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Technical Datasheet

EZcount™ Sulphorhodamine B Cell Assay Kit

Product Code: CCK052

1. Introduction

Cell proliferation and death are essential processes for tissue generation and regeneration, organ development etc. in mammals and are usually under stringent control of extra and intracellular factors. Non-physiological alterations in levels of these factors lead to anomalous cytogenetic behavior of cells which in turn leads to cell transformation, uncontrolled cell growth - the initiating event for cancer development. Pharmaceutical research is hence largely focused on effects of drugs, cytotoxic agents and biologically active compounds which affect cytogenetics.

Multiple procedures are available for determination of cell proliferation and cytotoxicity. Simple and cheap methods for estimating cell viability (or death) are Trypan Blue exclusion and Erythrocin B staining. However, these methods are not sensitive enough and cannot be used for high throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Sulphorhodamine B (SRB) assay is used for determination of cell proliferation and cytotoxicity based on the measurement of cellular protein content. The results can be read on a multi-well scanning spectrophotometer or a standard ELISA reader and show a high degree of precision.

2. About the Assay

The EZcount™ Sulphorhodamine B cell assay kit is designed for determination of cell viability and cell proliferation and/or effect of cytotoxic agents. This kit is based on the quantitative measurement of cellular protein. SRB is a bright pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues in proteins under mild acidic conditions. Under mild basic conditions the dye can be extracted from the cells. Due to the stoichiometric binding of SRB, the amount of dye extracted from the cells is directly proportional to the cell mass. Intensity of the colour can be measured spectrophotometrically at 490nm.

3. Applications

- **Cell proliferation:** Quantification of changes in proliferative activity of cells caused by trophic factors, cytokines, and growth promoters
- **Cell cytotoxicity**: Evaluation of effects of inhibitors or inducers of apoptosis, cytotoxic reagents, carcinogens and toxins
- **Drug discovery**: High-throughput screening of various anti-cancer drugs

4. Kit contents

The reagents supplied in the kit are sufficient for 1000 assays (Ten 96-microwell plates).

Code	Description	Quantity	Storage
CCK052(A)	Sulphorhodamine B solution	1 x 50ml	RT
CCK052(B)	Fixing solution	1 x 25ml	RT
CCK052(C)	Washing solution	1 x 100ml	RT
CCK052(D)	Solubilization solution	1 x 100ml	RT

5. Materials required but not provided in the kit

- Cells in appropriate medium without phenol red
- Adjustable pipettes and a repeat pipettor
- Flat-bottom 96-well microtiter plate for culturing the cells
- 96-well plate reader capable of measuring the absorbance at 490nm and >630nm

6. General guidelines

It is important to optimize experimental factors like cell density, incubation time, media composition and concentration of the agents under investigation prior to use of EZcount™ Sulphorhodamine B Cell Assay Kit. Procedure for optimizing cell density is outlined in section 7.2.

Assay controls

- Include appropriate assay controls i.e.
  1. Medium control (medium without cells)
  2. Cell control (medium with cells but without the experimental drug/ compound)
  3. Vehicle control (medium containing the experimental drug or compound but no cells)

Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a repeating pipettor is recommended to pipette reagents. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.
- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

Incubation period

- Different cell lines may have different rates of metabolic activity and doubling time and hence respond to SRB differently. For this reason, plating density and incubation period for every cell line should be optimized to obtain results in linear range.

Culture Medium

- Phenol red may interfere with the measurement of absorbance; therefore the cell culture media used for this assay should not contain phenol red.

Temperature

- Temperature affects the performance of the assay because of its effect on enzymatic rates. It is crucial to run the assay at a uniform temperature to ensure reproducibility across a single plate or among stacks of several plates. Since absorbance is measured at room temperature, it is important to ensure adequate equilibration of assay plates after removal from a 37ºC incubator to avoid differential temperature gradients. Stacking large numbers of assay plates in close proximity should be avoided to ensure complete temperature equilibration.

Measurement of absorbance

- Absorbance can be read with a filter in the wavelength range of 490nm (primary wavelength) and >630nm as a reference wavelength.

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1. Preparation of cells

Always use freshly harvested cells for assay. Seed the cells in a cell culture flask or dish in an amount appropriate for the assay and incubate at 37ºC in a 5% CO_2 environment. Allow the cells to grow for up to 24 hours or till confluence is reached. Harvest the cells and use for assay.

Note: The quantity of the cell suspension to be seeded in the medium depends upon doubling time of individual cell lines and seeding density to be used in the assay.

7.2. Pre-assay optimization procedure

Pre-assay optimization procedure needs to be performed once for each cell line to determine optimum plating density and incubation time.

1. Harvest the cells as explained in section 7.1.
2. Adjust the cell density to 1 x 10^6 cells /ml.
3. Serially dilute the cell suspension from 1 x 10^6 to 1 x 10^3 cells/ml using appropriate culture medium.
4. Seed 100µl of each dilution in 96-well microtiter plate in triplicate.
5. Add medium control in triplicate.
6. Incubate the cells under appropriate conditions depending on the cell line under study.
7. Remove the culture plates from incubator.
8. Gently layer 25µl of cold fixative solution CCK052(B) on top of culture medium in each well.
9. Incubate the plate at 2 – 8ºC for 1 hour.

   Note: Incubation period of 1 hour is sufficient for complete fixation of strongly adhered cell lines.

   For effective fixation of loosely attached cells or suspension cells, prolonged fixation period may be required. Centrifugation may also enhance cell fixation.

   We recommend to perform a trial experiment with suspension cells or loosely attached cells to determine optimum fixation conditions.

