EdU staining

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

> 5-Ethynyl-2'-deoxyuridine
> 252.22 g/mol
> Maximum solubility: 25 mM in water and up to 100 mM in DMSO.

- "**ddH_2O**" means deionized ultrapure water;
- "**complete medium**" means the medium used to cultivate the samples, for example, half-MS medium in this case.

Procedure

Step 1: Labeling

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

- For example, if we have five groups of samples, add **100 μL 10 mM Component A** into 100 mL half-MS medium for the experiment. Then, dispense the diluted 10 μM Component A to new falcon tubes, each tube takes around 20 mL for each sample (make sure the root tips can be fully submerged in the solution).

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

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

Step 2: Fixation

1. Prepare **200 μL fixative solution** for each sample (per eppendorf);

2. Cut the root tip (around 5 mm) and fully immerse in the fixative solution;

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

3. Incubate **30 minutes** under **room temperature**;

4. Remove fixative solution and add **400 μL 1X PBS** to wash the roots. Gently pipetting 5-10 times (avoiding contact with the root tip), then leave on the benchtop for 10 mins. Repeat this wash procedure three times (**3 × 10 mins**).

- **You can keep the samples in 1X PBS solution for at least 24 hours in 4°C protected from light after removing the fixative solution.**

Step 3: Detection

Prepare the cocktail

1. Dilute the **Component C** and **Component E** from 10X to **1X** using **ddH_2O**. Calculate the total usage amount according to the following tables.

- 10X Component C is stored at 4°C in the kit box.
- 10X Component E is stored at -20°C in JK's white box.
- Prepare the Component C and E as much as necessary only for that day's experiments, and use on the same day.

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.***
- ***Use the cocktail 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 197 μL

Incubation

3. Remove wash solution and add **197 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 200 μL of Click-iT® reaction rince buffer (**Component F**);
6. **Wash 3 times** with 1X PBS (**3 × 10 mins**);
7. Mount on slide with **Fluoromount-G anti-fade** solution[†]. Proceed to confocal imaging and analysis.

[†] *Using ddH_2O is also fine if the laser intensity is low and the laser exposure time (image capturing time) is short.*

Step 4: Confocal parameters

Parameters	Theoretical value	Our machine
Excitation peak	495 nm	488 nm
Emission peak	519 nm	499 ~ 539 nm
Magnification		10X
Laser intensity (**488 nm**)		1.0%
Master Gain		700 V
Digital Gain		1.0
Pinhole		≈ 32 μm
Z-stack interval		2 μm
Scan speed		7
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.

Step 6: Data analysis

Finished !!!

Materials Provided by the kit

Component	Material name	C10351 *	Concentration
A [α]	EdU **working solution**	525 µL	10 mM
B	Alexa Fluor® azide 488	330 µL	1X
C [β]	Click-iT® EdU **reaction buffer**	15 mL	10X
D	$CuSO_4$	1 vial	100 mM
E [γ]	Click-iT® EdU **buffer additive**	400 mg	10X
F	Click-iT® reaction **rinse buffer**	125 mL	1X

* **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 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.

[γ] **Component E**: Add 2 mL ddH_2O to the vial of the Component E, mix until fully dissolve the powder to 10X solution. 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.

Materials NOT Provided

Material	Concentration	Storage location
PBS (Phosphate buffer saline) δ	1X	
Fixative solution ε	4%	
Fluoromount-G	--	4°C (D cabinet)

δ 1X 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 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

- 10X PBS ⟶ Adjust to pH 7.4 ⟶ Autoclave ⟶ Dilute to 1X (100 mL 10X PBS + 900 mL ddH_2O)

ε 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		