For Research Use Only. Not for use in diagnostic procedures.

**RiboCluster Profiler™**

**BRIC Kit**

20 assays

CODE No. RN1007/RN1008
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1. Introduction

Please read these instructions carefully before beginning the assay.

It is very important to isolate “high-quality BrU-labeled RNA” from various materials to validate experiments such as reverse transcription quantitative polymerase chain reaction (RT-qPCR) and gene expression analysis based on deep sequencing technology because experimental results may be sensitive to RNA quality. In order to obtain “high-quality BrU-labeled RNA”, and reduce the chance of RNase contamination, gloves should be worn when proceeding BRIC, and RNase-free microcentrifuge tubes and pipette tips should be used for the assay.

1. Background and Introduction

Regulation of gene expression by RNA degradation is a critical step for the coordination of various physiological processes. Also, RNA degradation pathways play important roles in mRNA surveillance systems: nonsense-mediated mRNA decay (NMD), nonstop mRNA decay (NSD) and no-go decay (NGD). In order to analyze RNA degradation, transcriptional inhibitor such as Actinomycin D (ActD) has been widely used so far. However, the transcriptional inhibitors are known to influence cellular physiology, including splicing, polyadenylation and other mRNA processing events. Furthermore, ActD alters the localization and stability of RNAs, especially in the case of long noncoding RNA (lncRNA).

Recently, genome-wide RNA decay analysis in mammalian cells under inhibitor-free condition has been developed. To perform the analysis, endogenous RNA should be transcriptionally pulse-labeled with modified nucleosides, such as 4-thiouridine (4sU), 5-ethynyl uridine (EU) and 5-bromouridine (BrU), followed by purification of newly labeled RNA. Each modified uridine has both advantages and disadvantages in this analysis. BrU is less harmful for cells than other modified uridines and BrU-labeled RNA can be easily immunoprecipitated by anti-bromodeoxyuridine (BrdU) antibody through its cross-reactivity, while BrU-labeling takes 24 hours for effective cellular incorporation. In contrast to immunopurification by anti-BrdU antibody, another method, which depends on “click chemistry” utilizing EU-labeled RNA, requires biotinylation of the labeled RNA to specifically collect the biotin-labeled RNA by streptavidin beads. There is a crucial problem that the biotinylation step via click reaction can be a trigger for RNA degradation. In the case of 4sU, 4sU permits base-pairing with guanine instead of adenine, which causes base-changes in the RNA sequence. Unlike EU- and 4sU-based analyses, 5-bromouridine immunoprecipitation chase (BRIC) method has no undesirable effects for downstream applications. Thus, BRIC will be the most suitable method for the analysis of RNA decay.

It is thought that the half-life of RNA closely relates to its own physiological function. mRNAs derived from major housekeeping genes generally have long half-lives, while mRNAs of many regulatory genes, which encode functional proteins required for only a limited time in cell – such as cell cycle regulators, cytokines, transcriptional factors and oncoproteins – typically have short half-lives. In addition, the genome-wide analysis by BRIC-deep sequencing (BRIC-seq) has lately revealed that majority of non-coding RNAs (ncRNAs) with long half-lives involved in housekeeping functions, whereas the subsets of ncRNAs with short half-lives included known regulatory ncRNAs. The result implies that the subsets of
ncRNAs may play central roles in the important biological processes. Thus, BRIC can be a powerful tool for analysis of RNA metabolism which will lead us to the discovery of novel ncRNA biomarkers and drug targets.

2. Product Description

**BRIC Kit** enables determination of RNA stability by chasing chronological decreases of BrU-labeled RNA under physiologically undisturbed conditions. In the BRIC protocol, cells are pulse-labeled with BrU for constant period and washed with PBS to remove the BrU-containing medium, and then cells are chronologically harvested, followed by preparation of total RNA including newly BrU-labeled RNA. The BrU-labeled RNA can be specifically immunoprecipitated with *Anti-BrdU mAb* provided by MBL, followed by isolation of BrU-labeled RNA from immunocomplex on carrier material, such as protein G magnetic beads. The isolated BrU-labeled RNA can be analyzed to determine its own stability and half-life by various methods in molecular biology – RT-qPCR, deep sequencing or microarray.

