1. Introduction

Cell Counting Kit-8 (CCK-8) provide a more convenient and sensitive method for the research of cell number determination and cell proliferation/cytotoxicity assay than previous ways such as MTT. Our kit employs a highly water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt], which produces a water-soluble formazan dye upon reduction in the presence of an electron mediator, as shown in Figure. 1.

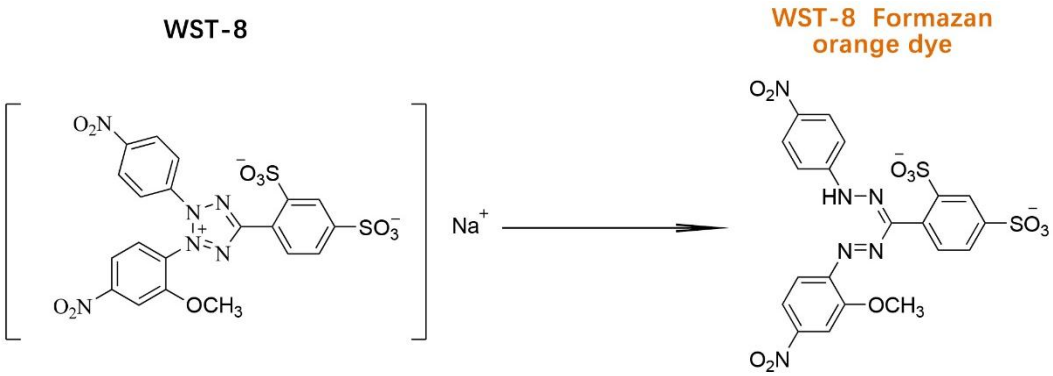

Figure.1 Structures of WST-8 and WST-8 formazan

CCK-8 is a one-bottle solution which has already been mixed with electron mediator. WST-8 will be reduced by dehydrogenases abundant in viable cells and transformed to formazan, which is an orange colored dye soluble in the culture medium (Figure. 2). The quantity of the formazan generated by dehydrogenases is directly in proportion to the numbers of living cells.

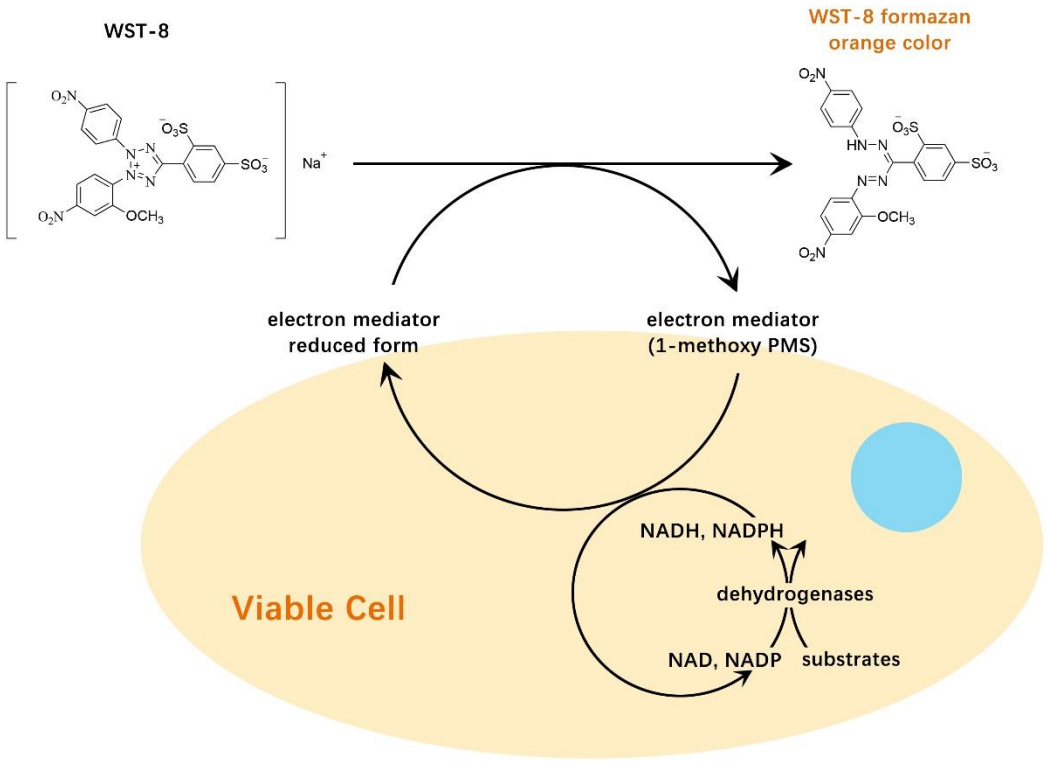

Figure.2 Principle of the detection

CCK-8 kit affords sensitive colorimetric assays for the detection of the number of viable cells in cell proliferation assays and cytotoxicity assays. Previous researches demonstrate that the detection sensitivity by CCK-8 is higher than other tetrazolium salts such as MTT, XTT, MTS or WST-1. Since WST-8 formazan is water soluble, so the adding of organic solvents such as DMSO is no more needed. The absorbance of WST-8 formazan at 450 nm is proportional to the number of viable cells in the medium, the viable cell number can be determined by the absorbance value and the standard curve. CCK-8 assay can also be substituted for the [3H]-thymidine incorporation assay.

2. Required Equipment and Materials not supplied

Plate reader (450 nm filter)
96-well plate
10 µl, 100-200 µl and multi-channel pipettes
CO_2 incubator

3. Cell Number Determination

3.1. Inoculate cell suspension (100 µl/well) in a 96-well plate. Pre-incubate the plate in a humidified incubator (e.g., at 37°C, 5% CO2).
3.2. Add 10 µl of the CCK-8 solution to each well of the plate. Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.
3.3. Incubate the plate for 1 - 4 hours in the incubator.
3.4. Measure the absorbance at 450 nm using a microplate reader.

