA extraction according to manufacturer’s protocol. Resuspend the RNA pellet in 42 μl of RNase-free water. Digest residual DNA by adding 2 μl of RQ1 DNase and 5 μl 10× DNase buffer and incubate at 37 °C for 1 h. Stop the DNase reaction by adding 1 ml of Tri-reagent and proceed to extraction of RNA. The RNA pellet in 70% ethanol can be stored at –80 °C up to several weeks.
2. Resuspend the RNA pellet in 12 μl of RNase-free water and dose RNA concentration on a NanoDrop spectrophotometer. Use 10 μl of the RNA solution (up to 10 μg, *see Note 4*) as an input for ribosomal RNA depletion with RiboMinus kit, following manufacturer’s recommendations. The RNA, depleted from rRNA, can be stored at –80 °C up to several weeks.

3. Resuspend the RNA pellet in 16 μl of RNase-free (DEPC-treated) water and dose RNA concentration on a NanoDrop spectrophotometer. About 2–4 μg is typically recovered after rRNA depletion from 10 μg of total RNA. Label rRNA-depleted RNA with biotin using “Click-it Nascent RNA Capture Kit” following manufacturer’s protocol and the following parameters: 0.5 mM azide-biotin concentration in 50 μl reaction volume per sample. Do not resuspend precipitated RNA pellet at this stage. The RNA pellet can be stored at $-80\text{ }^{\circ}\text{C}$ up to several weeks.

3.5 Purification of EtU-Labeled RNA

1. Resuspend the RNA pellet in 50 μl of RNase-free water. Since the whole sample will be subjected to pull-down, RNA dosage on NanoDrop at this step is optional. Proceed to the pull-down of biotinylated RNA with the “Click-it Nascent RNA Capture Kit” following manufacturer’s protocol with the following modifications: *see* **Notes 5–7**.
2. Elute RNA from streptavidin beads in 200 μl elution buffer at $95\text{ }^{\circ}\text{C}$ for 3 min. This incubation ensures both RNA elution and RNA fragmentation for NGS applications. Remove elution buffer immediately and cool it at $4\text{ }^{\circ}\text{C}$. Precipitate RNA by adding 1 μl GlycoBlue, 20 μl 3 M sodium acetate (pH 5.2), and 500 μl 100% ethanol and incubate overnight at $-20\text{ }^{\circ}\text{C}$. Centrifuge for 20 min at $16,000 \times g$. Wash the pellet in 70% ethanol and centrifuge for 5 min at $2000 \times g$. Repeat the ethanol wash and spin down again. The RNA pellet can be stored at $-20\text{ }^{\circ}\text{C}$ up to several weeks. Resuspend the RNA pellet in 20 μl of RNase-free water. Dose the RNA concentration on a Qubit fluorometer, suitable for the quantification of low concentration of RNA. Assess RNA size distribution on a Bioanalyzer device using the RNA Pico kit according to manufacturer’s protocol. Typically, RNA size distribution should be around 200–300 nt, and the recovered amount of labeled RNA should be around 10–50 ng.

3.6 Transcriptome Sequencing and RNA-seq Analysis

1. For each time-point, a strand-specific library preparation is highly recommended to efficiently detect antisense transcripts. The constructed libraries are subjected to deep-sequencing on a suitable platform.
2. The alignment of sequencing reads to an assembled human genome can be done using splice-aware transcriptome-based aligners such as HISAT2, STAR, or Tophat.
3. Following the alignment process, the number of reads per annotated transcripts can be quantified using featureCounts from “subread package” or HTSeq-count from “HTSeq package.” An appropriate mapping quality cutoff should be considered by avoiding multimapping reads.