A) For adherent cells

i. After fixation, discard fixative by gentle pipetting.
ii. Gently add 100µl of washing solution CCK052(B) to each well and swirl the plate.
iii. Discard the rinsate by gentle pipetting.
iv. Repeat this step ii and iii three more times to remove traces of fixative and serum proteins.

10. B) For suspension cells or loosely attached cells
    i. After fixation, centrifuge the plate at 1500rpm for 5 minutes.
    ii. Discard the supernatant by gentle pipetting.
    Note: Attachment of the suspension cells to plate bottom after fixation is extremely sensitive to movement. It is recommended to handle the plate carefully at this stage.
    iii. Gently add 100µl of washing solution CCK052(B) to each well and swirl the plate.
    iv. Centrifuge at 1500rpm for 5 minutes.
    v. Discard the rinsate by pipetting.
    vi. Repeat the steps iii, iv and v three times more to remove traces of fixative and serum proteins.
11. Observe the plates under the microscope to confirm fixation of cells to the bottom of plate.
    Note: Microscopic observation also gives an idea about cell loss during washing steps.
12. Air dry the plates after fixing and store until required.
13. For immediate use, add 50µl of Sulphorhodamine B staining solution CCK052(B) to each well and incubate at room temperature for 20 – 30 minutes.
    Note: Precipitates in SRB staining solution are normal. Filter the dye before use.
14. Discard the staining solution by pipetting.
15. Add 25µl of washing solution to each well to remove unbound stain from the wells.
16. Swirl the plate quickly and discard the washing solution.
17. Repeat the washing step three more times.
    Note: Washing should be performed as quickly as possible. Prolonged exposure of cells to washing solution leads to leaching of the stain into solution thereby resulting in erroneous readings.
    It is recommended to determine wash times empirically.
18. Allow the culture to air dry uniformly. Non-uniform drying may result in random absorbance readings.
19. Add 100µl of solubilization solution CCK052(D) to each well to extract the incorporated stain.
20. Allow the plate to stand at room temperature for 5 – 10 minutes.
21. Mix the extracted stain by trituration or stirring on gyratory shaker.
22. Measure the absorbance at 580nm (primary wavelength) and >630nm (reference wavelength).
23. If intensity of colour is too high and erroneous reading are obtained at 580nm, measure the absorbance at suboptimal wavelength i.e. 490 – 530nm (primary wavelength) and >630nm (reference wavelength).
24. Determine the average absorbance values from triplicate readings and subtract average values of medium control (blank) from each.

    Specific absorbance = Absorbance (test) – Absorbance (blank)

25. Plot specific absorbance against cell density.
26. Number of cells to be used in the cell proliferation assay should lie within linear portion of the plot.

7.4 Assay procedures

1. Seed 100µl of cell suspension in a 96-well microtiter plate at the required cell density, with or without the cell growth modifying agent.
   Note:
   a) If the cell growth modifying agent is a cytokine, metabolite, growth factor or any other compound, add its required quantity in the culture system.
   b) If the cell growth modifying agent is any kind of radiation or waves, treat the cells with them for required period of time.
2. Incubate the plate at 37°C in a 5% CO_2 atmosphere for the required period of time.
3. After the incubation period, remove the plates from incubator and follow the procedure mentioned in section 7.2, step 8 onwards.
4. Determine the average absorbance values from triplicate readings and subtract average values of medium control (blank) from each.

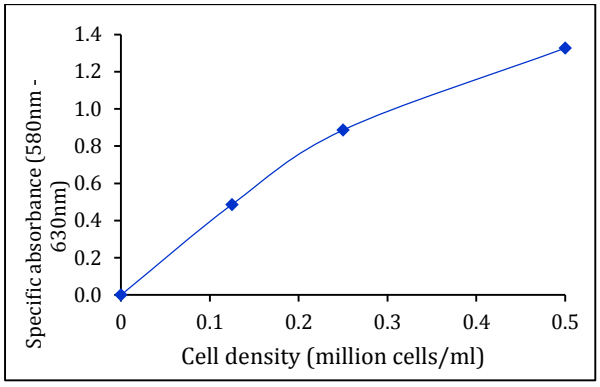
   Specific absorbance = Absorbance (test) – Absorbance (blank)

5. Plot the specific absorbance values on the Y-axis and your experimental parameters on the X-axis.
6. Test values higher than control values indicate increase in cell proliferation and viability and vice versa.

8. Storage and shelf life

Store all the reagents at temperature mentioned on product label.
Use before expiry date given on the label.

9. Performance characteristics



The sensitivity of Sulphorhodamine B to detect changes in cell number has been determined by plotting the graph of normalized absorbance values versus cell number.

CHO cells were serially diluted and seeded in a 96-well microtiter plate to perform Sulphorhodamine B assay.

10. Advantages

- **Reproducibility**: Entire assay can be performed in a single plate. Cells and reagents need not be transferred. This facilitates reproducibility of the results.
- **Safety**: No radioisotopes are involved.
- **Fast**: Use of multi-well ELISA plates allows the processing of large number of samples.
- **Flexibility**: Sulphorhodamine B works on adherent as well as suspension cell lines.

11. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Very high absorbance values	High cell densities	Repeat the assay with reduced cell densities
	Microbial contamination	Discard. Repeat the assay with new media and reagents
Very low absorbance values	Very low cell density	Repeat the assay with high cell densities
	Improper selection of filter for reading the absorbance	Choose appropriate filters
Random absorbance values/ poor consistency of replicates	Inaccurate pipetting technique or inaccurate equipment	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents
	Test compound under study is responsible for improper response of the cells to Sulphorhodamine B	Refer to the pharmacological properties of the compound
Blank/ medium control (i.e. medium without cells) give high absorbance readings	Microbial contamination	Discard. Repeat the assay with new media and reagents.

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