Protocol Booklet

Product Code(s)	HB7375
Product Name	H_2DCFDA - Cellular ROS Assay Kit
Purpose	Measurement of reactive oxygen species (ROS) in cultured cells

Please note: This product is for RESEARCH USE ONLY and is not intended for therapeutic or diagnostic use. Not for human or veterinary use

Contents

Product Overview

H_2DCFDA ROS Assay Kit is designed to measure levels of reactive oxygen species (ROS) within cultured cells. H_2DCFDA is a non-fluorescent cell permeable small molecule probe that is cleaved by intracellular esterases then subsequently oxidised by ROS into a fluorescent product. By measuring the fluorescence intensity within populations of cells treated with H_2DCFDA then the level of ROS activity can be calculated.

Components & Storage

This kit contains:

- Lyophilised H_2DCFDA (100 tests / 300 tests)
- 10x assay buffer (10ml)
- Pyocyanin (200µl of 10mM once reconstituted)

Note: Please ensure that all components are only used in sterile conditions to prevent contamination of the stock solutions. Store kit at -20°C until use.

This kit additionally requires:

- Sterile dark clear-bottomed 96-well microplates (for microplate assays)
- Cell culture media without phenol red
- Microplate reader, flow cytometer or fluorescence microscope
- Centrifuge for pelleting cells
- Centrifuge tubes
- Ethanol

Protocol

Preparing reagents and general advice

Before carrying out any experiments it is necessary to prepare the reagents:

- Reconstitute the pyocyanin by adding 200µl of ethanol to make a 10mM solution - this can be subsequently aliquoted and stored at -20°C
- Dilute the 10x assay buffer down to 1x with sterile dH2O, warm to 37°C before use - this can be subsequently aliquoted and stored at -20°C

It is best to assay all samples in a minimum of duplicate and it is possible to use media without phenol red in place of assay buffer if desired. Only use live cells as fixed cells are not compatible with this assay.

Non-Adherent Cell Microplate Assay

Non-adherent cells can be easily assessed for ROS production using this H_2DCFDA ROS Assay Kit. This protocol is based upon a working volume of 100uL a test in a 96 well microplate.

1. Culture cells following established protocols such that around $1.5x10^5$ cells are available per well of a 96-well plate for the experiment.
2. Freshly prepare a working solution of H_2DCFDA by diluting the 20mM stock in 1x assay buffer to a 20µM working concentration (10µL stock per 10ml of 1x assay buffer). Make fresh and do not store or re-use. The concentration of H_2DCFDA may need optimising as it depends upon the cell line used but a good starting range is 10-50µM.
3. Freshly prepare a working solution of pyocyanin (positive control) by diluting the 10mM stock in 1x assay buffer to a 1mM working concentration. Make fresh and do not store or re-use.
4. Collect cells into a centrifuge tube then pellet and wash once with sterile PBS before re-pelleting.
5. Resuspend cells in 20µM H_2DCFDA working solution at a concentration of $1x10^6$ cells / ml.
6. Incubate for 30 minutes in the dark at 37°C.
7. Pellet cells, remove supernatant then add 1X assay buffer (same volume as previously used for the H_2DCFDA working solution) before pelleting again.
8. Remove supernatant then resuspend in 1X assay buffer.
9. Add cells to a dark clear bottomed 96-well microplate using 100µl (100,000 cells) per well.

i. At this point test-compounds can be added as a 2x stock diluted 1:1 in each well. Incubation time will depend upon the compounds used but we recommend an incubation of 1-2 hours when using pyocyanin.
ii. To add the pyocyanin positive control then use a 67µM final concentration by adding 13.4µl of 1mM stock and 86.6µl of 1x assay buffer to each well to give a final volume of 200µl.
iii. Do not wash cells following treatment.

10. Measure the microplate immediately in a microplate reader (excitation at 485nm and emission at 535nm).

Adherent Cell Microplate Assay

Adherent cells can be easily assessed for ROS production using this H_2DCFDA ROS Assay Kit. This protocol is based upon a working volume of 100uL a test in a 96 well microplate.

1. Seed a dark clear-bottomed 96-well microplate with 50,000 cells per well then culture overnight under standard conditions using standard media.
2. Freshly prepare a working solution of H_2DCFDA by diluting the 20mM stock in 1x assay buffer to a 20µM working concentration (10µL stock per 10ml of 1x assay buffer). Make fresh and do not store or re-use. The concentration of H_2DCFDA may need optimising as it depends upon the cell line used but a good starting range is 10-50µM.
3. Freshly prepare a working solution of pyocyanin (positive control) by diluting the 10mM stock in 1x assay buffer to a 1mM working concentration. Make fresh and do not store or re-use.
4. Aspirate the media then add 100µl per well of 1x assay buffer.
5. Aspirate the 1x assay buffer then add 100µl per well of 20µM H_2DCFDA working solution. Incubate in the dark for 45 minutes at 37°C.
6. Aspirate the H_2DCFDA working solution and add 100µl per well of 1x assay buffer.
    i. At this point the assay buffer can be aspirated and exchanged for 100µl per well of 1x buffer containing test-compounds. Incubation time will depend upon the compounds used but we recommend an incubation of 1-2 hours when using pyocyanin.
    ii. To add the pyocyanin positive control then use a 67µM final concentration by adding 6.7µl of 1mM stock and 93.3µl of 1x assay buffer to each well to give a final volume of 100µl.
    iii. Do not wash cells following treatment.
7. Measure the microplate immediately in a microplate reader (excitation at 485nm and emission at 535nm).