It is reported that the RNA labeling efficiency of BrU is less effective than that of EU in HeLa cells under serum-starved condition. On the other hand, we confirmed that *Anti-BrdU mAb* (MBL) can immunoprecipitate both BrU- and EU-labeled RNAs because of its cross-reactivity. Thus, RNA stability can be analyzed by both BrU and EU with BRIC Kit.

[References]

3. Kit Components

20 assays

**[RN1008 / Store at 2-8°C]**

1. RNA-IP buffer 18 mL × 2 bottles
2. Wash buffer 41 mL × 3 bottles
3. mi-Solution I 240 μL × 1 vial: enzyme solution
4. mi-Solution II† 5.8 mL × 1 vial: diluent for Solution I
5. mi-Solution III‡ 3.6 mL × 1 vial: protein dissolvent
   Solution III can dissolve proteins and dissociate immunocomplex.
6. mi-Solution IV 90 μL × 1 vial: co-precipitator
   Solution IV can increase RNA precipitation efficiently.
7. Protein G-Magnetic beads* 1.5 mL × 4 vial
   1% beads slurry (mouse IgG binding capacity: 7 μg/mg beads)
4. Storage and Stability

The component 10 of BRIC Kit (Spike-in control) should be divided into appropriate aliquots to avoid freeze-thaw cycles when you receive, and stored at -80ºC or below until just before use because the component 10 is an RNA synthesized by in vitro transcription. The components 8 (BrU solution) and 9 (Anti-BrdU mAb) should be stored at -20ºC or below. Other components should be stored at 4ºC (Do not freeze). These components are stable for one and a half years from the date of manufacture when stored at the indicated conditions.

5. Materials Required but Not Provided

Equipments
1. Microcentrifuge capable of 15,000 × g
2. Microcentrifuge tubes (1.5 mL or 2 mL) (Nuclease-free)
   (Recommendation; use low-adhesion tube for RNA-IP)
3. Centrifuge capable of 2,000 × g
4. Centrifuge tubes (15 mL or 50 mL)
5. Pipettes (5 mL, 10 mL or 25 mL) (Nuclease-free)
6. Pipette tips (10 µL, 20-100 µL, 200 µL, and 1,000 µL) (Nuclease-free) (Recommendation; use low-adhesion pipette tip for RNA-IP)
7. Ultra low temperature freezer (-80°C)
8. Freezer (below -20°C)
9. End-over-end rotator
10. Vortex mixer
11. Magnetic stand
12. Gloves

Reagents
13. RNase inhibitor*
14. Dithiothreitol (DTT)*
15. 100% Ethanol (molecular biology grade)
16. 100% 2-Propanol (molecular biology grade)
17. Nuclease-free PBS
18. Nuclease-free water
19. Reagents for reverse transcription**
20. Primer set for Spike-in control***
21. Reagents for qPCR****

Note:  * Recommended concentration of each reagent is shown in Appendix.
** Commercially available reagents confirmed to work with BRIC Kit are shown in Appendix.
*** The recommended primer sequences to analyze the Spike-in control levels by RT-qPCR are described below:

Forward primer : 5’-AACTCTGGCTCACAGTACGC-3’
Reverse primer : 5’-TGGCGGTTAATTGCCAACG-3’

**** Commercially available reagents confirmed to work with BRIC Kit are shown in Related Products.
II. BRIC Kit Procedure

1. Procedure Summary

Overview of entire process

Cell

Pulse-labeling with BrU

Harvesting cells at each time point

Total RNA isolation

Immunopurification

Spike in BrU-labeled RNA (Spike-in control)

Anti-BrdU mAb

Wash / Elution

RT-qPCR / deep sequencing / microarray
BRIC process by step

Metabolic labeling of RNA with BrU

Seed cells at 1-2 x 10^6 cells/10 cm dishes

Add 100 μL of 100x concentrated BrU solution to 10 mL of culture medium, mix, incubate at 37°C with 5% CO₂ for 24 hours

Wash & Change medium (Removal of BrU)

Wash & Harvest at each time points (e.g., 0, 4, 8, 12, 24 hours)

0 hour  4 hours  8 hours  12 hours  24 hours

RNA extraction

Total RNA

• BrU-labeled RNA (Large)
• BrU-labeled RNA (Small)
• Non-labeled RNA

Proceed to RNA-IP: Step C
BRIC process by step-cont.

