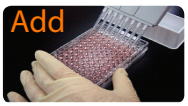
Featured Products

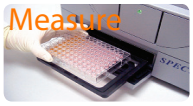
Simplify Your Cell Base Assay

Cell Counting Kit-8 (CCK-8) .....page 5

- One Bottle Solution

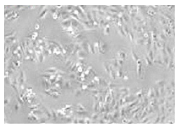
- Simple Procedure



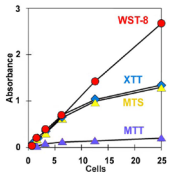


- No Cytotoxicity to the Cell

  Cells are Reusable for Additional Assays



- Contains Highest Sensitive Dye, WST-8



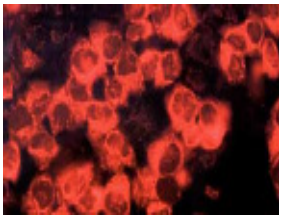
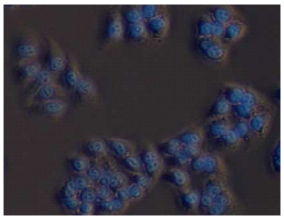
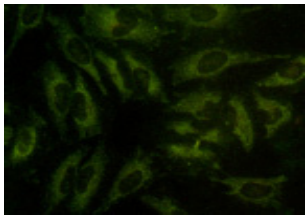
301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Contents

For Mammalian Cells

Contents

For Microbial Cells

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Measuring Cell Viability / Cytotoxicity

Introduction

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. Fig. 1 indicates various reagents used for cell viability detection. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Many have established methods such as Colony Formation method, Crystal Violet method, Tritium-Labeled Thymidine Uptake method, MTT, and WST methods, which are used for counting the number of live cells.

Trypan Blue is a widely used assay for staining dead cells. In this method, cell viability must be determined by counting the unstained cells with a microscope or other instruments. However, Trypan Blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions. In the Colony Formation method, the number of cell colonies are counted using a microscope as a cell viability indicator. In the Tritium-Labeled Thymidine Uptake method, [^{3}H]-thymidine is involved in the cell nucleus due to the cell growth, and the amount of the tritium in the nucleus is then measured using a scintillation counter. Though the Tritium-labeled thymidine uptake assay is sensitive to determine the influence on the DNA polymerization activity, it requires radioisotope which causes various concerns.

The ^{51}Cr method is highly sensitive, and is commonly used to determine low levels of cytotoxicity. However, the use of ^{51}Cr also causes problems in handling, storage, and disposal of the material.
Cellular enzymes such as lactate dehydrogenase, adenylate kinase, and glucose-6-phosphate dehydrogenase are also used as cell death markers, and there are several products available on the market. However, adenylate kinase and glucose-6-phosphate are not stable and only lactate dehydrogenase does not lose its activity during cell death assays. Therefore, cell death assays based on lactate dehydrogenase (LDH) activity are more reliable than other enzyme-based cell death assays.

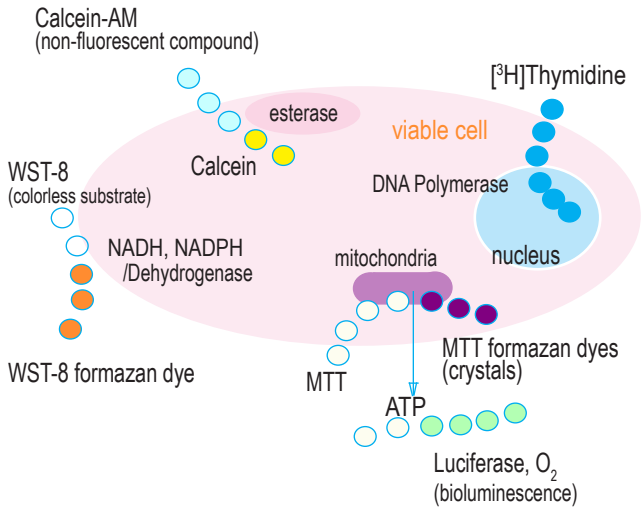


Fig. 1 Reagents for cell viability detection

Enzyme-based methods using MTT and WST rely on a reductive coloring reagent and dehydrogenase in a viable cell to determine cell viability with a colorimetric method. This method is far superior to the previously mentioned methods because it is easy-to-use, safe, has a high reproducibility, and is widely used in both cell viability and cytotoxicity tests. Therefore, this method is suitable for those who are just beginning such experiments. Among the enzyme-based assays, the MTT assay is the best known method for determining mitochondrial dehydrogenase activities in the living cells. In the method, MTT is reduced to a purple formazan by NADH. However, MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error.

Dojindo developed highly water-soluble tetrazolium salts called WSTs. WSTs produce water-soluble formazans and are suitable for cell proliferation and cytotoxicity assays. WSTs receives two electrons from viable cells to generate a yellow, orange, or purple formazan dye. WST-8, a highly stable WST, is utilized in Cell Counting Kit-8 (CCK-8). The electron mediator used in this kit, 1-Methoxy PMS, is also highly stable. Therefore, CCK-8 is stable for at least 6 months at the room temperature and for one year at 0-5 °C. Since WST-8, WST-8 formazan, and 1-Methoxy PMS have no cytotoxicity in the cell culture media, additional experiments may be carried out using the same cells from the previous assay.

Dehydrogenase-based assays reflect cell conditions with more sensitivity than the other assays because they depend on several elements including dehydrogenase, NAD(H), NADP(H), and mitochondrial activity. The major difference between CCK-8 and the MTT assay, other than MTT's toxicity, is the enzymes involved. The CCK-8 assay involves most of the dehydrogenase in a cell. On the other hand, MTT only involves mitochondrial dehydrogenase. Therefore, the MTT assay depends on mitochondrial activity, not the cell itself. Additionally, CCK-8 is far more sensitive than the MTT assay. Since WST-8 formazan is water soluble, it does not form crystals like MTT. Therefore, after 1-4 hours of incubation with the CCK-8 solution, measurement of O.D. at 450 nm gives the number of viable cells. No extra steps are required.

Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-8

Product Description

Cell Counting Kit-8 is a colorimetric assay for the determination of viable cell numbers and can be used for cell proliferation assays as well as cytotoxicity assays. Cell Counting Kit-8 uses a tetrazolium salt, WST-8, which produces the water soluble WST-8 formazan. Since this orange colored formazan does not require dissolving, no solubilizing process is required. Results are obtained after 3 simple steps: 1) add, 2) incubate, and 3) read. This kit is applicable for 96-well microplate assays and can also be applied to High-Throughput Screening such as a 384-well microplate. WST-8 is not cell permeable, which results in low cytotoxicity. Therefore after the assay it is possible to continue further experiments using the same cells.

