# Cystine Uptake Assay Kit

*Technical Manual*

## General Information

The cystine transporter xCT is a crucial transporter that maintains cellular redox balance by regulating glutathione synthesis via cystine uptake. xCT is highly expressed in several types of cancer cells, and is expected to be a therapeutic target for cancer. For a long time, the conventional method used to assess xCT activity has been a cystine uptake assay using radioisotopes. However, this method requires special handling facilities and disposal of radioactive materials.
The Cystine Uptake Assay Kit allows a simple fluorescence-based assay for cystine uptake via the xCT. The Cystine Analog (CA) in this kit can be taken up into cells via xCT, and the incorporated CA can be specifically detected using the Fluorescent Probe and Reducing Agent. Thus, the xCT activity can be measured easily.

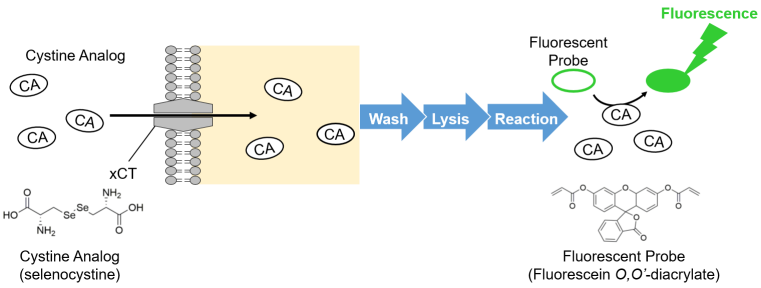


Figure 1. Principle of Cystine Uptake Assay Kit (Patent pending)

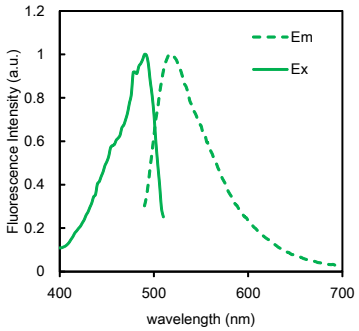


Figure 2. Excitation and emission spectra of the Fluorescent Probe

## Kit Contents

| | 20 tests | 100 tests |
|---|---|---|
| Cystine Analog Solution | 45 µl x1 | 225 µl x1 |
| Fluorescent Probe | x1 | x1 |
| Reducing Agent | x1 | x2 |
| Assay Buffer | 5 ml x1 | 25 ml x1 |

## Storage Condition

Store at -20°C

## Required Equipment and Materials

- Fluorescence microplate reader (Ex/Em = 490/535 nm)
- 96-Well black microplate
- Plate seals
- Incubator (37°C)
- 20-200 and 100-1000 µl micropipettes
- 20-200 µl multichannel pipette
- Medium (cystine-free, serum-free)
- Phosphate-buffered saline (PBS)
- Methanol
- Dimethylsulfoxide (DMSO)

## Precaution

Please tap the tube before opening, and open it with care. The content may have relocated from the bottom of the tube during shipping.
Multiple measurements (e.g., in triplicate) are recommended to obtain accurate data.

## Preparation of Solutions

### 1. Preparation of CA uptake solution

Dilute Cystine Analog Solution 100-fold with cystine-free, serum-free medium, and incubate at 37°C.

| | 20 tests (4 ml) | 50 tests (10 ml) | 100 tests (20 ml) |
|---|---|---|---|
| Cystine Analog Solution | 40 µl | 100 µl | 200 µl |
| cystine-free, serum-free medium | 4 ml | 10 ml | 20 ml |

Note: Prepare CA uptake solution fresh each day. Pre-warm the CA uptake solution in an incubator (37°C) – cystine uptake into cells may be affected by the temperature of the CA uptake solution.

### 2. Preparation of Probe solution

Add DMSO to a tube of Fluorescent Probe and dissolve by pipetting and vortex mixing.

| | DMSO |
|---|---|
| 100 tests | 50 µl |
| 20 tests | 10 µl |

Note: Centrifuge the tube briefly before opening to remove all content from the walls and cap.
Store the Probe solution at −20°C and protect it from light. The Probe solution is stable for 2 months.