Adherent Cell Fluorescence Microscopy assay

Adherent cells can also have ROS production visualised and measured using this H_2DCFDA ROS Assay Kit through fluorescence microscopy. This protocol is based upon a working volume of 100uL a test in a 96 well microplate or equivalent.

1. Seed a dark clear-bottomed 96-well microplate with 50,000 cells per well then culture overnight under standard conditions using standard media.
    i. Additional growth substrates are possible to be used such as glass coverslips or chamber slides, but reagent volumes used will need to be adjusted.
    ii. Avoid using media containing phenol red as this can cause high background fluorescence.
2. Freshly prepare a working solution of H_2DCFDA by diluting the 20mM stock in 1x assay buffer to a 20µM working concentration (10µL stock per 10ml of 1x assay buffer). Make fresh and do not store or re-use. The concentration of H_2DCFDA may need optimising as it depends upon the cell line used but a good starting range is 10-50µM.
3. Freshly prepare a working solution of pyocyanin (positive control) by diluting the 10mM stock in 1x assay buffer to a 1mM working concentration. Make fresh and do not store or re-use.
4. Aspirate the media then add 100µl per well of 1x assay buffer.
5. Aspirate the 1x assay buffer then add 100µl per well of 20µM H2DCFDA working solution. Incubate in the dark for 45 minutes at 37°C.
6. Aspirate the H_2DCFDA working solution and add 100µl per well of 1x assay buffer.
    i. At this point the assay buffer can be aspirated and exchanged for 100µl per well of 1x buffer containing test-compounds. Incubation time will depend upon the compounds used but we recommend an incubation of 1-2 hours when using pyocyanin.
    ii. To add the pyocyanin positive control then use a 67µM final concentration by adding 6.7µl of 1mM stock and 93.3µl of 1x assay buffer to each well to give a final volume of 100µl.
    iii. Do not wash cells following treatment.

7. Image cells using the FITC filter set of a fluorescence microscope adapted for live cell microscopy (heated to 37°C with CO_2 supplementation).
8. Use a low light intensity during imaging to prevent bleaching of the probe.

Flow cytometry assay

Both adherent and non-adherent cells can be assessed for ROS production using this H_2DCFDA ROS Assay Kit using flow cytometry.

1. Culture cells following established protocols such that around $1.5x10^4$ cells are available per experimental condition.
    i. Cells should not be denser than $1x10^6$ cells/ml
2. Freshly prepare a working solution of H_2DCFDA by diluting the 20mM stock in complete culture media to a 20µM working concentration (10µL stock per 10ml of media). Make fresh and do not store or re-use. The concentration of H_2DCFDA may need optimising as it depends upon the cell line used but a good starting range is 10-50µM.
3. Freshly prepare a working solution of pyocyanin (positive control) by diluting the 10mM stock in 1x assay buffer to a 1mM working concentration. Make fresh and do not store or re-use.
4. Pellet cells through centrifugation after ensuring a uniform cell suspension
    i. For adherent cells: trypsinize to get into solution then quench the trypsin with FBS supplemented media
    ii. For non-adherent cells: pipette up and down
5. Resuspend cells in 20µM H_2DCFDA working solution and incubate for 30 minutes in the dark at 37°C.
6. Pellet cells, remove supernatant then add 1X assay buffer.
    i. At this point test-compounds can be added by pelleting the cells again and resuspending in 1x assay buffer containing test compounds. Incubation time will depend upon the compounds used but we recommend an incubation of 1-2 hours when using pyocyanin.
    ii. To add the pyocyanin positive control then use a 67µM final concentration.
    iii. Do not wash cells following treatment.
7. Ensure the cell suspension is uniform before analysing on a flow cytometer (excitation at 488nm and emission at 535nm).

Guidelines, precautions, troubleshooting

Please follow the below table to resolve any problems encountered when using this kit. For any problems not listed or for any further advice please contact our technical support team at technicalhelp@hellobio.com.

Problem	Potential Cause
Low signal	Too low cell density. Try repeating the experiment with a higher cell density or test a range of cell densities to find an optimal density for your experimental conditions.
	Too low H_2DCFDA concentration. Try repeating the experiment with a higher H_2DCFDA concentration or conduct a concentration response to find the optimal concentration for your experimental conditions.
	Too long incubation time. H_2DCFDA has only been tested for stability up to 6 hours. For experiments requiring longer incubation times with test compounds instead pre-incubate the cells with the compounds before then incubating with H_2DCFDA for 30 minutes and measuring with a microplate reader.
High background	Too high cell density. Try repeating the experiment with a lower cell density or conduct a dilution series of cells to find an optimal density for your experimental conditions.
	Too high H_2DCFDA concentration. Try repeating the experiment with a lower H_2DCFDA concentration or conduct a concentration response to find the optimal concentration for your experimental conditions.

Observe safe laboratory practice and consult the safety datasheet. Please see the datasheet on our website for general guidelines, precautions, limitations on the use of the assay kit.

Contact

For customers in the UK, Europe and Rest of the World

Customer Care	customercare@hellobio.com
Technical support	technicalhelp@hellobio.com
By telephone:	+44(0)117 318 0505
By fax:	+44(0)117 981 1601
Opening hours:	8.30 am - 5.00 pm GMT weekdays

For customers in the USA, Canada and South America

Customer Care	customercare-usa@hellobio.com
Technical support	technicalhelp@hellobio.com
By telephone:	+1-609-683-7500
By fax:	+1-609-228-4994
Opening hours:	9.00 am - 5.00 pm EST weekdays