Applications: cell counting, cell proliferation experiments, cytotoxicity tests, drug sensitivity tests

Product Information

Cell Counting Kit-8

Product code	Unit	Components
CK04-01	100 tests	1 ml bottle x 1
CK04-05	500 tests	5 ml bottle x 1
CK04-11	1,000 tests	5 ml bottle x 2
CK04-13	3,000 tests	5 ml bottle x 6
CK04-20	10,000 tests	100 ml bottle x 1

One test corresponds to one well of the 96-well plate.

Required Materials

Devices, Tools

- Microplate Reader with a 450 - 490 nm filter
- 96 well microplate, sterilized clear plate for cell assay
- Multi-channel pipette (8 or 12 channel: 10-100 μl)
- Pipette tips for 10-100 μl
- CO_2 incubator
- Clean bench
- Hematocytometer or cell counter
- Centrifuge and rotor for a 15 ml centrifuge tube

Reagents

- Cell Counting Kit -8 [product code: CK04]
- Cell culture media
- Material to be tested
- PBS or other buffers for the preparation of material solutions if cell culture medium cannot be used.

Preparation

Cell Counting Kit-8

- Ready-to-use one solution
- Stable for 12 months when stored at 4 °C

If you use Cell Counting Kit-8 frequently, store in a refrigerator. The solution is stable for one year at 4 °C. The solution is also stable at room temperature for 6 months.

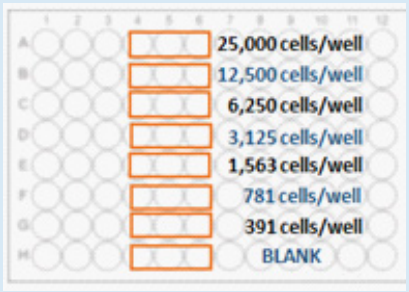
If you plan not to use the Cell Counting Kit-8 for more than a year, aliquot the Cell Counting Kit-8 solution and store in a freezer at -20 °C to avoid repeated freeze and thaw.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-8

Optimization of Assay Condition

When using Cell Counting Kit-8 for proliferation and cytotoxicity assays, it is necessary to have a proportional relationship between absorption and viable cell numbers. It is desirable to start with a set number of cells, and then determine the suitable incubation time for color development. Below, the method and conditions for using Cell Counting Kit-8 are described.

Procedure	Precautions & Tips
Recover the cells to be assayed from a culture flask. Adjust the concentration of the cell suspension to $5x10^5$ cells per ml using a hematocytometer or cell counter.	For adherent cells, recover the cells using trypsin to detach cells, and use a cell scraper if necessary.
Perform Serial Dilution by… a. Using an 8 channel multi-pipette, add 100 µl of media to each well of a 96 well microplate. b. Add 100 µl of a $5x10^5$ cells per ml solution to the wells of first triplicate row. c. Take 100 µl from the first triplicate row, add it to the next well and mix. The process is repeated as indicated in the figure 2. Reserve the final well for the negative control (Blank). This well should contain media only (no cells) for measurement of the background.	Refer to experimental example on the next page for instruction on serial dilution. 
Add 10 µl of Cell Counting Kit-8 reagent to each well on the 96-well microplate.	When using a plate or petri dish other than a 96 well plate, please add reagent equal to 1/10 the media volume. Due to the low volume of reagent added, it is recommended to place the pipette tips against the well wall to add the reagent (below picture). If the reagent sticks to the well wall, tap the plate lightly to mix with the media.
Place in a CO2 incubator for 1-4 hours to react.	If performing the experiment for the first time, we recommend taking readings every hour at 1, 2, 3 and 4 hours. Be aware that cell number after 24-48 hours of incubation may surpass the initial number of cells counted. In order to establish a relationship between cell number and absorbance, add the reagent before the cells proliferate, and take a reading.
Measure an absorbance on a microplate reader using a 450 nm filter.	Since the amount of formazan produced will differ with each cell types, the amount of coloration will differ even if the time between adding the reagent and taking a reading is the same. (See HeLa cell and HL60 cell charts on the next page)
Establish your standard curve by plotting the number of cells on the x -axis and the absorbance on the y-axis.	Since bubbles can cause an error, make sure there are no bubbles in the each well. If there are bubbles, use a pipette tip to remove or use a toothpick to pop them.

Experimental Example

Make serial dilutions of 2.5 x 10^4 , 1.25 x 10^4 , 6.2 x 10^3 ….0 cells per well to each well of a 96 well plate using HeLa cells (human cervical cancer cells) or HL60 cells (promyelocytic leukemia cells) suspensions as indicated in Fig. 2.

Serial Dilution Procedure

Using an 8 channel multi-pipette, add 100 µl of media to each well of a 96 well microplate. Next, add 100 µl of a 5 x 10^5 cells/ml solution to the first well and pipette to mix. This well will have the maximum number of cells. Next, take 100 µl from the first well and add it to the next well, and mix. The process is repeated as indicated in the figure. Take 100 µl from the last well which contains the minimum number of cells and discard.

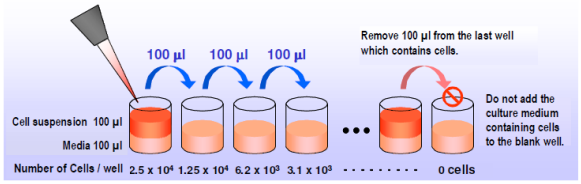


Fig. 2 Serial dilution process

Even when the cell number is the same, HeLa cells (Fig. 3) and HL60 cells (Fig. 4) have quite different cell activities. So, in a preliminary experiment, it is recommended to determine the suitable concentration of cells for each cell type and the time of coloration. In addition, for experiments involving drugs, consider the drug's properties which increases the cell proliferation, cell toxicity, reducing activity, and exposure time to drugs.

