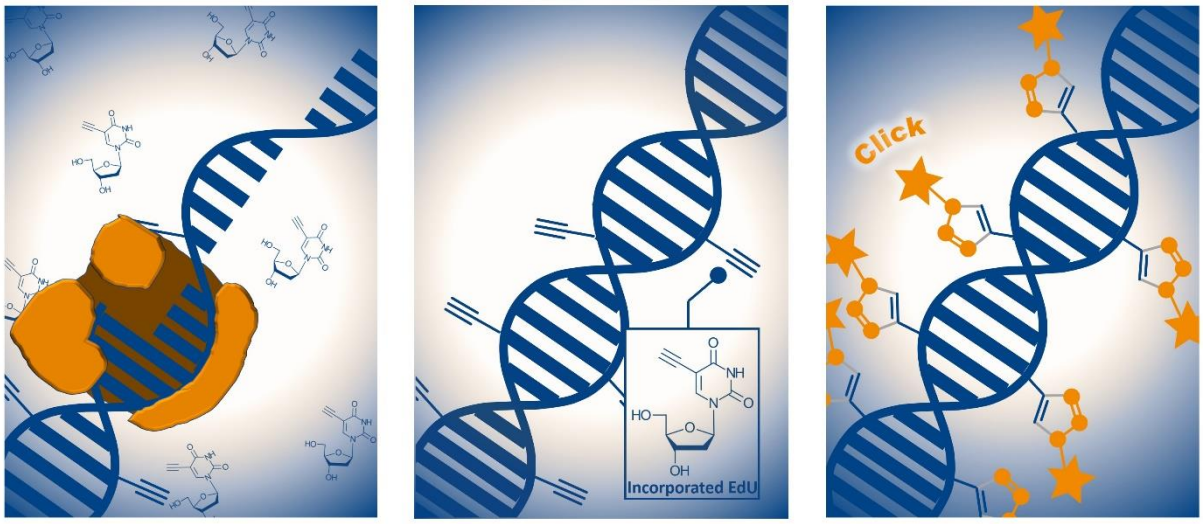
User Manual

ClickTech EdU Cell Proliferation Kit

for High-throughput Screening



Version 7.0

Ordering information

(for detailed kit content see **Table 2**)

ClickTech EdU HTS Kits ready for 2 x 96 well plate assays:

Product number	20X EdU in PBS	Used fluorescent dye
BCK-EdU488HTS2	2 mL	6-FAM Azide (λ_{abs}=496/λ_{em}=516)
BCK-EdU555HTS2	2 mL	5-TAMRA-PEG3-Azide (λ_{abs}=546/λ_{em}=579)

The Kit contains sufficient material for 2 x 96 well plate assays

EdU HTS Kits ready for 4 x 96 well plate assays:

Product number	20X EdU in PBS	Used fluorescent dye
BCK-EdU488HTS4	2x 2 mL	6-FAM Azide (λ_{abs}=496/λ_{em}=516)
BCK-EdU555HTS4	2x 2 mL	5-TAMRA-PEG3-Azide (λ_{abs}=546/λ_{em}=579)

The Kit contains sufficient material for 4 x 96 well plate assays

For References, FAQs and ordering please see online or contact us:

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ClickTech EdU Cell Proliferation Kit for High-throughput Screening

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.

The baseclick *EdU HTS Kits* overcome these limitations, providing a superior alternative to BrdU and [^{3}H]thymidine assays for directly measuring DNA synthesis of adherent cells in 96 well plates. 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 HTS Assays* are not antibody based and therefore do not require DNA denaturation for detection of the incorporated nucleoside. Instead, the *EdU HTS Kits* 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. In principle, the *EdU HTS Assays* can be performed also on suspension cultures, but this will require centrifugation at all incubation/washings step, which will decrease their practicability with HTS formats.

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Please read the material safety data sheets (MSDS) provided for each product/component.

Literature Citation: When describing a procedure for publication using this product, please refer to it as *baseclick ClickTech EdU HTS Kit*.

The baseclick *ClickTech EdU HTS Kit* 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.

Cautions:

The rinse buffer (**Component R**): contains hazardous components. Use with appropriate precautions. Keep away from acids to avoid dangerous gases.

Handle reagents containing the rinse buffer using equipment and practices appropriate for the hazards posed by such materials. Use gloves. Dispose of the reagents in compliance with all related local arrangements. For the correct handling we refer you to the MSDS which can be downloaded from our webpage www.baseclick.eu

This solution is stored at RT and will crystallize at lower temperatures. If crystallized, the solution has to be brought to RT, mixed thoroughly and can then, once homogenously dissolved, be used without further considerations. The activity of this compound is not affected hereby.

MSDS: the appropriate MSDS can be downloaded from our website www.baseclick.eu.