**RNA-IP: Step A**
Preparation of antibody-immobilized beads

1. Dispense Protein G-Magnetic beads
2. Magnetic separation & Add Wash buffer (+) & mix
3. Magnetic separation & Aspirate the sup.
4. Dispense Anti-BrdU mAb
5. Mix & Incubate for 30 min at 4°C

Proceed to Step C

**RNA-IP: Step B**
Washing antibody-immobilized Protein G magnetic beads

- Add RNA-IP buffer (+)

Proceed to Step C

**RNA-IP: Step C**
Preparation of antibody-immobilized beads-RNA complex

1. Spike-in control
2. Input total RNA
   - : BrdU-labeled RNA (Large)
   - : BrdU-labeled RNA (Small)
   - : Non-labeled RNA
3. Denature for 2 min at 80°C & Quench for 5 min on ice
4. Transfer the Input RNA to the tube prepared in Step B
5. Mix & Incubate for 3 h at 4°C

**RNA-IP: Step D**
Washing antibody-immobilized beads-RNA complex

- Magnetic separation & Aspirate the sup.

- Wash 4 times with 1 mL of Wash buffer (+)

- Magnetic separation & Aspirate the sup.

Proceed to RNA Isolation
BRIC process by step-cont.

RNA Isolation

Separation method

Add Master mix solution & Add mi-Solution III

Vortex, spin down & Magnetic separation

Transfer the sup. to tube containing 2 µL of mi-Solution IV

mi-Solution IV

Add ice-cold 2-Propanol, mix, Incubate for 20 min at -20°C or below, then centrifuge

Supernatant (Small RNAs)

Transfer the sup. to tube containing 2 µL of mi-Solution IV

mi-Solution IV

Add ice-cold 2-Propanol, mix, Incubate for 20 min at -20°C or below, then centrifuge

Pellet (Large RNAs)

Rinse twice with ice-cold 70% Ethanol, then centrifuge

Dry up precipitates & Reconstitute in Nuclease-free Water

BrU-labeled large RNAs (mRNAs, etc.)

Aspirate the sup. & Rinse twice with ice-cold 70% Ethanol, then centrifuge

Dry up precipitates & Reconstitute in Nuclease-free Water

BrU-labeled small RNAs (miRNAs, etc.)

Proceed to Analysis of target RNAs

Analysis of target RNAs

Deep sequencing

RT-qPCR

Microarray
2. Buffer Preparation

1. RNA-IP buffer
   Add appropriate concentrations of RNase inhibitor and dithiothreitol (DTT) to RNA-IP buffer just before use. RNA-IP buffer containing these reagents is described as **RNA-IP buffer (+)** in the following protocols. The optimal concentration of each reagent for BRIC is shown in Appendix.

2. Wash buffer
   Add appropriate concentration of dithiothreitol (DTT) to Wash buffer just before use. Wash buffer containing DTT is described as **Wash buffer (+)** in the following protocols. The optimal concentration of the reagent for BRIC is shown in Appendix.

3. Protocols for 5-Bromouridine Immunoprecipitation Chase (BRIC)
   The following protocol is for the RNA decay analysis by BRIC: labeling various cell lines with BrU, extracting total RNA from BrU-labeled cells, immunoprecipitating BrU-labeled RNA using Anti-BrdU mAb and isolating BrU-labeled RNA. The cell harvest should be performed at several time points after changing medium to analyze the RNA half-lives [e.g., 0, 4, 8, 12 and 24 hours]. The quantity and quality of isolated BrU-labeled RNAs can be checked by a spectrophotometer and/or Bioanalyzer. Then, the stabilities and half-lives of RNAs can be analyzed by deep sequencing, RT-qPCR and microarray.

   ◆ **Metabolic labeling of RNA with BrU**
      Note: In some cell lines, BrU-labeling efficiency into newly synthesized RNA depends on the cell conditions. In order to obtain reproducible results, prior to BRIC procedure, continue to culture the cryopreserved cells for about 1 week until the viability is recovered and pay much attention on the overgrowth of the cells. Labeling efficiencies of several cell lines are described in the table below.