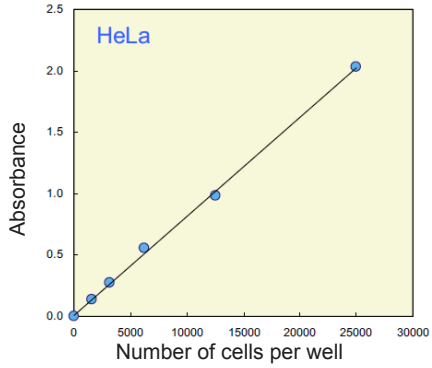


Fig. 3 Color development using HeLa cells
Media: DMEM (10% FBS)
Incubation: 37 °C, 3 hr, 5% CO_2 incubator
Measurement Wavelength: 450nm

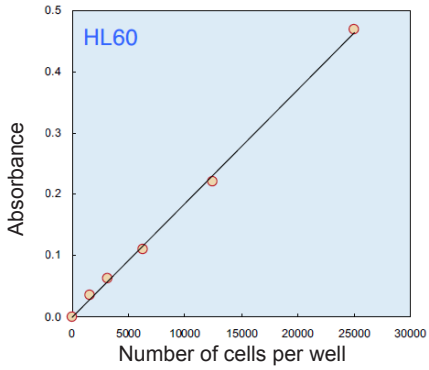


Fig. 4 Color development using HL60 cells
Media: RPMI1640 (10% FBS)
Incubation: 37 °C, 3 hr, 5% CO_2 incubator
Measurement Wavelength: 450nm

As indicated in Fig. 5, there is a good correlation between the Cell Counting Kit-8 assay and [^{3}H]-Thymidine Uptake assay.

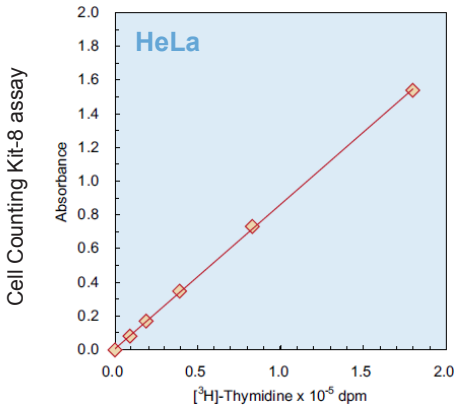
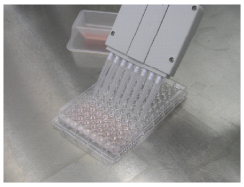
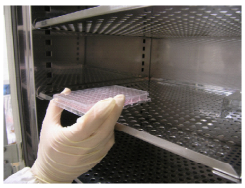
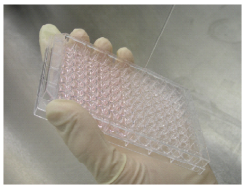
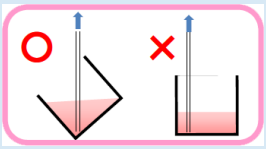
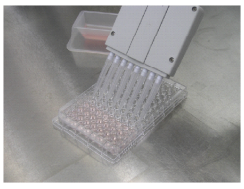
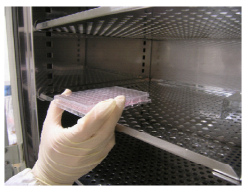


Fig. 5 Correlation between CCK-8 assay and [^{3}H]-Thymidine Uptake assay

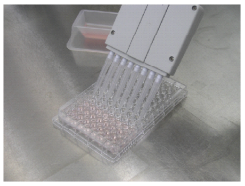
301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Cell Proliferation and Cytotoxicity Protocol

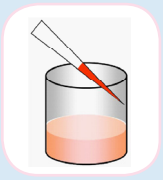
Procedure	Precautions & Tips
Recover the cells to be assayed from a culture flask.	For adhesive cells, recover the cells using trypsin, and use cell scrapers if necessary.
Count the cells and adjust the concentration to desired cell numbers* of the cell suspension. (cell conc.: _____cells/ml)	Use a hemacytometer or a cell counter. *Please see "Optimization of Assay Condition" on Page 6.
Add 100 µl of a cell suspension to each well in a 96 well microplate using serial dilution. Make a well of only media to measure the background. 	For floating type cells, please use a V bottom plate. The upper limit for the microplate reader may be surpassed if too many cells are present. The concentration of the cell should be adjusted based on whether the cells are promoted or inhibited by the test materials.
Incubate for 24-48 hrs. in a CO_2 incubator (start time: _____ end time: _____) 	If the time from starting incubation to taking a measurement is over 48 hrs, it is necessary to exchange the media.
If media change is necessary, remove media and add 100 µl of new media to each well including wells for a background measurement. 	Tilt the plate when removing the media to avoid touching the cells with the tip of the pipette as shown in the below diagram. 
Add 10 µl of media containing different concentrations of the test substances to each well. 	For floating type cells, centrifuge a V bottom plate with a microplate rotor, and then remove the media after the cells settle out of the solution with care not to suck in cells. Add the same amount of test substance to the blank wells (no cells) to measure the background absorbance. For negative control, add 10 µl of media to a well that does not contain the test substance. For dissolving the test substance, it is possible to use PBS or saline solution other than media.
Incubate for set periods of (6, 12, 24, 48 hrs) in a CO_2 incubator. (start time:_____ end time:_____) 	The exposure time depends on the test substance and purpose of the experiment. If the substance is highly toxic to the cell and destroys the cell membrane or reacts directly with proteins or DNA, short exposure time will be appropriate. If the substance slowly affects cell function, longer exposure time may be appropriate.

Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-8

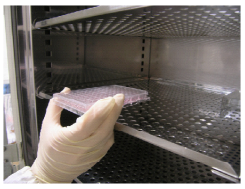
Add 10 μl of Cell Counting Kit-8 to each well in a 96 well microplate.



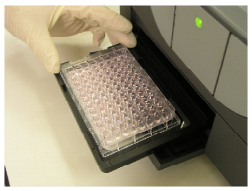
When using a plate or petri dish other than a 96 well plate, please add reagent equal to 1/10 the media volume.
Due to the low volume of reagent added, it is beneficial to touch the tip of the pipette to the well of the wall and when adding the reagent as indicated the figure below. If the reagent sticks to the well wall, tap the plate lightly to mix with the media.



Place in a CO_2 incubator for 1-4 hours to react.
(start time: _____ end time: _____)



Measure the absorbance at 450 nm with a microplate reader.



Since bubbles can cause error, make sure there are no bubbles in each well. If there are bubbles, use a pipette tip to remove or use a toothpick to pop them.