1. Materials provided with the Kit and storage conditions

Table 2: Contents of the kit and storage conditions

Component code	Amount for 2 assays / well plates	Amount for 4 assays / well plates	Component	Component long term storage	Kit storage
Component E yellow	2 mL	2 x 2 mL	5-Ethynyl-deoxyuridine (5-EdU)	– 20 °C	2 - 8 °C Dark Do not freeze
Component D red	130 µL	2 x 130 µL	• 6-FAM-Azide • 5-TAMRA-PEG3-Azide	– 20 °C dark	
Component RP	40 mL	40 mL	Reaction buffer	RT	
Component C green	1 mL	1 mL	Reactor System	RT	
Component B	200 mg	2 x 200 mg	Buffer additive	RT / – 20 °C*	
Component R	6 mL	2 x 6 mL	Rinse buffer (10X)	RT	

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

* When dissolved the component has to be kept at – 20 °C for long-term storage.

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, D-PBS or TBS
- Fixative solution (4% Paraformaldehyde in PBS)
- Permeabilization solution optimised for your cell line (for example, 0.5% Triton® X-100 in PBS, or a 0.5% saponin-based solution)
- Appropriate cell culture medium
- 1% BSA (bovine serum albumin) in PBS, pH 7.1 - 7.4
- Deionized water or 18 MΩ purified water

3. Workflow

The following protocol was developed using a final 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.

Workflow scheme for the *EdU HTS Assay*

Incubate sample with EdU

↓

Optional: Treat cells with antibodies to cell surface antigens

↓

Fix and permeabilize cells*

↓

Optional: Treat cells with antibodies to intracellular antigens

↓

Detect / Label EdU

↓

Wash cells well with rinse buffer

↓

Optional: Treat cells with cell cycle stain

↓

Imaging and Analyse Cells

* At this point the sample can be stored safely

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 20 µM stock solution of EdU (2X EdU), add the appropriate amount of aqueous solution (1X PBS) to EdU (**component E**) 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 aqueous solution needed to dissolve EdU to a final concentration of 20 µM

EdU HTS Kit	20X EdU solution	In dilution Volume for 2X EdU solution in PBS
2 x 96 well plates	2 mL	18 mL
4 x 96 well plates	4 mL	36 mL

4.3. For the preparation of a stock solution of the buffer additive, add the appropriate amount of deionized water (see **Table 4**) to the **component B** 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. We recommend preparing aliquots to avoid repeated thaw and freeze cycles!

Table 4: Amounts of aqueous solution needed to dissolve the buffer additive to the final work solution

EdU HTS Kit	Buffer additive (solide)	Dilution volume of deionized water
2 x 96 well plates	200 mg	2.5 mL
4 x 96 well plates	400 mg	5 mL

5. Labeling of cells with EdU

5.1. Plate cells in appropriate tissue culture medium to obtain optimal cell growth conditions.

5.2. Please note that, if the cells are washed prior to incubation with EdU or all of the medium is exchanged with EdU-containing one, cell growth may slow down due to transient perturbation (e.g., temperature decrease, dryness). We recommend applying a final concentration of 10 µM by adding a volume of pre-warmed medium containing an accordingly higher concentration of EdU without removing any of the medium in the culture vessel, i.e., half the volume (relative to the volume already in the vessel) of medium containing 30 µM EdU. We recommend to incubate for 1 - 4 hours as a starting point. Use higher EdU concentrations for a shorter incubation time. A longer incubation time requires lower EdU concentrations in order to avoid genotoxicity.

5.3. The incubation of the cells with EdU should be performed under the optimal conditions for your cell type, the number of cells plated 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. If performing antibody surface labeling, proceed to step **6**, otherwise continue to step **7**.

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

6.1. Wash cells in each well with 100 µL of 1% BSA in PBS.

6.2. Remove the wash solution and add again 100 µL of 1% BSA in PBS to the cells.

6.3. Add surface antibodies and mix well but gently.

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

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

6.5. Proceed to step **7**.

7. Cell fixation and permeabilization

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

7.1. Remove the incubation media and wash the cells, each well with 100 µL of 1% BSA in PBS. Afterwards remove the wash solution.

7.2. Add 100 µL of the fixative solution to the cells in each well. Incubate for 15 minutes at room temperature. Protect from light.

7.3. Remove the fixation solution and wash the cells in each well twice with 200 µL of 1% BSA in PBS. 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.

NOTE: At this point of the procedure the probes can be stored safely.

7.4. Remove the wash solution and add to each well 100 µL of permeabilization solution. Mix well but gently, incubate for 20 minutes at room temperature and proceed to step **8** for the click reaction.