<table>
<thead>
<tr>
<th>Labelling efficiency</th>
<th>Cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>HeLa, K562</td>
</tr>
<tr>
<td>Moderate</td>
<td>293T, HT29</td>
</tr>
<tr>
<td>Low*</td>
<td>Jurkat, HEK293, T-47D, PC3</td>
</tr>
<tr>
<td>Very Low**</td>
<td>MCF-7, ZR-75-1, KATO III, MEG-01, WERI-Rb-1</td>
</tr>
</tbody>
</table>

   Note: * By labelling with 500 µM BrU, BrU-labeled RNA can be quantified with NanoDrop.
      ** It may be difficult to analyze the RNA stability in these cell lines because BrU-labeled RNAs prepared from these cell lines pulse-labeled with 500 µM BrU could not be quantified with NanoDrop.

   Make 100× concentrated BrU solution by diluting 100 mM BrU stock solution (component 8) in nuclease free PBS (e.g., dilute 100 mM BrU stock solution to 15 mM with nuclease-free PBS).

   Note: For pulse-labeling the cell, the recommended final concentration of BrU in culture media is 150 µM. If desired, higher concentrations of BrU are also available for BRIC, but please note that higher concentrations of BrU can be toxic to cell. Pulse-labeling with 500 µM of BrU is more efficient, but this concentration may affect growth rate of cells and/or other biological processes.
2. Seed cells at 1–2 × 10^6 cells/10 cm dishes.
   **Note:** Timing of pulse-labeling might affect the BrU-labeling efficiency of RNA in some cell lines. If desired, pre-culture the cells in a humidified incubator at 37°C with 5% CO₂ for 24 hours in order to more stabilize the cellular conditions. Please pay much attention on the overgrowth of the cells.
3. Add 100 µL of 100× concentrated BrU solution to 10 mL of culture medium, then gently mix.
   **Note:** The recommended final BrU concentration, 150 µM, is mild condition for the cells and exhibits moderate pulse-labeling efficiency.
4. Incubate the cells in a humidified incubator at 37°C with 5% CO₂ for 24 hours. Protect from intense light exposure.
5. Remove BrU-containing medium (pulse media), and gently wash the cells 3 times with 5 mL of PBS.
6. Remove PBS, and add 10 mL of fresh medium (chase media).
7. Incubate the cells at 37°C with 5% CO₂ for appropriate hours [e.g., 0, 4, 8, 12 and 24 hours].
8. Wash the cells 3 times with 5 mL of PBS at each time point.
9. Detach the cells from the culture dish by pipetting or using a cell scraper.
10. Centrifuge the cell suspension at 300 × g for 5 minutes at 4°C to pellet the cells. Carefully remove and discard the supernatant.
11. Prepare the total RNA using commercially available RNA extraction reagent [e.g., RNAiso Plus, Takara Bio]. Finally, reconstitute the pellet at each time point in 50 µL of nuclease-free water.
   **Note:** If desired, reconstitution volume can be changed from 50 µL, but it is important to fix the reconstitution volume at each time point. The details are described in the Step C in next section (RNA Immunoprecipitation).
12. Quantify the extracted total RNA with NanoDrop (Thermo Fisher Scientific Inc.), and check the quality and quantity of total RNA.
13. Store at -80°C or below until starting RNA immunoprecipitation. For the preparation of input total RNA, please check the Step C in next section (RNA Immunoprecipitation).

**Immunoprecipitation of BrU-labeled RNA (RNA Immunoprecipitation)**

(A. Preparation of Anti-BrdU mAb-immobilized protein G magnetic beads)

**Note:** Immunoprecipitation protocol described below is designed only for the Protein G -Magnetic beads (provided by MBL). In case of using protein G agarose beads, centrifuge the tube (2,000 × g for 1 minute at 4°C) during washing step instead of magnetic separation with magnetic stand.

