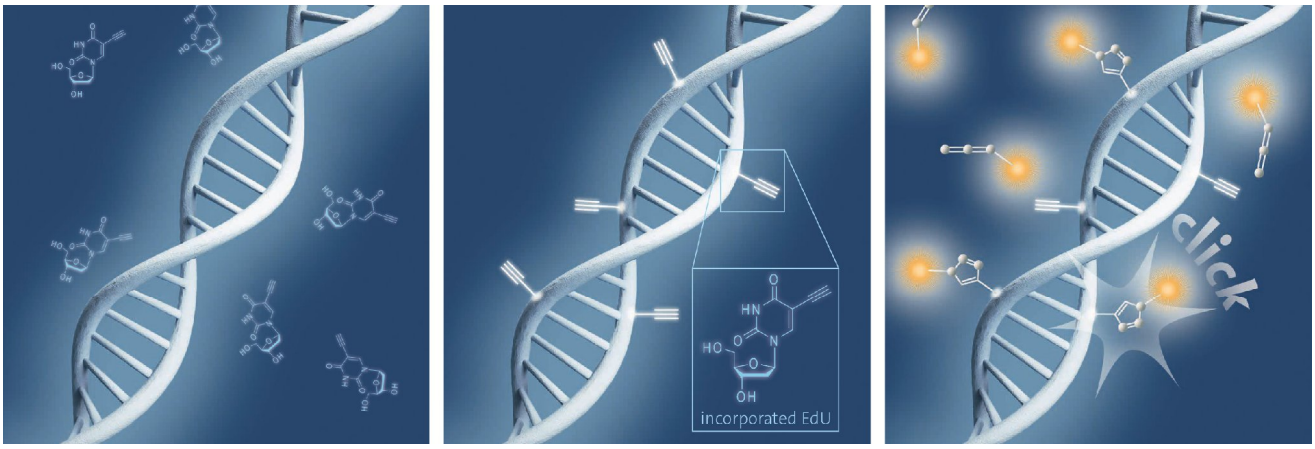
USER MANUAL

EdU Click FC

ROTI®kit for Flow Cytometry



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Introduction and product description:

The detection of cell proliferation is of utmost importance for assessing cell health, determining genotoxicity or evaluating anticancer drugs. This is normally performed by adding nucleoside analogs like [^{3}H]thymidine or 5-bromo-2'-deoxyuridine (BrdU) to cells during replication, and their incorporation into DNA is detected or visualized by autoradiography or with an anti-BrdU-antibody respectively. Both methods exhibit several limitations. Working with [^{3}H]thymidine is troublesome because of its radioactivity. Autoradiography is slow and thus not suitable for rapid high-throughput studies. The major disadvantage of BrdU staining is that the double-stranded DNA blocks the access of the anti-BrdU antibody to BrdU units. Therefore samples have to be subjected to harsh denaturing conditions resulting in degradation of the structure of the specimen.

Roth's *EdU Click FC* assays overcome these limitations, providing a superior alternative to BrdU and [^{3}H]thymidine assays for directly measuring DNA synthesis. EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. In contrast to BrdU assays, the *EdU Click FC* assays are not antibody based and therefore do not require DNA denaturation for detection of the incorporated nucleoside. Instead, the *ROTI®kits for Flow Cytometry* utilize click chemistry for detection in a variety of dye fluorescent readouts. Furthermore, the streamlined detection protocol reduces both the total number of steps and significantly decreases the total amount of time. The simple click chemistry detection procedure is complete within 30 minutes and is compatible with multiplexing for content and context-rich results.

Standard flow cytometry methods are used to determine the percentage of S-phase cells in the population (**Figure 1**).