8. EdU detection

8.1. Prepare the click assay cocktail in the same order as described in **Table 5**. 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 5: Click assay cocktails

Material	Component code	Number of well plates		
		1	2	4
Reaction buffer	**RP**	9.635 mL	19.27 mL	38.54 mL
Reactor System	**C - green**	220 µL	440 µL	880 µL
Dye Azide (10 mM)	**D - red**	55 µL	110 µL	220 µL
Buffer additive (prepared in 4.3)	**B**	1.1 mL	2.2 mL	4.4 mL
Total Volume	-	**11.01 mL**	**22.02 mL**	**44.04 mL**

8.2. Remove permeabilization solution from step **7.4** and add 100 µL of the click assay cocktail to each well and mix well but gently to distribute the assay solution evenly.

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

8.4. From the 10x rinse solution prepare a 1x rinse solution by applying following table (**Table 6**). Add the appropriate amount of PBS (1X) (see **Table 6**) to the **component R** and mix well (To prevent crystallization, keep **component R** at room temperature at all times. If **component R** has crystalized, please warm up to dissolve again. Please see also "cautions"). This additional wash step with this special rinse buffer reduces unspecific, cell number dependent background signal.

Table 6: Amounts of aqueous solution needed to dissolve the rinse buffer to the final work solution

EdU HTS Kit	Volume of 10X rinse buffer	Dilution volume of 1X PBS
2 x 96 wellplate Kit	5.8 mL	52.2 mL
4 x 96 wellplate Kit	11.5 mL	103.5 mL

Remove Click assay cocktail and wash the cells in each well twice with 150 µL with the 1X rinse solution prepared above.

8.5. Remove rinse solution. 100 µL of 1% BSA in PBS is then given to the cells in each well.

8.6. If performing antibody surface or intracellular labeling, proceed to step **9**, otherwise continue to step **10**.

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 cells for the time and temperature required for antibody staining. Protect from light.

9.3. Wash each well twice with 100 µL permeabilization solution. Remove the solution. Add again 100 µL of 1% BSA in PBS to the cells.

9.4. Proceed with step **10** for analyzing the cells.

10. Imaging and analysis

10.1. Close the 96 well plate by using a sealing film, if desired.

10.2. Fluorescence is quantified by scanning the plate using an automated imaging platform equipped with filters appropriate for the dye used. Images of each well can be taken by microscopy.

The Excitation and emission maxima of the available dyes are listed in **Table 7**.

Table 7: Emission and excitation maxima of the available dyes.

Product number	Dye	Excitation (nm)	Emission (nm)	Filter
BCK-EdU488HTS	6-FAM Azide	496	516	**green**
BCK-EdU555HTS	5-TAMRA-PEG3-Azide	546	579	**yellow**

11. Example of the data derived from an EdU HTS Kit based experiment:

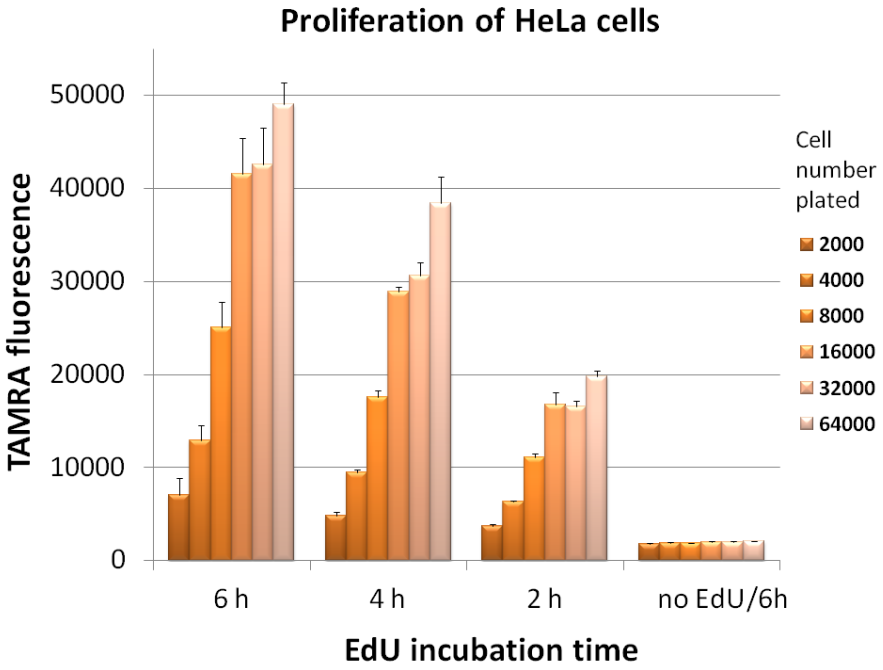

Figure 1: Detection of EdU incorporation depending on cell number and EdU incubation time.

HeLa cells were seeded in a transparent 96 well cell culture plate with indicated cell numbers per well. After 42 h cells were incubated with or without 10 µM EdU for 2, 4 or 6 h and subsequently EdU incorporation was detected using the ClickTech EdU-HTS Assay Kit and an automated imaging platform. Mean and SD values from quadruplicates are shown.