Calculating the Cell Survival Rate

Enter the absorbance reading from each well in the equation below to calculate the cell survival rate.

$$\text{Survival rate (\%)} = \frac{A_{\text{sample}} - A_b}{A_c - A_b} \times 100$$

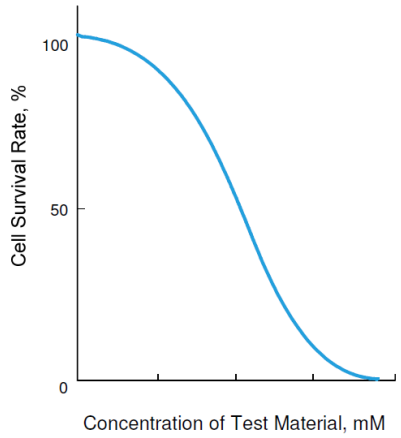


Fig. 6 Typical cell survival curve.

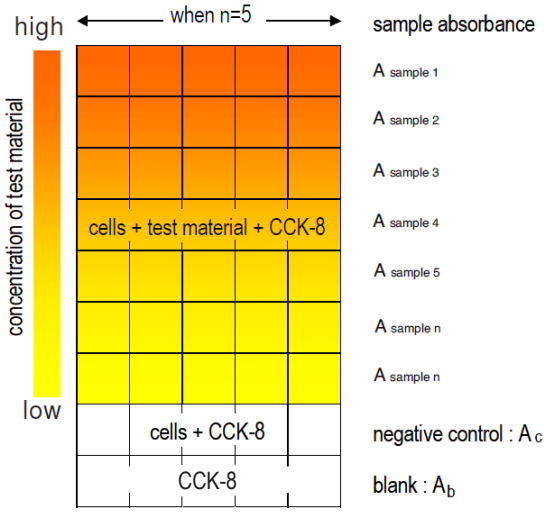


Fig. 7 Example of the plate arrangement and color development.

Does reducing material interfere with the Cell Counting Kit-8 assay?

On occasion when using the Cell Counting Kit-8 for cytotoxcity tests, cells that have been treated with the test material and should be dead may seem to show coloration. In this case, the test material is showing signs of reducing properties and it is possible that it has reduced the WST-8. So, before beginning your experiment, mix the test material and Cell Counting Kit-8 using media to confirm that the material does not react with Cell Counting Kit-8. If there is a significant coloration after the incubation, remove the media and wash the cells with media or PBS (-) to remove the test substance and add the same volume of fresh media to each well prior to adding Cell Counting Kit-8 solution.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Troubleshooting

Problem	Possible Cause	Solution
The absorbance reading exceeds the upper limit of the machine.	Too many cells per well.	The number of viable cells may increase during the pre-incubation. Prepare a microplate with a lower number of cells for the assay. For each cell type, determine the relationship between cell number and O.D readings (please refer to " Optimization of Assay Condition" on page 6).
	Too long of an incubation time.	Shorten the incubation time.
The color development occurs even though cells are clearly dead.	WST-8 is reduced by the test substance or materials which are generated in the culture media during the assay.	Mix Cell Counting Kit-8 with the substance to test whether the substance reacts with the Cell Counting Kit-8. If there is coloration, follow either of the following procedure: 1) Before adding Cell Counting Kit-8, exchange the culture media to remove the test substance or materials. 2) Use Cell Counting Kit-F.
The absorbance of the well with a toxic substance is higher than that of the well with no substance.	Toxic substances in low concentrations sometimes stimulate cell activity. Since cells have functions to protect themselves from the exposure of toxic substances, enzymatic activity of cells may increase at the initial stage. Then, the cell starts to die after a certain concentration.	If determining the LD_{50} of the substance, just ignore the area of increased absorbance.
		Try another method, such as Cell Counting Kit-F, to determine toxicity of the substance.
There is a high variation in the data.	The assay condition of the outer-most wells has changed due to the edge effect.	Do not use the outer-most wells for the assay. Just add media to these wells.
	Cell Counting Kit-8 has not been mixed well with the media.	Lightly tap the outside of the well in order to get the Cell Counting Kit-8 that is on the well wall to fall into the media.
	There are bubbles on the surface of the media.	Remove the bubbles using a pipet tip or a toothpick.
No color or less color development even though the number of cells seems to have increased.	Cell viability of each cell has been lowered because of too many cells.	Reduce the number of cells.

Q&A

Questions about reagents used in the kit

Q: *What causes color development in Cell Counting Kit-8?*
A: WST-8 is reduced to an orange-colored formazan through electron mediator, 1-Methoxy PMS by NADH and NADPH activity which are generated by cellular activities as indicated in the Fig. 8. The amount of WST-8 formazen is dependent on the activity of cellular dehydrogenase, so WST-8/1-Methoxy PMS system can be used to determine the number of living cells and cell viability.

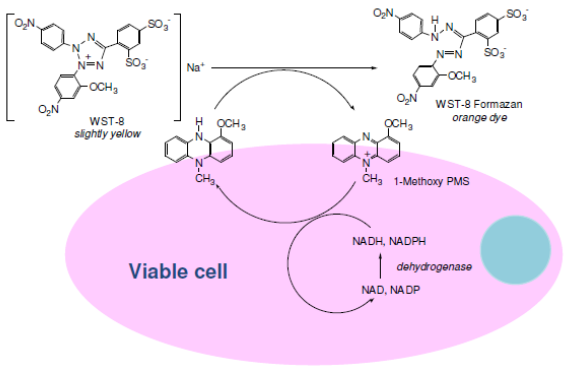


Fig. 8 Cell viability detection mechanism with CCK-8

Q: *Do WST-8 and 1-Methoxy PMS molecules enter into the cell?*
A: There is no clear evidence that these molecules do or do not enter the cell. Generally, a neutrally or positively charged organic molecule such as MTT can enter the cell. Therefore, it is estimated from their charges that 1-Methoxoy PMS can enter the cell, but WST-8 cannot. It is speculated that 1-Methoxy PMS receives an electron from NADH or NADPH at the membrane or inside of the cell and passes the electron to the WST-8 that is around the outer cell membrane.

Q: *What is the stability of the Cell Counting Kit-8?*
A: The Cell Counting Kit-8 is stable for over 6 months at ambient temperature. The kit is stable for over one year when stored in a refrigerator and over two years when stored in a freezer.

Q: *What is the toxicity of Cell Counting Kit-8 compared to MTT?*
A: Compared to MTT in which the cell cannot survive after the reagent has been added, the cell survival rate for Cell Counting Kit-8 is over 90% even after 24 hours incubation. Therefore, after the assay with Cell Counting Kit-8, those cells can be used for another experiments. However, it is necessary to wash the cells so that no dye remains on the cell surface.

Questions regarding cells and cell culture

Q: *What type of cells can be measured using Cell Counting Kit-8?*
A: Generally, Cell Counting Kit-8 can be used for mammalian cell lines, primary culture animal cells, and stem cells.