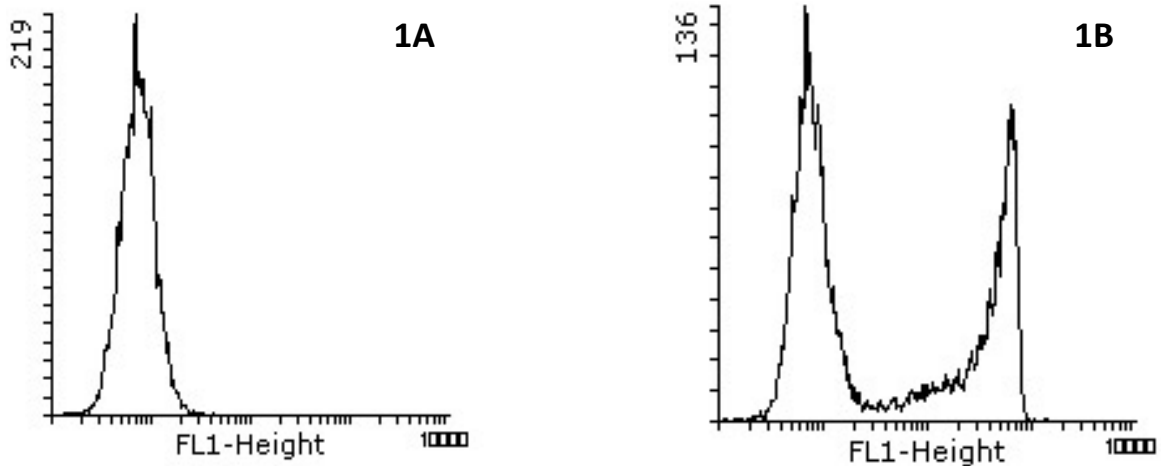

Figure 1: Fluorescence histograms of EdU-incorporation with the ROTI®kit for Flow Cytometry.
Samples of HeLa cells treated without (**1A**) or with EdU (**1B**) were incubated with 10 µM EdU for 2 hours. The click reaction using 6-FAM-Azide was performed according to the recommended staining protocol. Fluorescence intensity of 10.000 cells was measured by flow cytometry. The results are presented in form of histograms, showing the cell number in the y-axis and the FL1-Fluorescence in the x-axis. FL1 voltage setting was adjusted according to the fluorescence signal of the negative cell population (333 V with 6-FAM). **1A** represents the negative control of proliferating and non-proliferating cells without EdU incorporation. **1B** shows non-proliferating cells without EdU incorporation (left peak) and proliferating cells (S phase) which have incorporated EdU and are labelled with 6-FAM-Azide (right peak).

The ROTI®kit for Flow Cytometry is compatible with several cell cycle dyes. An example using 6-FAM-Azide is illustrated in **Figure 2**.

