Global RNA Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (BN00938)

(Catalog BN00938; 100 assays; Store at -20°C)

I. Introduction:

RNA plays crucial role in coding, decoding and regulation of genes, and protein expression in all living cells. The ability to detect newly synthesized RNA or changes in RNA levels under various physiological conditions, or resulting from disease, environmental damage, or drug treatments is an important aspect of toxicological profiling. Many anti-cancer drugs inhibit transcription, and most transcription inhibitors have useful pharmacological properties. Assay Genie's Global RNA Synthesis Assay Kit provides a simple and robust tool for detection of global RNA transcription temporally and spatially or changes in RNA levels directly in living cells. *De novo* synthesized RNA can be detected with a simple procedure without the use of radiolabeling or antibodies. Our approach relies on the incorporation of cell permeable 5-EU (Ethynyl uridine) into nascent RNA, but not into DNA, instead of its natural uridine analog. 5-EU can be used as a replacement for BrU (5-Bromo-uridine) to measure *de novo* synthesized RNA in proliferating cells. Modified RNA is detected by click chemistry with azide-containing dye that enables for multiplex analyses with other probes, or detection of RNA-interactive proteins for deeper biological insights. Our kit provides sufficient materials for 100 assays for analysis by FACS or detection by fluorescence microscope. We include Actinomycin D, an inhibitor RNA synthesis that serves as an experimental control.

II. Applications:

- Detection and quantification of RNA transcription *in vitro* and *in vivo* by fluorescence microscope and/or FACS
- Screening for genotoxic compounds and effectors of RNA biosynthesis in proliferating cells
- Evaluating effects of anti-cancer drugs and genotoxic agents

III. Sample Type:

- Suspension or adherent cell cultures

IV. Kit Contents:

Components	BN00938	Cap Code
Wash Buffer (10X)	25 ml	NM
Fixative Solution	10 ml	WM
Permeabilization Buffer (10X)	25 ml	NM/Blue
RNA Label (100X)	100 µl	Clear
Copper Reagent (100X)	100 µl	Blue
Fluorescent Azide (100X)	100 µl	Red
Reducing Agent (20X)	500 µl	Yellow
DAPI (1000X)	20 µl	Blue/Amber Vial
Actinomycin D (100X)	10 µl	Purple

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate culturing media; flow cytometry vessels
- Phosphate Buffered Saline (PBS, pH 7.4)
- Sterile 0.1% Gelatin Solution (optional, only required for adhering suspension cells to the surface)
- Flow cytometer equipped with laser capable of excitation at 488 and 530/590 nm emission filters respectively
- Fluorescence microscope capable of excitation and emission at 440/490 and 540/580 nm respectively

VI. Storage Conditions and Reagent Preparation:

Upon arrival, store the entire kit at -20°C protected from light. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **10X Wash Buffer and 10XPermeabilization Buffers:** Dilute the 10X stocks 1:10 in sterile water, mix well. Store at 4 °C.
- **Fixative Solution:** Ready to use, after opening store at 4°C, protected from light.
- **Remaining components:** Store at -20°C protected from light. While in use, keep on ice and minimize light exposure.

VII. Assay Protocol:

Notes:

This assay was developed with HeLa (adherent) and Jurkat (suspension) cells and can be modified for any cell line. The protocol below refers to a 96-well tissue culture plate; adjust volumes accordingly for other plate formats. The assay volume is 100 µl. Growth conditions, cell number per well and other factors may affect the incorporation rate of the RNA Label; therefore, optimize the assay for your cell type. We suggest an initial test of several RNA Label concentrations to find best conditions for tested cell type and experimental design. Avoid stressing the cells by washes or temperature changes prior to incubation with RNA Label. All steps should be carried out at room temperature (RT) unless otherwise specified; equilibrated all buffers to RT prior to the experiment.

1. Labeling of control and experimental cells:

a. Obtain cell suspension of desired density and seed directly into tissue culture vessels, or on coverslips for high resolution microscopy. **To immobilize suspension cells for microscopy**: add 100 µl of 0.1% gelatin solution directly into the wells, tilt the plate to cover the entire well surface and place it in a tissue culture hood for 1 hour. Gently remove the gelatin solution and seed your cells. Allow the cells to recover overnight before the treatment.

b. Next day, treat the cells with appropriate effectors according to your protocol; do not add treatment to the positive and negative control cells. **Negative control** (cells not exposed to the RNA Label or treatment), **positive control** (cells incubated with 1X RNA Label only). To use included Actinomycin D as an inhibitor of RNA synthesis, dilute it 1:100 directly into the culture medium and incubate the cells for 4 hours at 37°C.

c. Dilute RNA Label (100X) to 1X final concentration directly into the experimental, positive control, and Actinomycin D-treated cells respectively. *Do not add the RNA Label into the **negative control** cells*. To avoid potential reversibility of drug action on RNA synthesis, do not remove the drug-containing media during incubation with 1X RNA Label. Incubate the cells for additional hour in a 37°C incubator, or for the period of time required by your experimental protocol.