Q: *How long of a pre-incubation time is required prior to the assay?*
A: It depends on the cell type, but the cells should enter into the logarithmic growth phase. The average incubation time to enter into this phase is 24 hours to 48 hours. Please check cell databases to estimate the pre-incubation time.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Q: *Can Cell Counting Kit-8 be used for both adherent cells and suspension cells?*
A: Yes, it can be used for both types of cells. However, the color development for suspension cells will be lower compared to the coloration of the adherent cells, so it may be necessary to increase the incubation time or increase the number of cells for the assay using non-adherent cells.

Q: *When using Cell Counting Kit-8, what number of cells is appropriate?*
A: The appropriate number of cells depends on the type of cells and the type of your experiment. The amount of coloration will differ depending on the cell type, even if the cell number per well and incubation times are the same. When using a 96 well microplate, please check the absorbance level of 1,000-25,000 cells/well. If the experiment is for toxicity tests, 5,000-10,000 cells/well may be appropriate. If the number of cells are expected to increase during the assay, prepare a plate with 1,000-5,000 cells per well.

Q: *Is it necessary to pre-incubate?*
A: It is recommended to pre-incubate the adherent cells. When collecting the cells from a culture flask using Trypsin, the activity of the cells is not normal. Because of this, it is necessary to pre-incubate to get the cells back to their logarithmic growing phase to regain the viability prior the assay. For non-adherent cells, you can skip this step if the same culture medium is used for harvesting and resuspending cells for the assay.

Questions concerning the assay

Q: *Is it possible to do the assay in a 24 or 12 well plate? If so, how much Cell Counting Kit-8 solution should be used?*
A: Yes, it is possible to assay using plates other than a 96 well plate. Please add Cell Counting Kit-8 solution equal to 1/10 of the volume of the media (if the media is 1 ml, add 100 µl of solution)

Q: *What should be done to stop the color development reaction?*
A: Follow one of the below methods (volume is based on 96 well plates)

Method A) Add 10 µl of 1 % SDS (dissolve 0.1 g SDS with PBS buffer to prepare 10 ml solution)
Notes: Since bubbles on the surface causes errors when measuring the absorbance, be careful not to make bubbles when adding the SDS solution.

Method B) Add 10 µl of 0.1 mol/l acid such as Hydrochloric acid.
Notes: Be sure to take a reading within 24 hrs after stopping the reaction.
When using a media with a high buffering capacity, use a higher concentration of hydrochloric acid to stop the reaction.
Do not use alkaline solution to stop the color development reaction. WST-8 and other tetrazolium salts are not stable under alkaline condition.

Q: *How much incubation time is sufficient for color development?*
A: In general, the incubation time is 1-4 hrs. However, the absorbance will differ between cell types even if the number of cells/well and coloration time are the same. Set an appropriate incubation time to give a proportional relationship between the cell number and the absorbance.

Q: *Are there any materials that can affect the color development when using the Cell Counting Kit-8?*
A: Reducing agents and materials with reducing activity may react with WST-8 and give a false reading. If the material is considered having reducing activity, mix the material solution with Cell Counting Kit-8 and incubate to check whether the material reacts with WST-8. If the material reacts with WST-8, remove the culture medium containing such material from cells and add new culture medium prior to adding Cell Counting Kit-8. Dye materials with absorbance around 450-490 nm will affect the reading. However, absorbance from such dyes can be subtracted as a blank. Such absorbance can be subtracted as a blank and does not affect assay data. For more detailed information, please refer to the following Q&A.

Q: *The cell culture is not clear, and has some turbidity.*
A: Measure the absorbance at 600-650 nm of the well as a reference. Then, the absorbance at 600-650 nm should be subtracted from the absorbance of the same well measured at 450 nm to eliminate the background that comes from turbidity.
Notes: If the turbidity comes from contamination, such as bacteria of fungi, just discard the plate and check the entire cell culture and the plate during the preparation process.

Q: *The cell culture in the well contains a material which has an absorbance around 450 nm, what should I do?*
A: Prepare the well for background measurement which contains all materials except for cells. Measure the background absorbance of the well at 450 nm, and then subtract the background absorbance from the absorbance of the sample well containing all materials and cells.
Notes: If the background absorbance from the material is too high to subtract, remove the culture medium and wash cells with fresh media and add the same volume of fresh media to the well prior to adding the Cell Counting Kit-8.

Q: *What should be done regarding materials that may increase the color development and interfere with the Cell Counting Kit-8 assay?*
A: Determine whether the material interferes with the assay. Add the Cell Counting Kit-8 to the solution which contains the material and incubate for a general assay period.
a)If there is no color development during the incubation, the material does not react with the Cell Counting Kit-8.
b)If there is color development during the incubation, the material does react with the Cell Counting Kit-8. Prepare a couple of wells for background absorbance measurement which contains all material except for cells. Measure the absorbance of the background well at 450 nm and subtract the background from the absorbance of the wells containing all materials and cells.
Notes: If the color development is too high to subtract, remove the culture media, wash the cells with fresh media, and then add the same volume of fresh media to the well prior to adding the Cell Counting Kit-8.

Q: *What should be done regarding materials which may inhibit the color development and interfere with the Cell Counting Kit-8 assay?*
A: Determine whether the material intereferes with the assay. Prepare 0.5 mM NADH solution with PBS. Prepare a couple of wells with and without the material solution. Add 10 μl of 0.5 mM NADH solution and 10 μl of the Cell Counting Kit-8 solution sequentially. Incubate the plate for 10 to 30 min.
a) If both wells with and without the material solution have the same absorbance, the material does not inhibit. Use the material for the assay without modification of the assay protocol.
b) If the well containing the material solution is lower than that of the well without the material solution, remove the culture media and wash the cells with fresh media and add the same volume of fresh media to the well prior to adding the Cell Counting Kit-8.