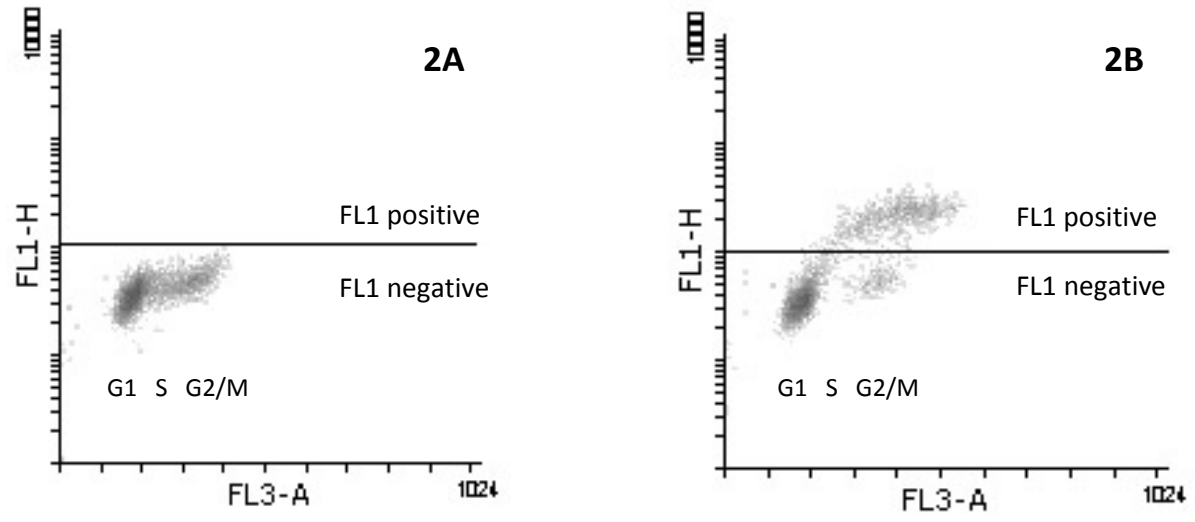

Figure 2: Density blots of Propidium Iodide (PI) stained samples.
Samples of HeLa cells treated without (**2A**) or with EdU (**2B**) were incubated with 10 µM EdU for 2 hours. The reaction cocktail carrying 6-FAM Azide was used. After the click reaction DNA was stained using PI (FL3 fluorescent channel). The y-axis presents the FL1-Fluorescence intensity, and the x-axis the content of DNA measured with FL3-area. Cell cycle phases are indicated as G1, S and G2/M phase.

The *ROTI®kit for Flow Cytometry* can be used with antibodies against surface and intracellular markers. To ensure the compatibility of your reagent or antibody, please refer to **Table 1**.

Table 1: EdU detection dye compatibility

Fluorescent molecule	Compatibility*
Organic dyes such as Fluorescein and Alexa dyes	Compatible
PerCP, Allophycocyanin (APC) and APC-based tandems	Compatible
R-phycoerythrin (R-PE) and R-PE based tandems	Use R-PE and R-PE based tandems **after** the EdU detection reaction
Quantum Dots	Use Quantum Dots **after** the EdU detection reaction
Fluorescent proteins (e.g. GFP)	Use anti-GFP antibodies* **before** the EdU detection reaction or use organic dye-based reagents for protein expression detection

* Compatibility indicates which involved components are unstable in the presence of copper catalyst for the EdU detection reaction (either the fluorescent dye itself or the detection method). Not all GFP antibodies recognize the same antigen site. Rabbit and chicken anti-GFP antibodies result in a good fluorescent amount. The mouse monoclonal antibodies tested are not recommended for this application because they do not generate an acceptable amount of fluorescence.

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Please read the material safety data sheets (MSDS) provided for this kit.

Cautions:

EdU (**Component A**): Danger H340-H360
P202-P280-P308+P313

Fixative solution (**Component D**): Warning H317-H351
P280-P302+P352a-P308+P313

Catalyst Solution (**Component F**): Warning H302-H315-H319-H400-H410
P280-P301+P312a-P302+P352a-P305+P351+P338

Saponin based permeabilization and wash reagent (**Component E**): contains sodium azide. This solution is orange.

MSDS: the appropriate MSDS can be downloaded from our website www.carlroth.com.

Literature Citation: When describing a procedure for publication using this product, please refer to it as *Carl Roth's ROTI®kit for Flow Cytometry (EdU Click FC)*.

1. Materials provided with the kit and storage conditions

Table 2: Contents of the kit and storage conditions

Vial-label	Amount for 50 assays	Component	Component long term storage	Kit storage*
Component A	10 mg	5-Ethynyl-deoxyuridine (5-EdU)	-20 °C	2 – 8 °C Dark Do not freeze Dry
Component B red	130 µL	6-FAM Azide (EdU Click FC-488) 5-TAMRA-PEG3-Azide (EdU Click FC-555) 5/6-Sulforhodamine101-PEG3-Azide (EdU Click FC-594) Eterneon-Red 645 Azide (Cyanine 5 Azide analogue) (EdU Click FC-647)	-20 °C dark	
Component C	5 mL	DMSO	RT	
Component D	5 mL	Fixative solution (4% Paraformaldehyde in PBS)	2 – 8 °C	
Component E	50 mL	Saponin-based permeabilization and wash reagent (10x solution)	2 – 8 °C	
Component F green	2 mL	Catalyst solution	RT	
Component G	400 mg	Buffer additive	-20 °C	

