EdU staining

Invitrogen™ Click-iT™ EdU Alexa Fluor™ 488 HCS Assay (Manual)

Invitrogen™ EdU (5-ethynyl-2'-deoxyuridine) (Manual)

> EdU (5-Ethynyl-2'-deoxyuridine)
> Molecular weight: 252.22 g / mol

Solubility:

Solvent	Maximum solubility	Concentration
DMSO	25 mg / mL	100 mM
water	6.25 mg / mL	25 mM
aqueous buffer	6.25 mg / mL	25 mM

Materials NOT Provided

Please refer to the last page to make the PBS 10X stock and fixative solution.

Material	Concentration	Storage location
Phosphate buffer saline (PBS)	1X	JK's bench
Fixative solution	4%	biosafety cabinet
Fluoromount-G	—	4°C (d)

Procedure

Step 1: Labeling

1. Prepare **incubation medium** containing **10 μM Component A (EdU)**;

- Component A (100 μM stock) is stored at 4°C (e-bottom-left) JK's green box (top-left corner side).
- Generally we make 40 mL incubation medium in 50 mL falcon tube for each treatment groups. For example, if we have 6 treatment groups, add 25 μL Component A stock (100 μM) into 250 mL medium and mix thoroughly. Then, dispense 40 mL to each tube.

> The manufacturer's manual suggests to dilute the EdU from 10 mM to 10 μM for *in vitro* experiment. When EdU was dissolved in DMSO, this concentration of DMSO might facilitate the entry of organic molecules into tissue. However, 0.1% of DMSO will affect root growth. So, consider to make higher concentration of EdU stocks, change solvent, or reduce the working solution concentration. Currently, this protocol uses higher concentration of EdU stock (100 μM), and the staining result is statisfiying.

2. Incubate the plants in the **incubation medium** in the same cultivation condition for **1 hour**.

- Directly immersed the roots into the solution, **DO NOT** cut the shoot part, the plant should still alive. Fully submerged the root into the solution. Do not over-pushing, avoid roots injury.

Step 2: Fixation

1. Prepare **400 μL fixative solution** in eppendorf for each group of sample;
2. Cut the root tip (around 1 cm) and fully immerse in the fixative solution;

- Better to do this in biosafety cabinet. If too many roots, cut the root on a rigid agar plate (~ 1%), keep the surface wet with 1X PBS to prevent the root dried.

3. Incubate **24 hours** at **room temperature**;

> - Incubation time can be varying from 30 minutes to 24 hours, depends on the sample condition. The longer the incubation time, the weaker the background autofluorescence and the EdU signal. When dealing with thick tissues, short incubation time may results incomplete penetration of the fixative solution, background autofluorescence, and too-strong EdU signal (false positive??). We incubate 24 hours to achieve clean background and optimal EdU signal intensity.

4. Remove fixative solution and add **400 μL 1X PBS** to wash the roots. Gently pipetting 5-10 times (avoid touching the root tip), then leave on the benchtop for 10 mins. Repeat this wash procedure around 5 times (**5 × 10 mins**) until no bubbles appear.

> - **Discard the fixative solution in the dark blue toxic carboy under the toxic cabinet;**

5. (optional) You can keep the samples in 1X PBS solution up to 48 hours in 4°C protected from light after washing out the fixative solution.

Step 3: Detection

Prepare the cocktail

1. Place all the components **on ice** and **protected from light**. Prepare the cocktail as much as necessary only for that day's experiments, and use on the same day.

> - Component B, C, and D are stored at 4°C, in the kit box.
> - Component E (10X stock) is stored at −20°C in JK's white box.
> - Component F is stored at 4°C, placed in front of the kit box.

2. Add the **Click-iT® reaction cocktail** ingredients **as follow**.

- **Add the incredients in the order listed in the table**; otherwise, the reaction will not proceed optimally.
- The cocktail must be **used immediately** after preparation. The Click-iT® reaction buffer additive is susceptable to oxidation and is the limiting factor to the Click-iT® reaction cocktail's effectiveness over time.

Component	Material name	Addition per sample	Note
ddH_2O	deionized ultrapure water	171 μL	
C	**10X** Click-iT® EdU reaction buffer	17 μL	
D	$CuSO_4$	8 μL	
B	Alexa Fluor® azide	0.5 μL	keep in dark
E	**10X** Click-iT® EdU buffer additive	2 μL	add before use
Total:		**198.5 μL**	**take 195 µL**

Incubation

3. Remove wash solution and add **195 μL** of **Click-iT® reaction cocktail** (prepare as the table above) for each sample;

4. Incubate for **30 minutes** at **room temperature**. Must be **protected from light**;

5. Remove the reaction cocktail and wash once with **400 μL** of **Click-iT® reaction rince buffer (Component F)** (1 × 15 mins);

6. **Wash 5 times** with 1X PBS (**5 × 15 mins**);

- You can keep the samples in 1X PBS solution or reaction rince buffer (Component F) up to 24 hours in 4°C and **protected from light** after washing out the cocktail. If kept more than 24 hours, background fluorescence could be quite obvious. Before mount on slide, wash the sample again with 1X PBS for at least twice (2 × 15 mins) to minimize background autofluorescence.

