GeneCopoeia™
Expressway to Discovery

9620 Medical Center Drive, Suite 101
Rockville, MD 20850, USA
www.genecopoeia.com
1-866-360-9531

iClick™ EU Andy Fluor 488 Imaging Kit
Catalog Number: A009

Table 1. Kit Components and Storage

Material	Amount	Concentration	Storage	Stability
EU (Component A)	**500 µL**	**100 mM in DMSO**	**-20 °C**	**The product is stable for at least one year when stored as directed.**
Andy Fluor 488 azide (Component B)	**100 µL**	**NA**	**-20 °C Protect from light**	
iClick EU reaction buffer (Component C)	**50 mL**	**1X**	**4 °C**	
$CuSO_4$ (Component D)	**1 mL**	**100 mM in H_2O**	**4 °C**	
iClick EU buffer additive (Component E)	**200 mg**	**NA**	**4 °C**	
Hoechst 33342 (Component F)	**70 µL**	**5 mg/mL in H_2O**	**4 °C**	

Number of assays: Sufficient material is supplied for 50 coverslips based on the protocol below.
Approximate fluorescence excitation/emission maxima, in nm: Andy Fluor 488 azide**:** 495/520; Hoechst 33342: 350/461, bound to DNA.

Introduction

The ability to detect newly synthesized RNA or changes in RNA levels resulting from disease, environmental damage, or drug treatments is an important aspect of toxicological profiling. Utilizing an alkyne-modified nucleoside, 5-ethynyl uridine (EU), and powerful click chemistry, newly synthesized RNA can be detected without the use of radioactivity or antibodies with a simple, two-step procedure. In step one, the alkynecontaining nucleoside is fed to cells or animals, and is actively incorporated into nascent RNA. The small size of the tag enables efficient incorporation of the modified nucleoside into RNA, but not into DNA. Detection utilizes the chemoselective ligation or “click” reaction between an azide and an alkyne where the modified RNA is detected with a corresponding azide-containing dye. With its dimunitive “footprint”, the iClick detection molecule can easily penetrate complex samples and leaves open the possibility of multiplex analyses with other probes, including antibodies for the detection of RNA-interactive proteins for deeper biological insights.

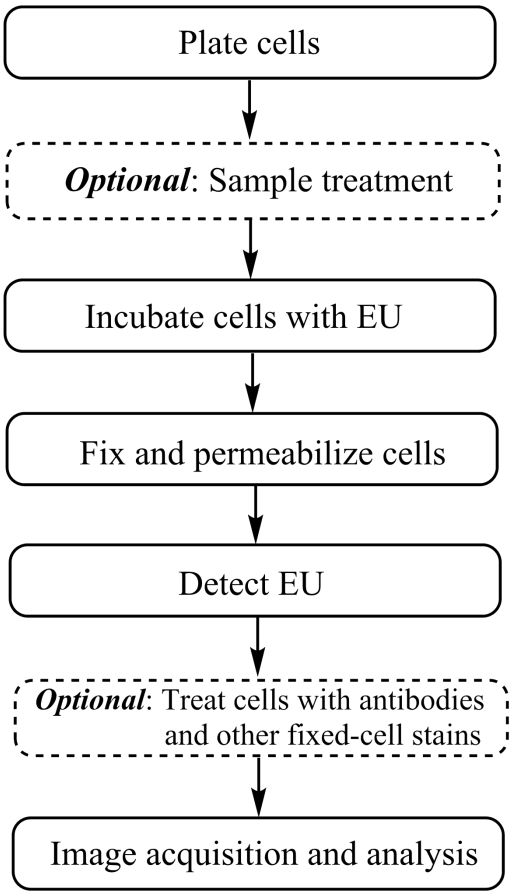
Click reactions have several general characteristics: the reaction is efficient, no extreme temperatures or solvents are required, the reaction is complete within 30 minutes, the components of the reaction are bioinert, and perhaps most importantly, no side reactions occur — the label and detection tags react selectively and specifically with one another. This final point is a key advantage of this powerful detection technique; it is possible to apply click chemistry-labeled molecules to complex biological samples and detect them with unprecedented sensitivity due to extremely low background.

The kits contain all of the components needed to label and detect newly synthesized RNA in whole cells. The kits are also supplied with blue fluorescent Hoechst 33342 dye as a nuclear counterstain or for DNA profiling. The kits include sufficient reagents for labeling 25, 18 × 18 mm coverslips using 500 µL reaction volume per well.

Materials required but not provided

- Phosphate-buffered saline (PBS, pH 7.2–7.6)
- Fixative (3.7% Formaldehyde in PBS)
- Permeabilization reagent (0.5% Triton® X-100 in PBS)
- 3% Bovine serum albumin (BSA) in PBS (3% BSA in PBS), pH 7.4
- Deionized water

Workflow diagram for the iClick EU Imaging Assay



Experimental Protocols

Labeling cells with EU

Note: The optimal EdU concentration varies with different cell types. It is recommended to start with EU concentration at 1 mM. Growth medium, cell density, cell type variations, and other factors may influence labeling. In initial experiments, we recommend testing a range of EU concentrations to determine the optimal concentration for your cell type and experimental conditions.

1.1 Plate the cells on coverslips at the desired density, then allow them to recover overnight before additional treatment.

1.2 *Optional*: Perform cell treatments as desired. To address the potential reversibility of drug action on RNA synthesis, we recommend that you do not remove the drug-containing media during EU treatment.

1.3 Prepare a 2X working solution of EU from the 100 mM stock solution (Component A) in pre-warmed complete medium. For example, for a 1 mM final EU treatment, prepare a 2 mM working solution.

