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EZClick™ Global RNA Synthesis Assay Kit

rev 07/20

(Catalog # K462-100; 100 assays; Store at -20°C)

I. Introduction:

RNA plays a crucial role in coding, decoding, 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. **BioVision's EZClick™ 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. The kit principle 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 using an azide-containing dye that enables for multiplex analyses with other probes, or detection of RNA-interactive proteins for deeper biological insights. The kit provides sufficient materials for 100 assays for quantitative analysis by microplate reader (Ex/Em= 494/521 nm) and/or by fluorescence microscope. The kit includes 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*
- 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	K462-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K462-100-1
Fixative Solution	10 ml	WM	K462-100-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	K462-100-3
EZClick™ RNA Label (100X)	100 µl	Clear	K462-100-4
Copper Reagent (100X)	100 µl	Blue	K462-100-5
EZClick™ Fluorescent Azide (100X)	100 µl	Green/Amber Vial	K462-100-6
Reducing Agent (20X)	500 µl	Yellow	K462-100-7
EZClick™ Total DNA Stain (1000X)	20 µl	Blue/Amber Vial	K462-100-8
Actinomycin D (100X)	10 µl	Purple	K462-100-9

V. User Supplied Reagents and Equipment:

- Tissue culture vessels and appropriate culturing media
- A 6-, 12-, 24-, or 96-well clear plates should be used only for cell culturing. The measurement of fluorescence should be performed in white opaque plates. Alternatively, sterile opaque plates can be used for both, culturing and measurements
- Phosphate Buffered Saline (PBS, pH 7.4)
- Multi-well spectrophotometer and Fluorescence microscope (optional) capable of measuring Ex/Em= 494/521 nm spectra

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 10X Permeabilization Buffers: Thaw at 37°C to dissolve completely.** Dilute the 10X stocks 1:10 in sterile water, mix well. Store at 4°C.
- **Fixative Solution: Divide into aliquots and store at -20°C, protected from light.**
- **Remaining components:** Store at -20°C protected from light. While in use, keep on ice and minimize light exposure.

VII. Global RNA Synthesis Assay Protocol:

Notes:

This assay was developed with Jurkat (suspension) and HeLa (adherent) cells and can be modified for any cell line. The following protocol has been optimized for a 96-well opaque plate at $1X10^6$ cells per well, using fewer cells per well or clear plate will result in decreased signal. We suggest testing growth conditions, cell number per well and several concentrations of the EZClick™ RNA Label, to find the best experimental design for your cell type. The assay volume is 100 µl; adjust volumes accordingly for other plate formats. Avoid stressing the cells by washes or temperature changes prior to incubation with EZClick™ RNA Label. All steps should be carried out at room temperature (RT) unless otherwise specified; equilibrate all buffers to RT prior to the experiment.

1. Labeling of control and experimental cells:

a. Plate suspension or adherent cells at a desired density and allow for overnight recovery before treatment. Ensure that adherent cells are sub-confluent. Include appropriate controls and account for cell loss during the processing. **Negative control** (cells not exposed to the Phospholipid Label or treatment), **Background control** (cells treated with EZClick cocktail only), **Positive control** (cells incubated with 1X RNA Label only).

b. Next day, remove the media and treat the cells with appropriate effectors according to your protocol; do not add treatment to the positive and negative control cells. For **suspension cells**: Centrifuge the plate at 500 x *g* (or the lowest centrifuge setting) for 5 minutes at RT to pellet the cells. Tilt the plate and ***gently*** remove the media with a pipette tip. Avoid excessive centrifugation speeds, which can damage the cells. *Use these centrifugation settings throughout the entire protocol!* 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 EZClick™ RNA Label (100X) to 1X final concentration with culture medium and add into the **Experimental**, **Positive control** and Actinomycin D-treated cells respectively. Incubate the cells for additional 1-24 hours, or time required by your experimental protocol in a 37°C incubator. *Do not add the EZClick™ RNA Label into the **Negative control** cells*. Do not remove the drug-containing media during incubation with 1X RNA Label to avoid potential reversibility of drug action on label incorporation.

d. Harvest the **suspension cells** by centrifugation. **Optionally**, detach **adherent cells** (e.g. trypsinize and quench with media), and harvest by centrifugation. Wash the cells once with 100 µl of PBS, discard the supernatant and proceed to the Fixation and Permeabilization.

2. Fixation and Permeabilization:

a. For adherent and suspension 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 once 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.Proceed to EZClick™ RNA reaction.