1. Suspend the Protein G-Magnetic beads slurry by vortex thoroughly, then spin-down briefly.
2. Resuspend the Protein-G Magnetic beads slurry by pipetting, Aliquot 300 µL of the beads slurry to each new microcentrifuge tube.
3. Place the tube on the magnetic stand to separate the beads from the solution.
4. After the solution becomes clear (about 1 minute), aspirate the supernatant carefully and remove the tube from the magnetic stand.
5. Add 1 mL of Wash buffer (+) to each tube and vortex thoroughly, then spin-down briefly.
6. Place the tube on the magnetic stand to separate the beads from the solution.
7. After the solution becomes clear (about 1 minute), aspirate the supernatant carefully.
8. Add 500 µL of Wash buffer (+) to each tube.
9. Add 20 µL of Anti-BrdU mAb to each tube, then vortex briefly but thoroughly.
10. Incubate the tube with constant rotation for at least 30 minutes at 4°C. If necessary, this incubation can be extended to overnight.

(B. Washing the antibody-immobilized protein G magnetic beads)
11. Spin-down briefly, and then place the tube on the magnetic stand to separate the beads from the solution.
12. After the solution becomes clear (about 1 minute), aspirate the supernatant carefully and remove the tube from the magnetic stand.
13. Add 1 mL of RNA-IP buffer (+) to each tube and vortex thoroughly, then spin-down briefly.
14. Place the tube on the magnetic stand to separate the beads from the solution.
15. After the solution becomes clear (about 1 minute), aspirate the supernatant carefully, and then remove the tube from the magnetic stand.
16. Add 500 µL of RNA-IP buffer (+) to each tube.
17. Leave the beads at 4°C or on ice and use the beads in Step 21.

(C. Preparation of antibody-immobilized protein G magnetic beads-BrdU-labeled RNA complex)
Note: When performing immunoprecipitations with constant quantity (µg) of total RNA at several time points, the percentage of BrU-labeled RNA to input total RNA gradually diminishes just after removal of pulse-labeling medium. This is due to both the decrease of BrU-labeled RNA and the increase of non-labeled total RNA, which are caused by cell proliferation during chase period. In order to accomplish more precise analysis of RNA half-life, please select one of the two “Normalization methods” described below:

A. Normalization by the quantity of stable transcripts
   In case of RT-qPCR or deep sequencing (RNA-seq) analysis, the expression level of each transcript at each time point can be normalized with several stable transcripts such as GAPDH. The analyses might be more accurate if the quantities of BrU-labeled RNA among the time points are normalized by the geometric mean of several transcripts (e.g., GAPDH, PGK1, GRN and TRAP1). When choosing this normalization method, prepare input total RNA of each time point to be 40 µg.

B. Normalization by Spike-in control
   The proportions of BrU-labeled RNA to total RNA at each time point can be normalized by fixing input total RNA volume (µL) for immunoprecipitation and utilizing the result of Spike-in control. In this case, each total RNA including BrU-labeled RNA, extracted from each time point sample, should be reconstituted in the equal volume of nuclease-free water (e.g., 50 µL each). The constant volume to all input total RNA samples should be calculated based on quantity of chase time 0 hour sample which is recommended to be 40 µg. Normalize the expression levels of various transcripts by expression data of Spike-in control.
18. According to “Normalization methods” above, prepare appropriate quantity (or volume) of input total RNA. Then add 2 μL of Spike-in control to the tube.

**Note:** Excess volume of input total RNA could affect immunopurification efficiency. The volume of input total RNA should be less than 100 μL.

19. Denature the input RNA by heating for 2 minutes at 80°C.
20. Quench the RNA 4°C or on ice at least 5 minutes.
21. Spin-down briefly, and then transfer the denatured input RNA into the tube prepared in step 17.
22. Incubate the tube with rotation for 3 hours at 4°C.

23. Spin-down the tube (prepared in step 22) containing antibody-immobilized protein G magnetic beads-RNA complex, and place the tube on the magnetic stand to separate the beads from the solution.
24. After the solution becomes clear (about 1 minute), aspirate the supernatant carefully.
25. Add 1 mL of Wash buffer (+) and vortex briefly but thoroughly.
26. Spin-down briefly, and place the tube on the magnetic stand to separate the beads from the solution.
27. After the solution becomes clear (about 1 minute), aspirate the supernatant carefully.
28. Wash the antibody-immobilized beads-RNA complex; repeat the steps 25-27 three times.