7. Proceed to confocal imaging and analysis.

Step 4: Confocal parameters

Mount on slide with **Fluoromount-G anti-fade** solution (located at 4°C (d)). Using ddH_2O is also fine if the laser intensity is low and the laser exposure time (image capturing time) is short.

Set the confocal parameters as follow:

Parameters	Theoretical value	Our machine
Excitation peak	495 nm	488 nm
Emission peak	519 nm	499 ~ 539 nm
Magnification		10X
Laser intensity (**488 nm**)		16.0%
Master Gain		700 V
Digital Gain		1.0
Pinhole		≈ 32 μm
Z-stack interval		4 μm
Scan speed		6
Scan direction		↔

Step 5: Image processing

1. Use **ImageJ** with **bioformats_package.jar** plugin to proceed the confocal images;

> - The plugin (bioformats_package.jar) can be downloaded from https://www.openmicroscopy.org/bio-formats/downloads/
> - The plugin should be placed in the ***"/ImageJ/plugins/jars"*** directory

2. Open the confocal .czi file with the ImageJ, the "**Bio-Formats Import Options**" will automatically pop up;

3. Select the options as follow:

Options	Choose
View stack with:	Hyperstack
Color mode:	Colorized
Autoscale	☑

4. Stack the image layers and perform max intensity projection along the Z-axis;

> - `Image` → `Stacks` → `Z project...` → `Projection type: Max Intensity`
> - Optional: Shows scale bar in the image.
> - `Analyze` ⟶ `Tools` ⟶ `Scale Bar...`

5. Save the images as TIFF format;

> - `File` → `Save as` → `Tiff...`

6. Select the region of interest (ROI) and measure.

Done !!!

Materials Provided by the kit

Component	Material name	C10351 *	Concentration	Location
A α	EdU **working solution**	525 µL	10 mM	4°C (c)
B β	Alexa Fluor® azide 488	330 µL	1X	4°C (c)
C γ	Click-iT® EdU **reaction buffer**	15 mL	10X	4°C (c)
D δ	$CuSO_4$	1 vial	100 mM	4°C (c)
E ε	Click-iT® EdU **buffer additive**	400 mg	10X	−20°C
F ζ	Click-iT® reaction **rinse buffer**	125 mL	1X	4°C (c)

* **C10351**: Catalogue number. All the raw materials in this kit should be stored at **2 ~ 6°C**, **dessiccated**, **protect from light**, and **DO NOT FREEZE**.

α **Component A**: This is the EdU chemical stock solution. Dilute to 10 μM in complete medium on the day of the experiment, and use immediately. The 10 mM stock solution is stored at −20°C (the EdU powder also put in −20°C, in JK's white box), and the 10 mM aliquots are stored at 4°C (in the EdU kit box, put together with the other components).

β **Component B**: Fluorescence probe. Protected from light.

γ **Component C**: Dilute from 10X to 1X using ddH_2O, *i.e.*, 15 mL 10X Component C + 135 mL ddH_2O.
The 1X solution could be stored at 2 ~ 6°C for 6 months.

δ **Component D**: Catalyze the reaction.

ε **Component E**: Add 2 mL ddH_2O to the vial of the Component E, mix until fully dissolve the powder to 10X solution. The stock should be dispense into several aliquots (400 μL each eppendorf for example) to avoid too frequent thawing and freezing, to prolong the storage lifespan. The **10X solution** could be stored at **≤ −20°C** for up to 1 year. If the solution develops a brown color, it has degraded and should be discarded.

ζ **Component F**: Used to wash cells after the Click-iT® reaction cocktail incubation step. This helps to remove unreacted components and reduce background. Located at 4 °C (c), in front of the EdU kit boxes.

Materials NOT Provided by the kit

Phosphate buffer saline (PBS) (pH 7.4)

- Make 10X PBS stock solution first as follow (10X stock located at 4°C bottom right). When in use, dilute to 1X PBS.
- 10X PBS ⟶ Adjust to pH 7.4 ⟶ Autoclave ⟶ Dilute to 1X (100 mL 10X PBS + 900 mL ddH_2O)

10X PBS contents	M.W. (g/mol)	Addition
NaCl	58.44	80.1 g
KCl	74.55	2.0 g
Na_2HPO_4	141.96	14.4 g
KH_2PO_4	136.09	2.7 g
ddH_2O		1 L

Fixative solution

Chemical	Addition	Final concentration	Storage location
Formaldehyde (38%)	105 µL	4%	Toxic cabinet D
Triton X-100 (100%)	1 µL	0.1%	IV
1X PBS	894 µL		
Total:	**1000 µL**		