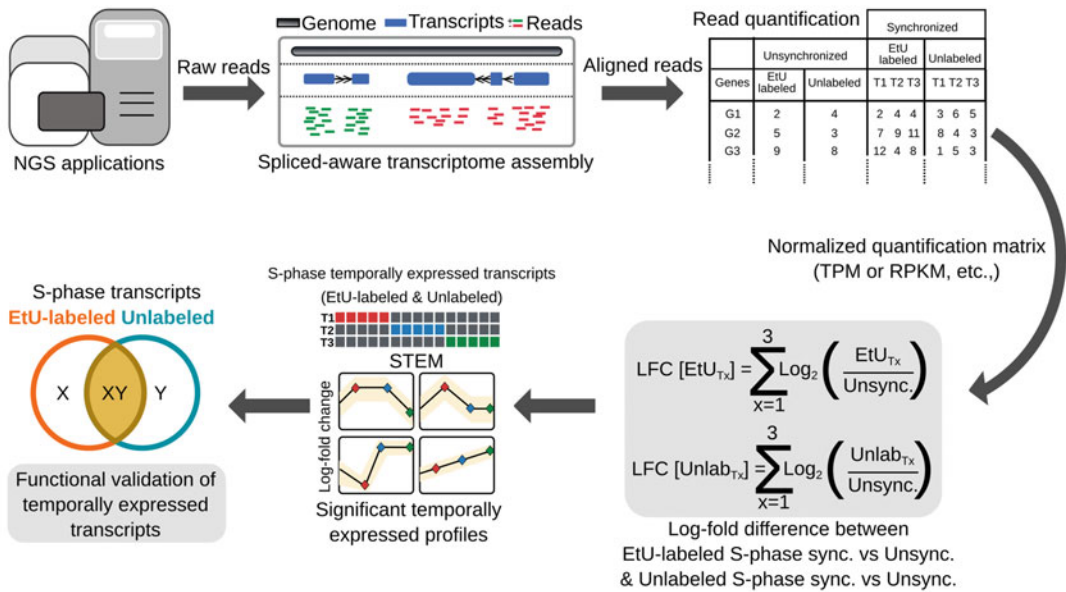


Fig. 2 Computational analysis workflow to identify S-phase temporally expressed transcripts. Workflow represents assembling raw reads to the transcripts from reference genome followed by transcript-wise read quantification for individual samples. Obtained quantification matrix are normalized using available normalization methods to calculate log-fold differences between S-phase synchronized samples (individual time-points) over unsynchronized samples. These normalized profiles are subjected to STEM analysis to find transcripts having significant temporal expression patterns. EtU-labeled transcripts having significant temporal expression patterns are then used for further functional validation experiments

- The obtained reads per transcript have to be normalized using Reads Per Kilobase Million (RPKM) with respect to transcript length and the library size (total number of reads) per sample. Other similar normalization methods can be implemented for this purpose such as TPM (Transcript Per kilobase Million).
- By comparing normalized read (expression) values from individual time-points of synchronized samples (both EtU-labeled and unlabeled) over unsynchronized samples, fold changes can be calculated and transformed to logarithmic (base 2) fold change ($\log_2\text{FC}$) values.
- Transcripts with significant fold difference can be used for further analysis using Short Time-series Expression Miner (STEM) clustering (Fig. 2). STEM is specifically designed to find significant temporal expression patterns among short time-series gene expression datasets. Transcripts with significant temporal expression patterns can be chosen for further functional experiments.

4 Notes

1. The cell cycle synchronization protocol is optimized for HeLa cells and the protocol can be easily adapted to other cancer cell lines with minor modifications. For example, single or double thymidine block can be used for cell synchronization, and the duration of block with these chemicals can be adjusted. As a general rule, one should always make sure that cells remain individualized from plating to harvesting, to avoid confluence-induced cell cycle exit. We advise a thorough examination of the cell cycle distribution over time with various synchronization methods to ensure a robust synchronization method before starting with the labeling experiments. Non-chemical methods such as mitotic shake-off or cell-sorting based on DNA content have also been described, but the low yield of cells in the desired phase or sub-phase of the cell cycle requires scaling up the experiment to a wide extent to get enough material for downstream analysis.
2. After ethynyl uridine supplemented medium is removed, labeled RNA transcripts will start to decay, depending on their respective half-lives and the newly transcribed RNAs will not be labeled. In order to minimize the loss of information, RNA from EtU labeled cells should be processed as fast as possible.
3. Because 2 h of EtU-incubation period is relatively short, only a small fraction of the total RNA is labeled. However, the fluorescent signal from EtU labeling is strong enough to be visible by fluorescence microscope. If signal is invisible, very low amounts of labeled RNA can be expected at the end of the experiment which might not be enough for downstream applications.
4. Due to the low proportion of labeled RNA in a two-hour period, and the low yield of RNA recovery at the end of the protocol, we strongly advice to scale up the experiments to obtain 10 μg RNA as input for RiboMinus reaction.
5. Use the whole RNA sample for pull-down, which should be in the range of 1–10 μg RNA. However, do not use more than 50 μl bead volume, because only a small fraction of RNA is labeled with the short (2 h) incubation times of ethynyl uridine.
6. During the 30 min incubation time on a vortex mixer (not tube rotator), make sure the beads remain suspended throughout the incubation time by gently pipetting the sample up and down every 10 min.
7. cDNA synthesis, for downstream NGS applications, can be directly performed on beads. However, these preparations

often gave poor quality RNA libraries. Therefore do not proceed to cDNA synthesis on the biotinylated RNA bound to streptavidin beads, instead proceed immediately to RNA elution, it is of primary importance for downstream NGS applications. However, if the sample is to be used for RT-qPCR, cDNA synthesis can be performed on beads. To perform RT on beads, we use Superscript II (SSII) according to manufacturer's instructions in 20 μ l reaction volume, except that we maintain beads in suspension by incubating in a vortex mixer (avoid tube rotator devices).

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