### 3. Preparation of Reducing agent (RA) solution

Add $ddH_2O$ to a tube of Reducing Agent and dissolve by pipetting and vortex mixing.

| | $ddH_2O$ |
|---|---|
| 100 tests | 250 µl |
| 20 tests | 100 µl |

Note: Centrifuge the tube briefly before opening to remove all content from the walls and cap.
Store the RA solution at −20°C and protect it from light. The RA solution is stable for 2 months.

## General Protocol

Use a multichannel pipette to reduce the time difference between loading each well.

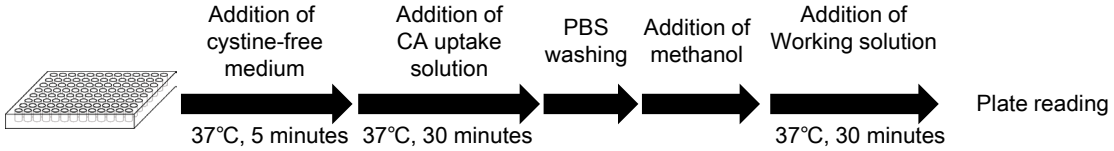


Figure 3. Protocol for plate reading

1. Seed cells in a 96-well microplate and culture the cells at 37°C overnight in a 5% $CO_2$ incubator.
2. Remove the culture medium and wash the cells three times with pre-warmed cystine-free, serum-free medium*1.
3. Add 200 µl of pre-warmed cystine-free, serum-free medium*1 and incubate at 37°C for 5 minutes in a 5% $CO_2$ incubator.
4. Remove the supernatant and add 200 µl of pre-warmed CA uptake solution*1 or pre-warmed cystine-free, serum-free medium*1 (for the blank).
5. Incubate at 37°C for 30 minutes in a 5% $CO_2$ incubator.
6. Remove the supernatant and wash the cells with 200 µl of ice-cold PBS three times.
7. Remove the supernatant and add 50 µl of methanol.
8. Prepare Working solution.*2

| | 20 tests (4 ml) | 50 tests (10 ml) | 100 tests (20 ml) |
|---|---|---|---|
| Probe solution | 8 µl | 20 µl | 40 µl |
| RA solution | 80 µl | 200 µl | 400 µl |
| Assay Buffer | 4 ml | 10 ml | 20 ml |

9. Add 200 µl of Working solution, mix by pipetting, and incubate at 37°C for 30 minutes. *3
10. Measure the fluorescence intensity using a fluorescence plate reader (Ex/Em = 490/535 nm) *4.
11. Subtract the measured value of the blank from the value measured for each sample.

*1 Pre-warm the culture medium and CA uptake solution in an incubator (37°C). Cystine uptake into cells may be affected by the temperature.
*2 Prepare Working solution immediately before use.
*3 When incubating, use a microplate seal to prevent evaporation.
*4 Normalize the fluorescence values by the protein concentration or by the number of cells (determined by nuclear staining), as needed.

## Experimental example

Inhibition of xCT activity by xCT inhibitor erastin (HeLa cells)

1. HeLa cells ($1.5\times10^4$ cells/well, 150 µl) in Minimum Essential Medium (MEM; 10% fetal bovine serum) were seeded in a 96-well black microplate (655090, Greiner) and cultured overnight at 37°C in a 5% $CO_2$ incubator.
2. After removing the supernatant, the cells were washed three times with 200 µl of pre-warmed Dulbecco's modified Eagle's medium (DMEM; cystine-free, serum-free medium, 2 mmol/l glutamine, 21013024: Thermofisher Scientific).
3. Pre-warmed DMEM (200 µl, cystine-free, serum-free medium, 2 mmol/l glutamine) containing 0 µmol/l (sample 1 and the blank) or 100 µmol/l erastin (sample 2) was added, and the cells were incubated at 37°C for 5 minutes in a 5% $CO_2$ incubator.
4. After removing the supernatant, 200 µl of pre-warmed CA uptake solution containing 0 µmol/l erastin (sample 1), 100 µmol/l erastin (sample 2) or DMEM (cystine-free, serum-free medium, 2 mmol/l glutamine, for the blank) were added, and the cells were incubated at 37°C for 30 minutes in a 5% $CO_2$ incubator.
5. After removing the supernatant, the cells were washed three times with 200 µl of ice-cold PBS.
6. After removing the supernatant, 50 µl of methanol were added.
7. Working solution (200 µl) was added, mixed by pipetting, and incubated at 37°C for 30 minutes. The plate was sealed.
8. The fluorescence intensity was measured using a fluorescence plate reader (Ex/Em = 490/535 nm; Infinite m200 PRO, Tecan Trading AG).
9. The fluorescence intensity derived from the incorporated Cystine Analog was calculated by subtracting the blank value from the value measured for each sample.