1.4 Prewarm the 2X EU solution, then add an equal volume of the 2X EdU solution to the volume of media containing cells to be treated to obtain a 1X EU solution. (For example, for a final concentration of 1 mM, replace half of the media with fresh media containing 2 mM of EU). We do not recommend replacing all of the media, because this could affect the rate of cell proliferation.

1.5 Incubate under normal cell culture conditions for 1 hour. The optimal incubation time depends on the cell growth rate and cell treatment, and needs to be determined experimentally.

Cell fixation and permeabilization

Note: This protocol is optimized with a fixation step using 3.7% formaldehyde in PBS, followed by a 0.5% Triton® X-100 permeabilization step. However, this protocol is also amenable to other fixation/ permeabilization reagents, such as methanol and saponin.

2.1 After incubation, remove the media and add 1 mL of 3.7% formaldehyde in PBS to each well containing the coverslips. Incubate for 15 minutes at room temperature.

2.2 Remove the fixative and wash the cells in each well twice with 1 mL of 3% BSA in PBS.

2.3 Remove the wash solution. Add 1 mL of 0.5% Triton® X-100 in PBS to each well, then incubate at room temperature for 20 minutes.

EU detection

Note: This protocol uses 500 µL of iClick reaction cocktail per coverslip. A smaller volume can be used as long as the remaining reaction components are maintained at the same ratios.

3.1 Make a 10X stock solution of the iClick EU buffer additive (Component E): Add 1 mL of deionized water to the vial, then mix until fully dissolved. After use, store any remaining stock solution at ≤–20°C. When stored as directed, this stock solution is stable for up to 1 year. If the solution develops a brown color, it has degraded and should be discarded.

3.2 Prepare 1X iClick EU buffer additive by diluting the 10X solution 1:10 in deionized water. Prepare this solution **fresh** and use the solution on the same day.

3.3 Prepare iClick reaction cocktail according to Table 2. It is important to add the ingredients in the order listed in the table; otherwise, the reaction will not proceed optimally. Use the iClick reaction cocktail within 15 minutes of preparation.

Table 2. iClick reaction cocktails.

Reaction components	Number of coverslips			
	1	2	4	10
iClick EU reaction buffer (Component C)	430 µL	860 µL	1.8 mL	4.3 mL
$CuSO_4$ (Component D)	20 µL	40 µL	80 µL	200 µL
Andy Fluor 488 azide (Component B)	2 µL	4 µL	8 µL	20 µL
1X Reaction buffer additive (prepared in step 3.2)	50 µL	100 µL	200 µL	500 µL
Total volume	500 µL	1 mL	2 mL	5 mL

3.4 Remove the permeabilization buffer, then wash the cells in each well twice with 1 mL of 3% BSA in PBS. Remove the wash solution.

3.5 Add 0.5 mL of iClick reaction cocktail to each well containing a coverslip. Rock the plate briefly to insure that the reaction cocktail is distributed evenly over the coverslip.

3.6 Incubate the plate for 30 minutes at room temperature, **protected from light**.

3.7 Remove the reaction cocktail, then wash each well once with 1 mL of 3% BSA in PBS. Remove the wash solution.

For nuclear staining, proceed to **DNA staining**. If no additional staining is desired, proceed to **Imaging and analysis**.

DNA staining

4.1 Wash each well with 1 mL of PBS. Remove the wash solution.

4.2 Dilute the Hoechst 33342 (Component F) solution 1:1000 in PBS to obtain a 1X Hoechst 33342 solution (the final concentration is 5 µg/mL).

4.3 Add 1 mL of 1X Hoechst 33342 solution per well. Incubate for 30 minutes at room temperature, **protected from light**. Remove the Hoechst 33342 solution.

4.4 Wash each well twice with 1 mL of PBS. Remove the wash solution.

Imaging and analysis

iClick EdU cells are compatible with all methods of slide preparation, including wet mount or prepared mounting media. See Table 3 for the approximate fluorescence excitation/emission maxima for Andy Fluor 488 dye and Hoechst 33342 dye bound to DNA.

Table 3. Approximate fluorescence excitation/emission maxima.

Fluorophore	Excitation (nm)	Emission (nm)
Andy Fluor 488	495	520
Hoechst 33342, bound to DNA	350	461

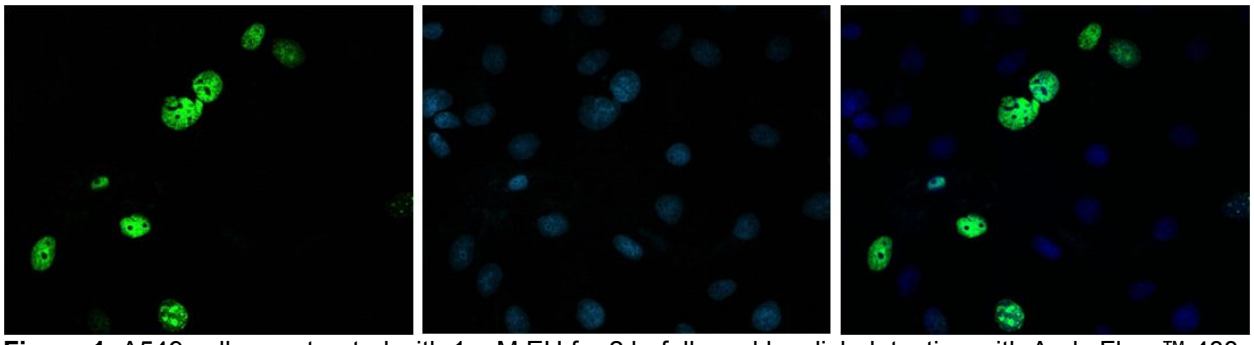


Figure 1. A549 cells was treated with 1 mM EU for 2 h, followed by click detection with Andy Fluor™ 488 Azide (green), nuclei are counterstained with Hoechst 33342 (blue).