d. Terminate the experiment by removal of the culture medium and rinse the cells once with 100 µl of PBS, discard the supernatant. **For immobilized suspension cells**: Centrifuge the plate at 300 x g (or the lowest centrifuge setting) for 5 minutes to deposit the cells onto the surface. Tilt the plate and ***gently*** remove the media with a pipette tip. It is important to avoid excessive centrifugation speeds, which can damage the cells. *Make note of the place that is used, and perform subsequent aspirations from the same place*. Proceed to the Fixation and Permeabilization. *Pellet the **suspension cells** at 300 x g for 5 min throughout the entire protocol!*

2. Fixation and Permeabilization:

a. **For adherent cells**: Add 100 µl of Fixative Solution to each well and incubate the cells for 15 min at RT protected from light. Remove the fixative and wash the cells twice with 100 µl of 1X Wash Buffer, remove the wash. Add 100 µl of 1X Permeabilization Buffer and incubate the cells for 10 min at RT. Remove the Permeabilization Buffer and replace it with a 20 µl of fresh aliquot.

b. **For suspension cells:** Re-suspend the cells in 100 µl of Fixative Solution and incubate for 15 min at RT protected from light. Remove the fixative and wash the cells twice with 0.5 ml of 1X Wash Buffer. Discard the supernatant and re-suspend the cells in 100 µl of 1X Permeabilization Buffer. Incubate the cells for 10 min at RT. Remove the Permeabilization Buffer and replace it with a 20 µl of fresh aliquot. Proceed to RNA reaction and total DNA staining.

3. RNA reaction and total DNA staining:

Reaction cocktail: Prepare 1X reaction cocktail according to the table below. Volumes should be multiplied by number of samples and reagents added in the exact order. Use the reaction cocktail within 15 minutes of preparation. *Cells should be protected from light during and following the reaction and DNA staining*.

	Amount per Reaction
PBS	93 µl
Copper Reagent (100X)	1 µl
Fluorescent Azide (100X)	1 µl
Reducing Agent (20X)	5 µl

RNA Reaction: Add 100 µl of 1X Reaction cocktail to each sample and incubate the cells for 30 min at room temperature protected from light. Remove the reaction cocktail and wash cells three times in 100 µl of Wash Buffer. Remove the wash and suspend the cells in 100 µl of PBS. Proceed to DNA staining. If no DNA staining is desired, proceed to Microscopic or FACS analysis. **DNA staining:** Prepare 1X dilution of DAPI Stain and add 100 µl per well. Incubate the cells for 20 minutes at room temperature or refrigerate at 4 °C protected from light. Remove the DNA stain solution; wash the cells once with 100 µl of PBS. Note: cells are compatible with all methods of slide preparation including wet mount or prepared mounting media.

4. Fluorescence Microscope Imaging: Analyze samples for red fluorescence generated by labeled RNA and nuclear DNA under the UV filter respectively. **FACS analysis:** Harvest the cells by preferred method and wash with 0.5 ml of ice-cold PBS. Re-suspend the pellets with 100 µl of ice-cold PBS. Transfer the cell suspension into flow cytometry vessels. Analyze samples in FL-2 channel for signal generated by RNA during click reaction. **Note:** Trypsin can be used to collect the adherent cells prior to FACS analysis.

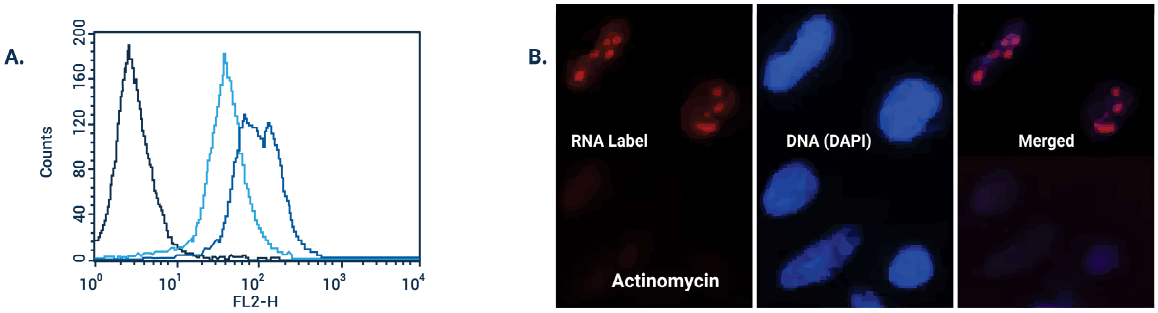

Figure: Analysis of RNA biosynthesis in presence of Actinomycin D. (A) Jurkat cells (1X10^6 cells/ml) were pre-treated with vehicle **(black line)** or 1 X Actinomycin D **(blue line)** for 4 hours at 37°C prior to 1-hour incubation with RNA Label **(red line)**. Cells were then processed for detection of *de novo* synthesized RNA according to the included protocol. Fluorescence measured in FL-2 channel clearly shows the inhibitory effect of Actinomycin D on RNA synthesis. **(B)** HeLa cells (10^5 cells/ ml) were pre-treated either with vehicle **(top)** or 1 Actinomycin D **(bottom)** for 4 hours at 37°C prior to 1-hour incubation with RNA Label. *De novo* synthesized RNA was detected by Fluorescence Microscope. Reduced red fluorescence in panel B confirms the inhibitory effect of Actinomycin D on RNA biosynthesis. Nuclear staining in both panels confirms that red fluorescence is the result of RNA Label incorporation.

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