Example images

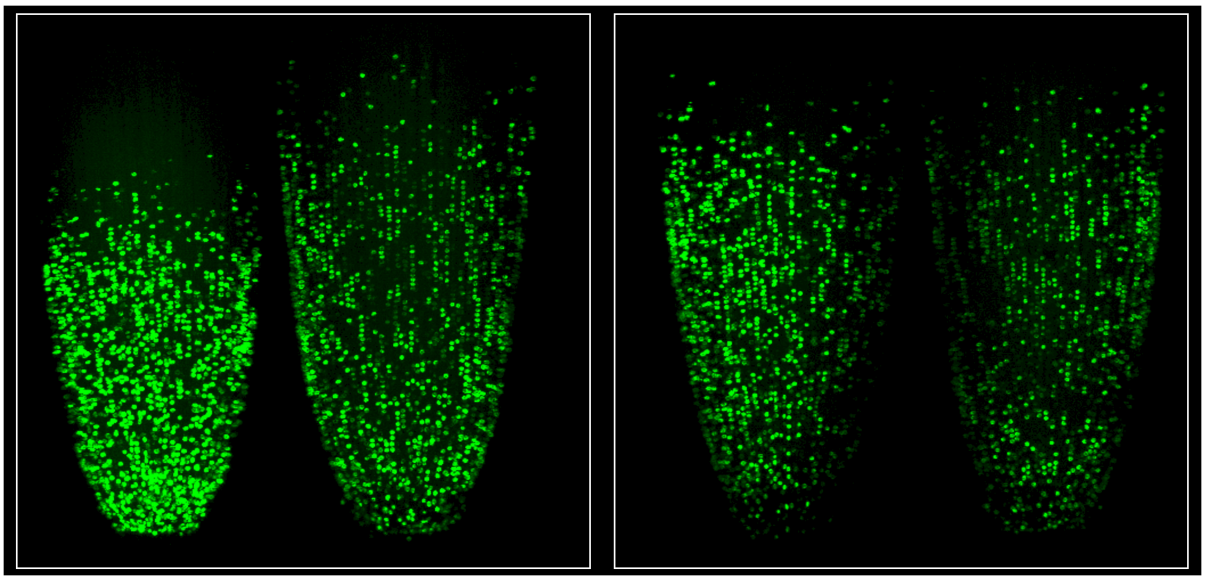


Figure 1. Example of EdU autofluorescence background (left panel) and incomplete stained (right panel).

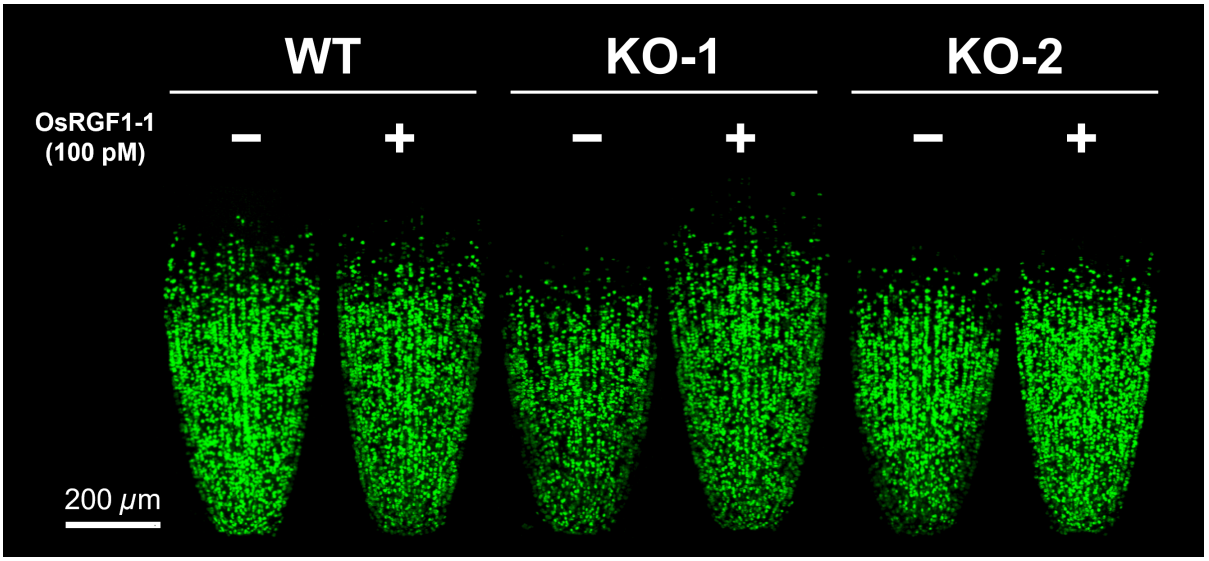


Figure 2. Example of normal EdU signal.