***This kit is stable up to 1 year after receipt, when stored as directed.

2. Required Material and Equipment not included in this kit

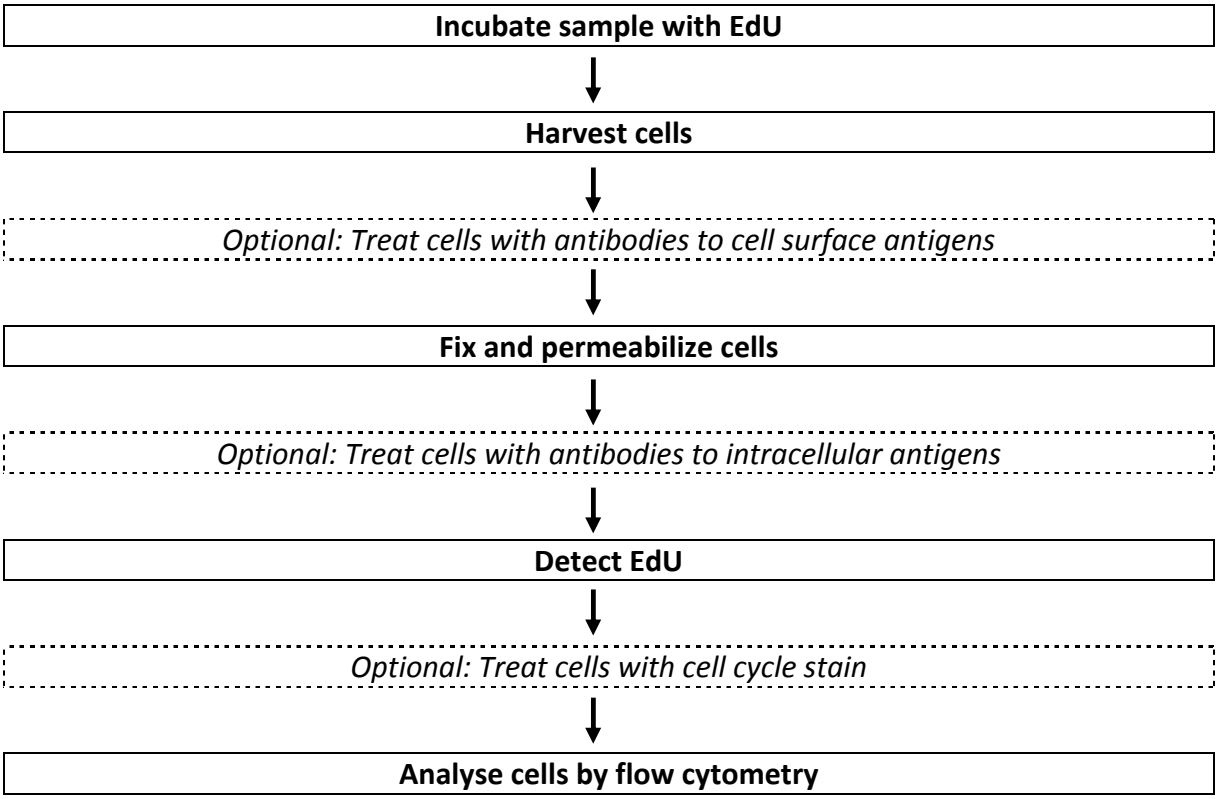
- Adherent cells
- Reaction tubes (size depends on the volume of reaction cocktail needed)
- Buffered saline solution, such as PBS, DPBS or TBS
- Appropriate cell culture medium
- 1% BSA (bovine serum albumin) in PBS, pH 7.1 – 7.4
- 18 MΩ purified water
- Flow cytometry tubes

3. Workflow

The following protocol was developed using an EdU concentration of 10 µM and can be adapted for any cell type. There are many factors which can influence the labeling such as the growth medium, the density and the type of cells. To determine the optimal concentration for your experiment, a range of EdU concentrations should be tested for your cell type and experimental conditions.

Principally, a similar concentration to BrdU can be used for EdU as a starting point. Heparin can be used as anticoagulant for collection, if a whole blood sample is used.