Q: *What should be done regarding test material which is a reducing agent?*
A: Determine whether the reducing agent interferes with the assay. Add the Cell Counting Kit-8 to the solution containing the reducing material and incubate for a general assay period.
a) If there is no color development during the incubation, the material does not react with the Cell Counting Kit-8.
b) If there is color development during the incubation, the material does react with the Cell Counting Kit-8. Prepare a couple wells which contains all materials except cells for a background absorbance measurement. Measure the absorbance of the background well at 450 nm and subtract the background from the absorbance of the wells that containing all materials and cells.
Notes: If the color development is too high to subtract, remove the culture media and wash the cells with fresh media and add the same volume of fresh media to the wells prior to adding the Cell Counting Kit-8.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

References

Origin	Cell Line	References
bovine brain microvascular endothelial	BBMVEC	T. Kitamuro, *et al., J. Biol. Chem.*, **278**, 9125 (2003)
dog canine kidney epithelial cell	MDCK	H. Shimura, *et al., Cancer res.*, **61**, 3640 (2001)
human anaplastic thyroid carcinoma	ARO	F. Furuya, *et al., Endocrinology*, **145**, 2865 (2004)
human B lymphoid	WSU-CLL	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)
human bladder carcinoma	T24	Y. Shibata, *et al., J. Biol. Chem.*, **277**, 746 (2002)
human bone marrow mesenchymal stem cell	BMMSC	M. Miura, *et al., Stem Cells*, **24**, 1095 (2006)
human bronchial epithelial cell	BEAS-2B	C. A. Reilly, *et al., Toxicol. Sci.*, **73**, 170 (2003)
		M. E. Johansen, *et al., Toxicol. Sci.*, **89**, 278 (2006)
human burkitt lymphoma	Daudi	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)
	Ramos	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)
human cervical carcinoma	C33A	W. Yang, *et al., Mol. Cancer Ther.*, **5**, 1610 (2006)
human colon carcinoma	HCT116	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
human colorectal adenocarcinoma	DLD-1	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
human embryonal carcinoma	NT2N	J. Tessier, *et al., Infect. Immun.*, **75**, 1895 (2007)
human epithelial carcinoma cell	A431	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
human esophageal squamous cell carcinoma	KYSE	I. Imoto, *et al., Cancer Res.*, **61**, 6629 (2001)
		K. Nakakuki, *et al., Carcinogenesis*, **23**, 19 (2002)
human gastric cancer cell	SH10TC	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
human gingival fibroblast	Gin-1	R. Takii, *et al., Infect. Immun.*, **73**, 883 (2005)
human glioblastoma	T98G	T. Kitamuro, *et al., J. Biol. Chem.*, **278**, 9125 (2003)
	U87MG	S. Kim, *et al., Clin. Cancer Res.*, **11**, 5965 (2005)
		S. Kim, *et al., Clin. Cancer Res.*, **12**, 5550 (2006)
human intrahepatic bile duct carcinoma cell	HuCCT1	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
human kidney carcinoma	293T	H. Fuda, *et al., J. Lipid Res.*, **48**, 1343 (2007)
human leukemia cell	Kasumi-1	G. Zhou, *et al., Blood*, **109**, 3441 (2007)
human lung adenocarcinoma	LK87	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
human lung cancer cell	H1299	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999)
		S. Semba, *et al., J. Biol. Chem.*, **281**, 28244 (2006)
human lymphoblast cell	SupT1	J. Melton, *et al., J. Biol. Chem.*, **279**, 14315 (2004)
	T-cell	I. Y. Lee, *et al., J. Immunol.*, **175**, 1658 (2005)
	Namalwa	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)
human medulloblastoma	Daoy	X. Li, *et al., Mol. Cancer Ther.*, **4**, 1912 (2005)
		S. Kim, *et al., Clin. Cancer Res.*, **12**, 5550 (2006)
human mesenchymal stem cell	hMSC	D. Huang, *et al., FASEB J.*, **19**, 2014 (2005)
		L. Song, *et al., Stem Cells*, **24**, 1707 (2006)
human monoblastic lymphoma	U937	R. Hori, *et al., J. Biol. Chem.*, **277**, 10712 (2002)
human multiple myeloma	AMO1	J. Inoue, *et al., Am. J. Pathol.*, **165**, 71 (2004)
	KMS-11	J. Inoue, *et al., Am. J. Pathol.*, **165**, 71 (2004)
human neural stem cell	HB1.F3	S. Kim, *et al., Clin. Cancer Res.*, **11**, 5965 (2005)
		S. Kim, *et al., Clin. Cancer Res.*, **12**, 5550 (2006)
human neuroblastoma	IMR32	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999)
	SK-N-SH	Y. Wang, *et al., J. Virol.*, **78**, 7916 (2004)

Origin	Cell Line	References
human neuroblastoma	SMS-KAN	A. Misawa, *et al., Cancer Res.*, **65**, 10233 (2005)
human non-small-cell lung cancer cell	LCSC#2	H. Ishibashi, *et al., Cancer Res.*, **65**, 6450 (2005)
	RERF-LC-OK	H. Ishibashi, *et al., Cancer Res.*, **65**, 6450 (2005)
human ovarial cancer cell	OVK18	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
human ovarian adenocarcinoma	HTOA	M. Furuya, *et al., Cancer Res.*, **65**, 2617 (2005)
human pancreatic cancer cell	Alexander cell	S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
	AsPC-1	T. Mori, *et al., Mol. Cancer Ther.*, **3**, 29 (2004)
		S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
	BxPC-3	S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
	MiaPaCa-2	A. Aghdassi, *et al., Cancer Res.*, **67**, 616 (2007)
	PBMC	T. Mori, *et al., Mol. Cancer Ther.*, **3**, 29 (2004)
		S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
		A. Aghdassi, *et al., Cancer Res.*, **67**, 616 (2007)
human peripheral blood mononuclear cell	PBMC	C. Chang, *et al., Stem Cells*, **24**, 2466 (2006)
		T. Lee, *et al., Mol. Cancer Ther.*, **5**, 2398 (2006)
human prostate carcinoma	LNCaP	D. J. Son, *et al., Mol. Cancer Ther.*, **6**, 675 (2007)
human pulmonary adenocarcinoma	H441	H. Shimura, *et al., Cancer res.*, **61**, 3640 (2001)
human skin mast cell	primary Mast cell	J. Tessier, *et al., Infect. Immun.*, **75**, 1895 (2007)
human T cell	Jurkat	T. Kasai, *et al., J. Biol. Chem.*, **277**, 5187 (2002)
		L. Lu, *et al., J. Biochem.*, **141**, 157 (2007)
mouse cortical neurons, primary		M. Ikonen, *et al., PNAS*, **100**, 13042 (2003)
mouse Macrophage		Y. Miyake, *et al., J. Immunol.*, **178**, 5001 (2007)
mouse embryonic fibroblast	Balb3T3	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999)
	3T3-L1	D. Huang, *et al., FASEB J.*, **19**, 2014 (2005)
mouse fibroblast	L929	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999)
	NIH3T3	R. Yu, *et al., Toxicol. Sci.*, **93**, 82 (2006)
mouse hippocampal cell	HT22	H. Sohn, *et al., FASEB J.*, **20**, 1428 (2006)
mouse insulinoma	MIN6	S. Oyadomari, *et al., PNAS*, **98**, 10845 (2001)
mouse macrophage	RAW 264	M. Shiga, *et al., Anesth. Analg.*, **92**, 128 (2001)
	RAW 264.7	S. Oyadomari, *et al., PNAS*, **98**, 10845 (2001)
		D. J. Son, *et al., Mol. Cancer Ther.*, **6**, 675 (2007)
mouse hepatocellular carcinoma	MH134	S. Shibata, *et al., J. Immunol.*, **177**, 3564 (2006)
mouse malignant melanoma	B16F1	S. Shibata, *et al., J. Immunol.*, **177**, 3564 (2006)
Wistar rats calvaria	osteoblast	E. Hinoi, *et al., FASEB J.*, **17**, 1532 (2003)