3. EZClick™ RNA reaction:

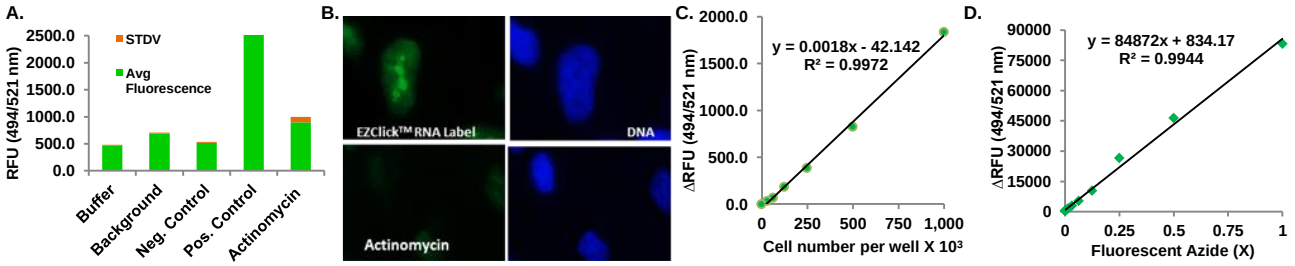
a. Reaction cocktail: Prepare 1X EZClick™ reaction cocktail according to the table below. Volumes should be multiplied by number of samples and reagents and added in the exact order. Use the reaction cocktail within 15 minutes of preparation. *Cells should be protected from light during, and following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

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

b. For Negative Control Cells: Add 100 µl of 1X PBS. **For Experimental Cells and Positive Control Cells:** Add 100 µl of 1X EZClick™ reaction cocktail to each sample and incubate the cells for 30 min at RT protected from light. Remove the reaction cocktail and wash cells three times in 100 µl of Wash Buffer. Suspend the cells in 100 µl of Wash Buffer.

c. Detection: Cells must be analyzed **immediately** in the plate reader at Ex/Em = 494/521 nm in end point mode to determine change in fluorescence of compounds and controls after background subtraction, or imaged with fluorescence microscope directly in the plate following DNA staining. Analyze samples for green fluorescence generated by *de novo* synthesized RNA.

d. Azide Fluorescence Curve: To increase the accuracy of your data, instantly prepare azide fluorescence curve for each condition and cell line using cells previously detected for green fluorescence (**Step 3C**). In a 96-well white opaque plate, prepare a series of dilutions of your cell suspension starting with the same volume and number of cells as in the experimental wells. Dilute the cells by factor of 2, prepare at least 6 dilutions. To minimize the error, aliquot at least 2 wells per dilution. Measure fluorescence and calculate average for each dilution, subtract the background value. Plot the Azide Fluorescence Curve to obtain fluorescence per cell number and the detection limit for your assay. **Optional:** Standard curve of Fluorescent Azide concentration per well can be prepared in the same manner to obtain the least detectable amount of azide for your experiment.



Figures: Analysis of RNA biosynthesis in presence of Actinomycin D. Jurkat cells (1X10^6 cells/well) or HeLa cells seeded at 10^5 cells/ ml were pre-treated with vehicle or 1 X Actinomycin D for 4h at 37°C prior to 24 hour incubation with EZClick™ RNA Label then processed for detection of *de novo* synthesized RNA according to the kit protocol. **(A)** Jurkat cells: plate reader analyses of controls and Actinomycin treatment; Avg fluorescence +/- standard deviation plotted for 3 replicates per condition. **(B)** HeLa cells: upper panel- green fluorescence of *de novo* synthesized RNA; bottom- panel cells treated with Actinomycin. Nuclear staining confirms that the green signal results from RNA Label incorporation. **(C) Azide Fluorescence Curve of Jurkat cells prepared for this assay.** Detection limit corresponds to about 31,250 of Jurkat cells per well. *Your results may not be identical to these. A new curve must be obtained for each experiment and the cell line*. **(D) Azide Fluorescence Curve in 0-1 X range**. *This is reference data and it should not be used to interpret actual results. Your data will depend on the cell type and tested compound.*

VIII. RELATED PRODUCTS:

EZClick™ Global RNA Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (K718)
EZClick™ Global Phospholipid Synthesis Assay Kit (FACS/Microscopy), Red Fluorescence (K717)
EZClick™ EdU Cell Proliferation/DNA Synthesis Kit (FACS/Microscopy), Red Fluorescence (K946)

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