**RNA Isolation**

(from antibody-immobilized protein G magnetic beads-RNA complex)

- mi-Solution II and mi-Solution III should be equilibrated to room temperature before use.
- The reagents should be briefly but thoroughly mixed before use.

**Note:** The following RNA isolation protocol in this section is almost identical to “Separation method” used in RIP-Assay kit for microRNA described at [http://ruo.mbl.co.jp/gtf/1/1/RN1005.pdf](http://ruo.mbl.co.jp/gtf/1/1/RN1005.pdf). In this method, large RNA and small RNA are divided into individual microcentrifuge tubes. If you do not intend to analyze small RNA, large RNA fraction prepared by Separation method is sufficient for analysis of large RNA. If simultaneous analysis of small and large RNAs is required, 2-step method in RIP-Assay kit for microRNA can be available, although 2-step method has a little higher background than Separation method.

**Comparative table of RNA isolation methods**

<table>
<thead>
<tr>
<th>Collectable RNA species</th>
<th>Separation method</th>
<th>2-step method</th>
<th>1-step method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>large RNA</td>
<td>large RNA</td>
<td>small RNA</td>
</tr>
<tr>
<td></td>
<td>small RNA (in individual tubes)</td>
<td>small RNA (in one tube)</td>
<td>(a small amount of large RNA)</td>
</tr>
<tr>
<td>Recovery rate for large RNA</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
<td>&lt;40%</td>
</tr>
<tr>
<td>Recovery rate for small RNA</td>
<td>&gt;80%</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Classification by nucleotide length</td>
<td>Yes (large RNA: &gt;60-80 nt) (small RNA: &lt;60-80 nt)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Assay time</td>
<td>75 min.</td>
<td>75 min.</td>
<td>45 min.</td>
</tr>
<tr>
<td>Background (silver staining)</td>
<td>Low</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Advantage</td>
<td>Available for multiple applications</td>
<td>High-recovery rate for large/small RNA</td>
<td>Short assay time</td>
</tr>
<tr>
<td>Disadvantage</td>
<td>A little loss in recovery of small RNA compared to the other 2 methods</td>
<td>Not suitable for visualization by silver staining following denaturing PAGE</td>
<td>Low-recovery rate for large RNA (~40% of large RNAs)</td>
</tr>
</tbody>
</table>
Separation method

1. Prepare Master mix solution by diluting 10 μL of mi-Solution I with 240 μL of mi-Solution II per sample.
2. Dispense 2 μL of mi-Solution IV to each new microcentrifuge tube for step 6.
3. Add 250 μL of Master mix solution to each tube containing antibody-immobilized protein G magnetic beads-RNA complex (prepared in RNA Immuno precipitation-step 28), vortex thoroughly, then spin-down.
4. Add 150 μL of mi-Solution III to each tube, vortex thoroughly.
5. Spin-down briefly, and place the tube on the magnetic stand to separate the beads from the solution.
6. After the solution becomes clear (about 1 minute), carefully transfer the supernatant to the tube containing 2 μL of mi-Solution IV prepared in step 2. (Avoid removing the protein G magnetic beads from the pellet. Contamination of the beads may affect following steps.)
7. Add 300 μL of ice-cold 2-propanol to each tube, vortex briefly but thoroughly, then spin-down.
8. Incubate the tube at -20°C or below for 20 minutes (or overnight, if necessary).
   * During the incubation, dispense 2 μL of mi-Solution IV to each new microcentrifuge tube for step 10. In case of purification of large RNA, this process is not necessary.
9. Centrifuge the tube at 12,000 × g for 10 minutes at 4°C. At this point, the pellet is mainly composed of large RNAs, while small RNAs remain in the supernatant.
10. Transfer the supernatant, which contains small RNAs, to the tube containing 2 μL of mi-Solution IV prepared in step 8. Isolation method for small RNAs from the supernatant is described in the following steps 11–19.
   In case of purification of large RNAs in the pellet, skip to step 14.