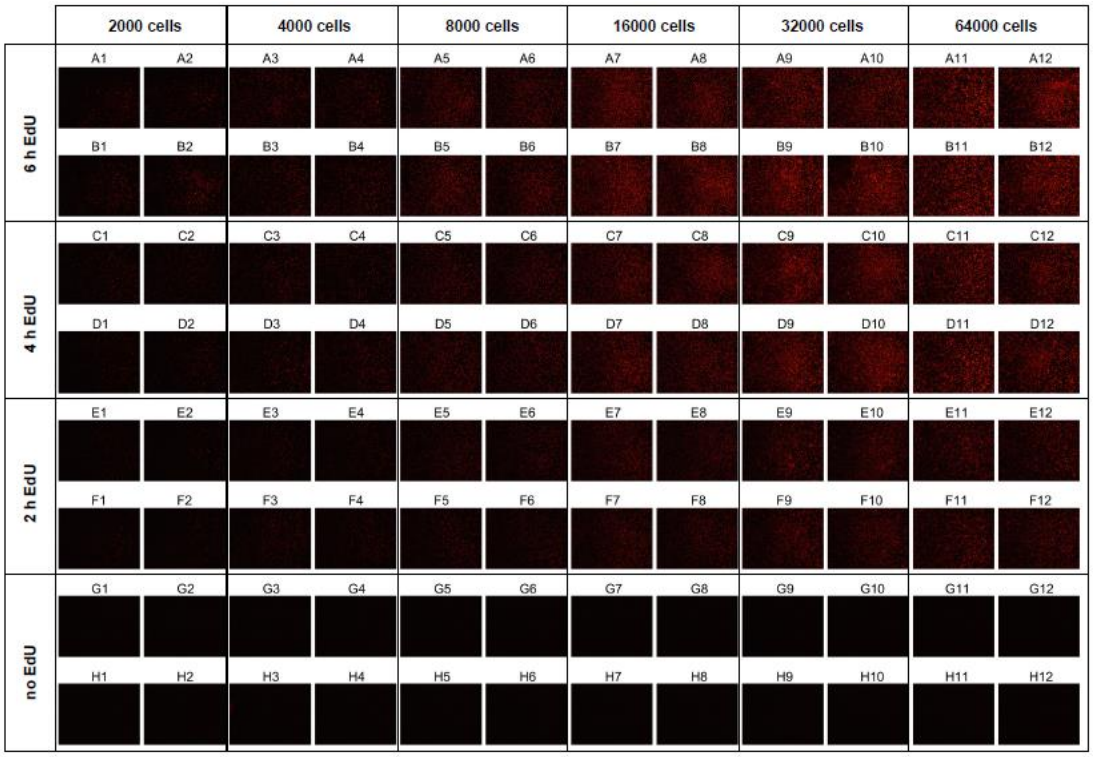

Figure 2: Detection of EdU incorporation via fluorescence microscopy.

A fluorescence photo (40x) of the center of each 96 well of the assay plate washed with rinse buffer. The picture was captured and presented using the Nikon NIS-elements software.

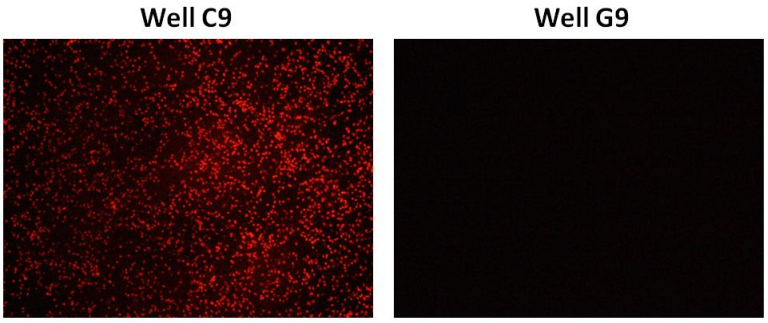

Figure 3: Zoom on the samples after Click reaction and washing (in Figure 2) cells, which do EdU proliferation in well C9 and cells, which have not received EdU, in well G9.

Analysis using a conventional plate reader or plate reader module is also possible, but raises two potential caveats, depending on the density of the cells: 1) sensitivity: if cell density is too low the signal will be too weak for meaningful results; 2) uneven distribution of the signal: microplate readers are designed for measuring dyes in relatively homogeneous solutions, typically with a few sampling spots being irradiated in each well (signals across each well are automatically averaged). If the cells are plated at low density, they may not be homogeneously distributed, potentially leading to unreliable results. To minimize potential bias, in addition to keeping cell density as high as possible, the instrument should be set to measure at a high number of sampling spots in each well.