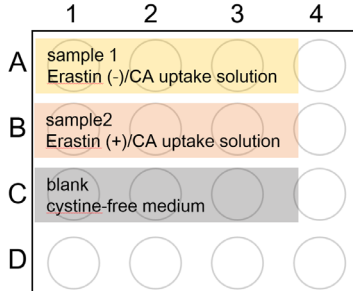


Figure 4. Plate layouts

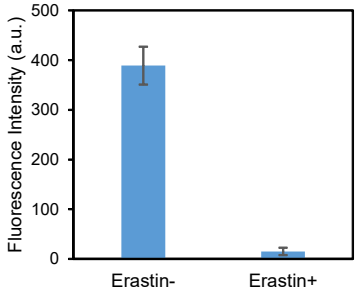


Figure 5. Inhibition of cystine transporter activity in HeLa cells by erastin

## Inhibition of xCT activity by erastin (HL60 cells)

1. HL60 cells were dispensed into nine tubes at $1.0\times10^6$ cells/tube (samples 1, 2, and blank; $n = 3$).
2. The cells were centrifuged at 300 x *g* for 3 minutes.
3. After removing the supernatant, 500 µl of pre-warmed DMEM (cystine-free, serum-free medium, 2 mmol/l glutamine, 21013024: Thermofisher Scientific) were added. The cells were resuspended and centrifuged at 300 x *g* for 3 minutes. This step was repeated twice.
4. Pre-warmed DMEM (500 µl) containing 0 µmol/l (sample 1 and the blank) or 100 µmol/l erastin (sample 2) was added, and the cells were resuspended and incubated at 37°C for 5 minutes in a 5% $CO_2$ incubator.
5. The cells were centrifuged at 300 x *g* for 3 minutes.
6. After removing the supernatant, 500 µl of pre-warmed CA uptake solution containing 0 µmol/l erastin (sample 1), 100 µmol/l erastin (sample 2) or DMEM (blank) were added. The cells were resuspended and incubated at 37°C for 30 minutes in a 5% $CO_2$ incubator.
7. The cells were centrifuged at 300 x *g* for 3 minutes.
8. After removing the supernatant, 500 µl of ice-cold PBS was added. The cells were resuspended and centrifuged at 300 x *g* for 3 minutes. This step was repeated twice.
9. After removing the supernatant, 100 µl of methanol were added and mixed by pipetting.
10. The solution from step 9 (50 µL) was transferred to a 96-well black microplate, and 200 µl of Working solution was added and mixed by pipetting. The plate was sealed and incubated at 37°C for 30 minutes.
    Note: Thoroughly suspend the solution in step 9 before transferring it to the microplate.
11. The fluorescence intensity was measured using a fluorescence plate reader (Ex/Em = 490/535 nm; Infinite m200 PRO).
12. The fluorescence intensity derived from the incorporated Cystine Analog was calculated by subtracting the blank value from the value measured for each sample.
13. RIPA Buffer (50 µl; 16488-34, Nacalai Tesque) was added to the remainder of the solution from step 9 and mixed by vortexing. The mixture was allowed to stand at room temperature for 30 minutes.
14. The solution from step 13 (15 µl) was used for protein quantification (by BCA assay), and the fluorescence intensity obtained in step 12 was normalized by the protein concentration.

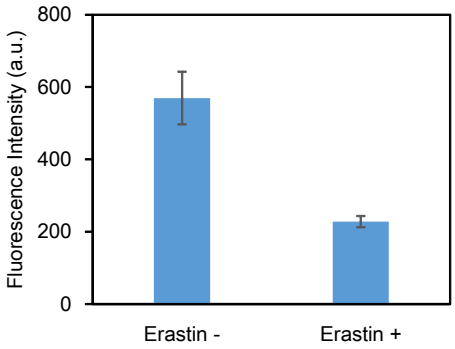


Figure 6. Inhibition of cystine transporter activity in HL60 cells by erastin