Workflow scheme for the *EdU Click FC Assay*



4. Preparation of the stock solutions

4.1 Allow all vials to warm to room temperature before opening.

4.2 For the preparation of a 10 mM stock solution of EdU, add the appropriate amount of DMSO (**Component C**) or aqueous solution (PBS) to EdU (**Component A**) according to **table 3** and mix until the compound is completely dissolved. After use, store any remaining solution at -20°C. When stored as directed, this stock solution is stable for up to one year.

Table 3: Amounts of DMSO or aqueous solution needed to dissolve EdU to a final concentration of 10 mM

EdU amount	DMSO/aqueous solution amount
10 mg	4 mL

4.3 For the preparation of a 10x stock solution of the buffer additive, add 5.5 mL of deionized water to the **Component G** and mix until the compound is dissolved completely. After use, store any remaining solution at -20°C. When stored as directed, this stock solution is stable for up to 6 months. If the solution starts to develop a brown colour, it has degraded and should be discarded. We recommend preparing aliquots to avoid repeated thaw and freeze cycles!

4.4 For the preparation of 500 mL of the 1x saponin-based permeabilization buffer and wash reagent (for 50 assays), add 50 mL of **Component E** to 450 mL of 1% BSA in PBS. For the preparation of 1 L of the 1x saponin-based permeabilization buffer and wash reagent (for 100 assays), add 100 mL of **Component E** to 900 mL of 1% BSA in PBS After use, store any remaining solution at 2 - 8°C.

Note: The saponin-based permeabilization and wash reagent contains sodium azide.

5. Labeling of cells with EdU

5.1 Suspend the cells in an appropriate tissue culture medium to obtain optimal cell growth conditions. Please note that the growth of the cells during incubation decelerates, if the temperature changes or the cells are washed prior to incubation with EdU.

5.2 For the desired final concentration, add the appropriate amount of EdU to the culture medium and mix well. We recommend using a concentration of 10 µM for 1-2 hours as a starting point. Use higher EdU concentrations for a shorter incubation time. A longer incubation time requires lower EdU concentrations.

5.3 The incubation of the cells with EdU should be performed under the optimal conditions for your cell type and for the desired length of time. Various DNA synthesis and proliferation parameters can be evaluated by altering the EdU incubation time or by subjecting the cells to pulse labeling with EdU. Effective time intervals for pulse labeling and the length of each pulse depend on the cell growth rate.

5.4 Harvest cells. If performing antibody surface labeling, proceed immediately to step **6**, otherwise continue to step **7**.

6. Staining cell-surface antigens with antibodies (optional)

6.1 Wash cells with 3 mL of 1% BSA in PBS. Centrifuge to pellet cells and remove supernatant.

6.2 Dislodge the pellet and resuspend cells in 1% BSA in PBS at 1 x 10^7 cells/mL.

6.3 Add 100 µL of cell suspension or whole blood sample to flow tubes.

6.4 Add surface antibodies and mix well.

Note: PE, PE-tandem or Quantum Dot antibody conjugates should not be used before performing the click reaction (step **8**).

6.5 Incubate the cells for the recommended length of time and temperature. Protect from light!

6.6 Proceed to step **7**.

7. Cell fixation and permeabilization

This protocol was developed with a fixation step using 4% Paraformaldehyde in PBS, followed by a saponin-based permeabilization step. The saponin-based permeablization and wash reagent can be used with cell suspensions containing red blood cells or whole blood as well as with cell suspensions containing different cell types. The morphological light scatter characteristics of leukocytes are maintained by the permeabilization reagent while red blood cells are lysed.

7.1 Remove the incubation media and wash the cells with 3 mL of 1% BSA in PBS. Pellet the cells and remove the supernatant.

7.2 Dislodge the cell pellet. Add 100 µL of the fixative solution (**Component D**) to the cells. Mix well and incubate for 15 minutes at room temperature. Protect from light.

7.3 Remove the fixation solution and wash the cells with 3 mL of 1% BSA in PBS. Pellet the cells and remove the supernatant. If red blood cells or haemoglobin are present in the sample, repeat the washing step. Remove all residual blood cell debris and haemoglobin before proceeding.