Visit our website, www.dojindo.com for additional cell line reference information. Simply type "CK04" on the search bar to see the product page.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-F

Introduction

The Cell Counting Kit-F is a fluorometic assay for the determination of viable cell numbers. Calcein-AM in this kit passes through the cell membrane and is hydrolized by the esterase in the cell to be converted to calcein, a fluorescence dye. Since the total esterase activity depends on the viable cell number, fluorescence intensity of the assay solution correlates with the viable cell number. The Cell Counting Kit-F has a higher sensitivity than tetrazolium-based assays such as Cell Counting Kit-8, MTT, and XTT assays. Since the Cell Counting Kit-F assay requires culture medium change prior to adding the assay solution, any test materials and medium can be used for this assay. However, this requirement is a disadvantage for assays using non-adherent type cells. A V-bottom plate and a plate rotor for centrifuge is necessary for non-adherent type cells.

Applications: cell counting, cell proliferation experiments, cytotoxicity experiments, drug sensitivity tests

Product Information

Cell Counting Kit-F

Product code	Unit	Components
CK06-10	500 tests	1 vial

One test corresponds to one well of the 96-well plate.

Required Materials

Devices, Tools

- Microplate Reader
  Filters excitation wavelength : 480-500 nm
  emission wavelength : 500-535 nm
- 96 well microplate (for cell culture, fluorescence assay)
- Multi-pipette (8 or 12 channel: 10-100 μl)
- CO_2 incubator
- Clean bench
- Hematocytometer or cell counter

Reagents

- Cell Counting Kit-F (product code: CK06)
- Cell Culture Media
- PBS(-): Phosphate Buffered Saline Solution (does not contain Ca^{2+}, Mg^{2+}), autoclave sterilized

Preparation

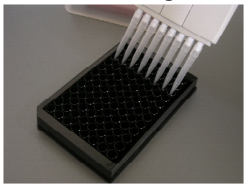
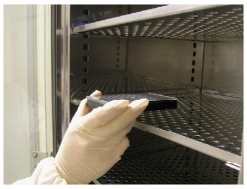
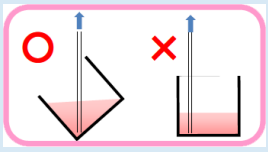
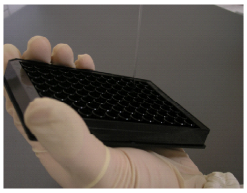
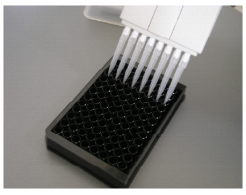
Cell Counting Kit-F solution

Dilute Cell Counting Kit-F by 50 times using PBS (-) to prepare an assay solution

The component of Cell Counting Kit-F is easily hydrolyzed. Prepare the assay solution right before use.

Optimization of Assay Condition

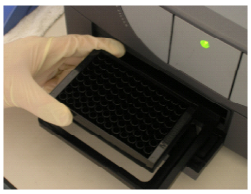
When using Cell Counting Kit-F for proliferation and cytotoxicity assays, it is necessary to have a proportional relationship between amount of fluorescence intensity and cell number. It is desirable to start with a set number of cells, and then roughly determine the incubation time and cell number. Below, the method and conditions for using Cell Counting Kit-F are described.

Procedure	Precautions & Tips
Recover the cells to be assayed from a culture flask.	Use a plate for fluorescent assays to prevent an increase in background.
Count the cells and adjust the concentration of the cell suspension. (cell concentration: _____cells/ml)	For adherent cells, recover the cells using trypsin to detach cells, and use a cell scraper if necessary.
Add a cell suspension of 100 μl to each well in a 96 well microplate using serial dilution. Make a well containing only medium for background measurement. 	Use a hematocytometer or a cell counter. Refer to experimental example on the next page for instruction on serial dilution.
Incubate for 24-48 hrs. in a CO_2 incubator (start time: _____ end time: _____) 	Be aware that cell number after incubation may surpass the initial number of cells counted. In order to establish a relationship between cell number and absorbance, add the reagent before the cells proliferate, and take a reading. Tilt the plate when removing the media to avoid touching the cells with the tip of the pipette as shown in the below diagram. 
After removing the media, add 100 μl PBS (-) which does not contain serum and phenol red to each well. 	For non-adherent cells, centrifuge a V-bottom plate using a microplate rotor and a centrifuge gather the cells on the bottom, and then remove the media with care not to suck in cells. Add 100 ul PBS(-) to each well and pipet to mix, and then transfer the cell suspension to a black or white plate.
Add 10 μl of Cell Counting Kit-F to each well of the 96 well microplate and allow to react at room temperature for 15-30 min. (start time: _____ end time: _____) 	When using a plate or petri dish other than a 96 well plate, add reagent equal to 1/10 the media volume. The 10 ul of the reagent is a very small volume. When adding the reagent, touch the wall of the well with the tip of the pipette, and then introduce the reagent along the wall. If the reagent sticks to the wall, tap the plate lightly to mix it into the culture medium.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-F

Measure the fluorescence intensity on a microplate reader.
Excitation wavelength: 480-500 nm
Emission wavelength: 500-535 nm



Since bubbles can cause an error, make sure there are no bubbles in each well. If there are bubbles, use a pipette tip to remove or use a toothpick to pop them.

Experimental Example

Make serial dilutions of 2.5 x 10^4 , 1.25 x 10^4 , 6.2 x 10^3 ….0 cells to each well in a 96 well plate using HeLa cells (human cervical cancer cells) or HL60 cells (promyelocytic leukemia cells) suspensions as indicated in Fig. 14. Following the method on the previous page to use Cell Counting Kit-F for counting the viable cells.