Additional protocol: isolation for small RNAs

11. Add 500 μL of ice-cold 2-propanol to the supernatant containing small RNAs prepared in step 10, vortex briefly but thoroughly, then spin-down.
12. Incubate the tube at -20°C or below for 20 minutes (or overnight, if necessary).
13. Centrifuge the tube at 12,000 × g for 10 minutes at 4°C, then aspirate the supernatant carefully.
14. Rinse the pellet with 500 μL of ice-cold 70% ethanol, and mix briefly.
15. Centrifuge the tube at 12,000 × g for 3 minutes at 4°C, then aspirate the supernatant carefully.
16. Rinse the pellet once again using steps 14–15.
17. Dry up the pellet by aspirating excess ethanol followed by evaporation for 5–15 minutes at room temperature. Avoid RNase contamination. (Evaporation in clean bench is recommended.)
18. Reconstitute the pellet containing large RNAs in 20 μL of nuclease-free water and the pellet containing small RNAs in 10 μL of nuclease-free water.
19. Store at -80°C or below until starting following analysis.
Additional Procedure: Analysis of isolated RNA

We recommend qualitative and quantitative analysis of isolated RNAs prior to downstream analysis such as RT-qPCR, deep sequencing and microarray. These technologies may be useful for profiling RNAs.

- Quality control for isolated RNAs
  
  It is very important for comprehensive analysis such as deep sequencing or RT-qPCR to retain high-quality RNA because experimental results may be sensitive to RNA quality. Please confirm the quality of RNAs with Bioanalyzer (Agilent Technologies, Inc.) after the quantification of the isolated RNAs with NanoDrop (Thermo Fisher Scientific Inc.).
III. Examples of BRIC Analysis

1. Loss of BrU-labeled RNA in 24 hours

HeLa, HEK293T (293T), Jurkat, K562, HT29, T-47D and HEK293 cells were pulse-labeled with 150 μM BrU for 24 hours. Then, the cells were washed and harvested at chase time 0 and 24 hours. After RNA extraction using RNAiso Plus (Takara Bio), BrU-labeled RNA was isolated by BRIC Kit. The isolated BrU-labeled RNA was quantified with a spectrophotometer (NanoDrop) according to manufacturer’s instructions (Thermo Fisher Scientific Inc.). HeLa cells were most effectively labeled with 150 μM BrU in 7 cell lines, while BrU-labeled RNA from Jurkat, T-47D and HEK293 were almost not detected by NanoDrop. Quantity of BrU-labeled RNA in HeLa, K562, and 293T drastically decreased in 24 hours.
2. Target RNA decay in 24 hours

HeLa, HEK293T (293T), Jurkat, K562, HT29, T-47D and HEK293 cells were pulse-labeled with 150 μM BrU for 24 hours. Then, the cells were washed and harvested at chase time 0 and 24 hours. After RNA extraction using RNAiso Plus (Takara Bio), BrU-labeled RNA was isolated by BRIC Kit. The isolated BrU-labeled RNAs were analyzed by RT-qPCR. RT-qPCR was carried out with PrimeScript® RT reagent Kit (Takara Bio) and qPCRBIO SyGreen Mix Lo-ROX (MBL) according to manufacturer’s instructions.

Each normalized expression data was calculated based on the expression level of Spike-in control. The RNAs derived from housekeeping genes such as 18S rRNA and ACTB were stable in most of cell lines, while HIF-1α and ADM were unstable. The results of Jurkat, HEK293 and T-47D cells seemed to be unsuccessful because the labeling efficiency was low.
3. Time-course analysis of BrU-labeled RNA with NanoDrop

HeLa cells were pulse-labeled with 150 µM BrU for 24 hours. Then, cells were washed and harvested at chase time 0, 4, 8, 12 and 24 hours. After RNA extraction by RNAiso Plus (Takara Bio), BrU-labeled RNA was isolated by BRIC Kit and quantified with a spectrophotometer (NanoDrop) according to manufacturer’s instructions (Thermo Fisher Scientific Inc.).

The percentage of isolated BrU-labeled RNA rapidly decreased to 60% in the first 4 hours and then gradually decreased in the next 20 hours. This result suggests that a lot of unknown functional ncRNAs may be buried in short-lived RNA population (T<sub>1/2</sub> < 4 hours).