7.4 Dislodge the cell pellet. Resuspend the cells in 100 µL of 1x saponin-based permeabilization buffer in PBS (prepared in **4.4**). Mix well and proceed to step **8.** for the click reaction.

8. EdU detection

8.1 Prepare the assay cocktail in the same order as described in **table 4**. If the ingredients are not added in the order listed, the reaction will not proceed optimally or might even fail.

Important: Once the assay cocktail is prepared, use it immediately, at least within the next 15 minutes!

Table 4: Click assay cocktails

Material	Component	Number of assays				
		1	2	3	5	10
PBS, DPBS or TBS	Not provided!	438 µL	875 µL	1.32 mL	2.19 mL	4.38 mL
Catalyst solution	**F - green**	10 µL	20 µL	30 µL	50 µL	100 µL
Dye Azide (10 mM)	**B - red**	2.5 µL	5 µL	7.5 µL	12.5 µL	25 µL
Buffer additive (10x) (prepared in **4.3**)	**G**	50 µL	100 µL	150 µL	250 µL	500 µL
Total Volume	-	**500 µL**	**1 mL**	**1.5 mL**	**2.5 mL**	**5 mL**

8.2 Add the appropriate amount of the assay cocktail to the cells and mix well to distribute the assay solution evenly.

8.3 Incubate the assay mixture for 30 minutes at room temperature. Protect from light!

8.4 Wash the cells with 3 mL of 1x saponin based permeabilization and wash reagent (prepared in **4.4**). Pellet the cells and remove the supernatant. Dislodge the cell pellet. If proceeding with intracellular antibody labeling in step **9**, resuspend the cells in 100 µL of 1x saponin-based permeabilization and wash reagent. Otherwise, add 500 µL of 1x saponin-based permeabilization and wash reagent and proceed with step **10** for analyzing the cells with a flow cytometer.

Important: Keep the samples protected from light during the whole procedure.

9. Staining intracellular or surface antigens (optional)

9.1 Add antibodies against intracellular antigens or against surface antigens that use RPE, PR-tandem or Quantum Dot antibody conjugates. Mix well.

9.2 Incubate the tubes for the time and temperature required for antibody staining. Protect from light.

9.3 Wash each tube with 3 mL 1x saponin-based permeabilization and wash reagent (prepared in **4.4**). Pellet the cells and remove the supernatant. Dislodge the cell pellet and resuspend the cells in 500 µL of 1x saponin-based permeabilization and wash reagent.

9.4 Proceed with step **10** for analyzing the cells with a flow cytometer.

10. Imaging and analysis

Use a low flow rate during acquisition, if a traditional flow cytometer with a hydrodynamic focusing is used to measure the total DNA content. The same collection rate and cell concentration should be used for each sample within an experiment. Detect the fluorescent signal generated by DNA content stains with linear amplification. The fluorescent signal generated by EdU labeling is best detected with logarithmic amplification.

The Excitation and emission maxima of the available dyes are listed in **table 5**.

Table 5: Emission and excitation maxima of the available dyes.

Product number	Dye	Excitation (nm)	Emission (nm)	Filter
7779.1	6-FAM-Azide	496	516	Green
7780.1	5-TAMRA-PEG3-Azide	546	579	Violet
7781.1	5/6-Sulforhodamine 101-PEG3-Azide	584	603	Orange
7783.1	Eterneon-Red 645 Azide (Cyanine 5 Azide analogue)	643	662	Red

***Your notes*:**

***Your notes*:**

***Your notes*:**

Ordering information:

(for detailed kit content see Table 2)

ROTI®kits for Flow Cytometry (for 50 assays):

Product number	Product	Used fluorescent dye
7779.1	EdU Click FC-488	6-FAM-Azide
7780.1	EdU Click FC-555	5-TAMRA-PEG3-Azide
7781.1	EdU Click FC-594	5/6-Sulforhodamine 101-PEG3-Azide
7783.1	EdU Click FC-647	Eterneon-Red 645 Azide (Cyanine 5 Azide analogue)

To place your order, please contact us:

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