To measure the absorbance later, add 10 µl of 1% w/v SDS or 0.1 M HCl to each well, cover the plate and store it with protection from light at room temperature. No absorbance change should be observed for 24 hours.

4. Cell Proliferation and Cytotoxicity Assay

4.1. Dispense 100 µl cell suspension (about 5000 cells/well) in a 96-well plate. Pre-incubate the plate for 24 hours in a humidified incubator (e.g., at 37°C, 5% CO2).
4.2. Add 10 µl of various concentrations of substances to be tested to the plate.
4.3. Incubate the plate for an appropriate length of time (e.g., 6, 12, 24 or 48 hours) in the incubator.
4.4. Add 10 µl of CCK-8 solution to each well of the plate. Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.
4.5. Incubate the plate for 1 - 4 hours in the incubator.
4.6. Before reading the plate, you can mix gently on an orbital shaker for homogenization. Measure the absorbance at 450 nm using a microplate reader.

To measure the absorbance later, add 10 µl of 1% w/v SDS or 0.1 M HCl to each well, cover the plate and store it with protection from light at room temperature. No absorbance change should be observed for 24 hours.

5. General principles for statistical analysis

There are several methods for statistical analysis by taking advantages of O.D. or quantities of cells, we provide one of them.

Cell viability (also called survival rate) (%) = [(As - Ab) / (Ac - Ab)] x 100

Cell inhibition (%) = [(Ac-As) / (Ac-Ab)] × 100

As= Absorbance of tested compound (Absorbance of well containing cell, culture medium, CCK-8 and tested compound)

Ab= Absorbance of blank (Absorbance of well containing culture medium and CCK-8)

Ac= Absorbance of control (Absorbance of well containing cell, culture medium and CCK-8)

6. Creating a standard curve

6.1. Count the number of cells in the cell suspension using a hemocytometer or cell counter

6.2. Proportionally dilute the cells with medium to a concentration gradient, generally 5-7 concentration gradient is necessary, several replicate wells per group is recommended. And then inoculate the cells. (*Pay attention to the numbers of cells per well. If you dilute a cell suspension in a tube, please be careful to mix the cells to homogenize once again before adding to the well of the plate. The volume of the cell suspension in each well of the plate should be consistent.*)

6.3. Incubate until cells adhering to the well (generally2-4 hours), then add 10 µL of CCK-8 per 100µL medium. Continue incubating the plate for 1 - 4 hours, measure the absorbance at 450 nm using a microplate reader. Constructing the standard curve by plotting the number of cells on the X -axis and the absorbance on the Y-axis.

The number of cells of the tested sample can be determined based on this curve. The prerequisite for using this standard curve is that the test conditions are identical.

7. Notes

7.1. Make sure that the tested substances and CCK-8 are uniformly distributed in the culture medium.

7.2. More cell proliferation means deeper color; more powerful cytotoxicity means lighter color.

7.3. For adhesive cells, at least 1000 cells per well (100 µl medium) are necessary. For leukocytes, at least 2500 cells are necessary per well (100 µl medium) on account of low sensitivity. The recommended maximum number of cells per well for the 96-well plate is 25000. If a 24-well or 6-well plate is used for this assay, please calculate the number of cells per well correspondingly, and **adjust the volume of the CCK-8 solution added to 10% of the total volume.**

7.4. Because the CCK-8 assay is based on the dehydrogenase activity detection in viable cells, conditions or chemicals that affect dehydrogenase activity may cause discrepancy between the actual viable cell number and the cell number determined using the CCK-8 assay.

7.5. WST-8 might react with reducing agents to generate WST-8 formazan. Please check the background O.D. if reducing agents are used in cytotoxicity assays or cell proliferation assays.

7.6. Typical background absorbance after 2 hours incubation is 0.1 - 0.2 absorbance units.

7.7. Care about not to introduce bubbles to the wells, since they interfere with the O.D. reading.

7.8. If you want to sterilize the CCK-8 solution, please filter the solution with a 0.2 µm membrane.

7.9. The incubation time varies by the type and number of cells in a well. Generally, leukocytes

give weak coloration, thus a long incubation time (up to 4 hours) or a large number of cells (~10^5 cells/well) may be necessary.

7.10. Measure and subtract the O.D. at 600 nm or higher from that of sample if there is a high turbidity in the cell suspension.

7.11. CCK-8 can't be taken for cell staining purpose

7.12. The absorption value of phenol red in a culture medium can be removed by subtracting the absorption value of a blank solution from the absorption value of each well. Therefore, a medium containing phenol red is usable for the CCK-8 assay.

7.13. The toxicity of CCK-8 is very low, the same cells might be used for other cell proliferation assays such as the crystal violet assay, neutral red assay or DNA fluorometric assay after the CCK-8 assay is completed. (Don't recommend.)

7.14. This kit can be utilized for E.coli, but not yeast cells.

7.15. Before reading the plate, you can mix gently on an orbital shaker for homogenization.

7.16. We recommend inoculating the cells near the center of the plate, culture medium in the most peripheral wells of the plate is easy to evaporate, you can fill these wells with PBS or water.

7.17. If you don't have a 450 nm filter. You can use filters with the absorbance between 430 and 490 nm, even though 450 nm filter gives the best sensitivity.

7.18. Measure the absorbance at 450 nm and **you can take the absorbance at 650 nm as a reference.**

7.19. The addition of a metal ion in the reagent may affect CCK-8 sensitivity.