4. Time-course analysis of BrU-labeled RNA with Bioanalyzer

HeLa cells were pulse-labeled with 150 µM BrU for 24 hours. Then, cells were washed and harvested at chase time 0, 4, 8, 12 and 24 hours. After RNA extraction by RNAiso Plus (Takara Bio), BrU-labeled RNA was isolated by BRIC Kit (RNA Isolation: Separation method) and analyzed on a Bioanalyzer RNA pico chip (Agilent Technologies, Inc.) according to manufacturer’s instructions.

The migration profile of the 0 hour showed several minor peaks with 2 main peaks around 2,000 and 4,000 nucleotides, corresponding to 18S and 28S ribosomal RNA, respectively. Several minor peaks corresponding to mRNA and rRNA precursor faded out over time. The low peak (indicated by arrowhead), probably corresponding to rRNA precursor, around the 28S rRNA, was not detected at time points after 8 hours. In contrast, the peak of 28S rRNA increased during the time. Furthermore, the heights of 2 main peaks were observed even at 24 hours. These results reveal the stability of processed rRNA and suggest that rRNA processing can be monitored by BRIC and Bioanalyzer.
5. Measurement of half-life of target RNA by RT-qPCR

HeLa cells were pulse-labeled with 150 μM BrU for 24 hours. Then, cells were washed and harvested at chase time 0, 4, 8, 12 and 24 hours. After RNA extraction by RNaIso Plus (Takara Bio), BrU-labeled RNA was isolated by BRIC Kit. Isolated BrU-labeled RNA was analyzed by RT-qPCR. RT-qPCR was carried out with PrimeScript® RT reagent Kit (Takara Bio) and qPCRBIO SyGreen Mix Lo-ROX (MBL) according to manufacturer’s instructions.

As expected, the transcripts derived from housekeeping genes, such as 18S rRNA and ACTB, showed relatively long half-lives, while HIF-1α and ADM* showed much shorter half-lives.

*ADM gene encodes a potent hypotensive peptide which plays important roles in both normal and disease conditions.

6. Comparison of BrU- and EU-labeled RNA recovery

HeLa cells were pulse-labeled with 150 μM of BrU or EU (Life Technologies Inc.) for 24 hours. Then, cells were washed and harvested without additional chase time. After RNA extraction by RNaIso Plus (Takara Bio), BrU- and EU-labeled RNA were isolated by BRIC Kit. These RNAs were quantified with a spectrophotometer (NanoDrop) according to manufacturer’s instructions (Thermo Fisher Scientific Inc.).

As mentioned above, Anti-BrdU mAb cross-reacted with EU-labeled RNA. This result indicates that the recovery of EU-labeled RNA was almost equal to BrU-labeled RNA.
IV. Related Products

RIP-Assay Kit
RN1001  RIP-Assay Kit (10 assays)

RIP-Assay Kit for microRNA
RN1005  RIP-Assay Kit for microRNA (10 assays)

RiboTrap Kit
RN1011/RN1012  RiboTrap Kit (10 assays)

Protein G magnetic beads
MJS002  Protein G-Magnetic beads

Magnetic stand
3190  Magnetic Rack

qPCRBIO SyGreen Mix
PB20-11-01  qPCRBIO SyGreen Mix Lo-ROX
PB20-11-05  qPCRBIO SyGreen Mix Lo-ROX
PB20-12-01  qPCRBIO SyGreen Mix Hi-ROX
PB20-12-05  qPCRBIO SyGreen Mix Hi-ROX

Anti-BrdU antibody
MI-11-3  Anti-Bromodeoxyuridine mAb

Isotype Control Antibody
M075-3  Mouse IgG1 (isotype control)

RIP-Certified Antibody and RBP Antibody
Various antibodies in “RNA-RNP network” are also available from MBL.
Please visit our website at http://ruo.mbl.co.jp/je/rip-assay/

V. Appendix

The following commercially available reagents have been confirmed to work with BRIC Kit at the indicated final concentration.

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<tr>
<th>Protein G beads</th>
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<td>MJS002</td>
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<tr>
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<td>Life Technologies</td>
<td>DB10004</td>
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<td>Immobilized Protein G Plus</td>
<td>Pierce</td>
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