Serial Dilution Procedure
Using an 8 channel multi-pipette, add 100 µl of media to each well of a 96 well microplate. Next, add 100 µl of a 5 x 10^4 cells/ml solution to the first well and pipette to mix. This well will have the maximum number of cells. Next, take 100 µl from the first well and add it to the next well, and mix. The process is repeated as indicated in the figure. Take 100 µl from the last well which contains the minimum number of cells and discard.

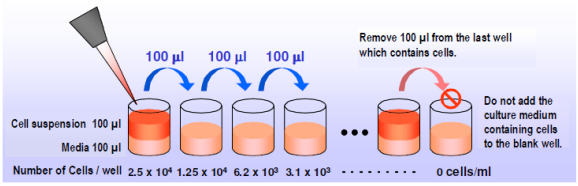


Fig. 14 Serial dilution process

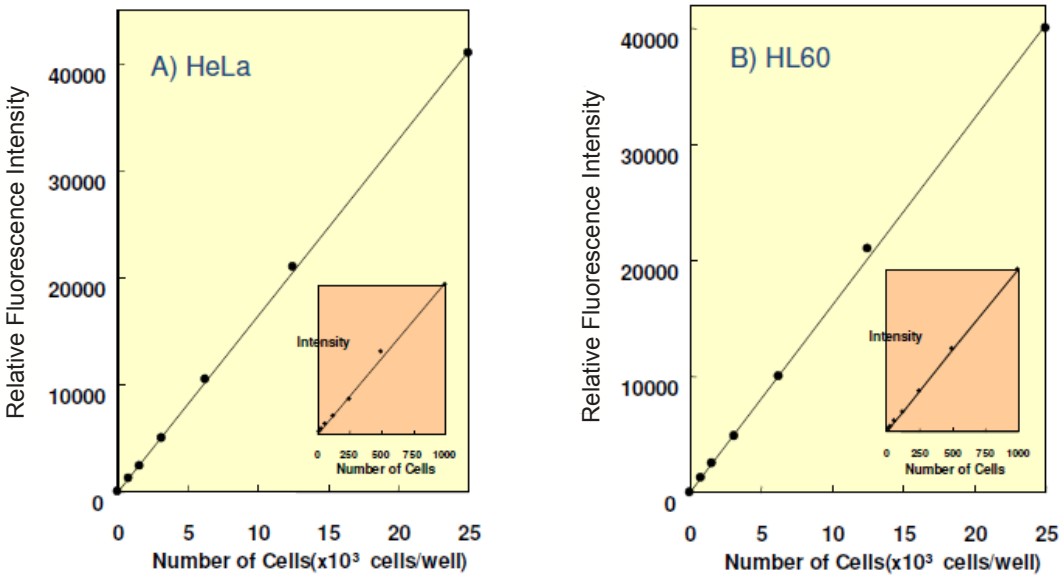
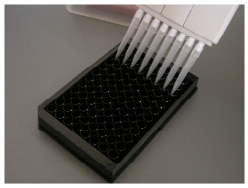
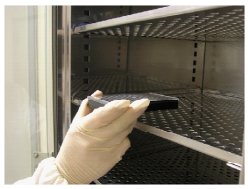
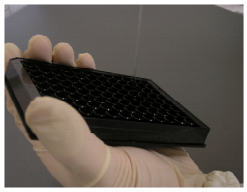
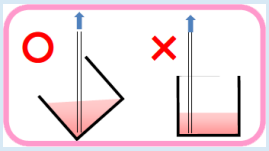
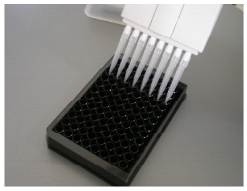
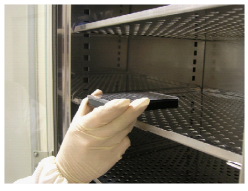


Fig. 15 Assay data of HeLa cells (A) and HL60 cells (B). Sensitivity of the Cell Counting Kit-8 for HL60 (non-adherent type cell) is about ten times lower than that for HeLa (adherent type cell). However, the sensitivity of the Cell Counting Kit-F for HL60 cells (non-adherent type cell) is almost the same as the HeLa cells.

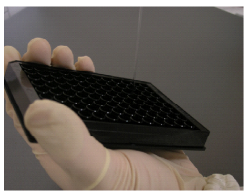
Cell Proliferation and Cytotoxicity Protocol

Procedure	Precautions & Tips
Recover the cells to be assayed from flask.	For adherent cells, recover the cells using trypsin to detach cells, and use a cell scraper if necessary.
Count the cells and adjust the concentration to desired cell numbers of the suspended cells. (cell concentration: _____cells/ml)	Use a hematocytometer or a cell counter. *Please see "Optimization of Assay Condition" on page 24.
Add a cell suspension of 100 µl to each well in a 96 well microplate using serial dilution. Make a well that contains only medium for a background measurement. 	Use a V bottom plate for suspension cells.
Incubate for 24-48 hrs. in a CO_2 incubator. (start time: _____ end time: _____) 	When incubating for more than 48 hrs, change the media.
Change media when necessary. Remove media and add 100 µl of new media to each well. Add only media to the well used for background measurement. 	Tilt the plate when removing the media to avoid touching the cells with the tip of the pipette as shown in the below diagram. 
Add 10 µl of media containing different amounts of the test substance to each well. 	Add the same amount of test substance to the blank wells (no cells). For negative control wells, add 10 µl of media that does not contain the test substance. It is possible to use PBS or saline solution other than media for dissolving the test substance.
Incubate for a set periods (6, 12, 24, 48 hrs) in a CO_2 incubator. 	The exposure time depends on the test substance and purpose of the experiment. If the substance is highly toxic to the cell and destroys the cell membrane or reacts directly with proteins or DNA, short exposure time will be appropriate. If the substance slowly affects cell function, longer exposure time may be necessary.

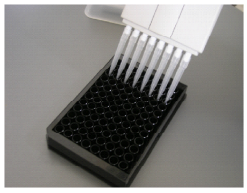
301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-F

After removing the media from each well, add 100 µl of PBS(-) which does not contain serum and phenol red to each well.

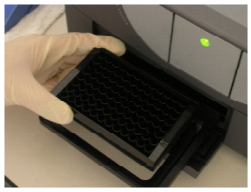


Add 10 µl of Cell Counting Kit-F to each well of the 96 well microplate.

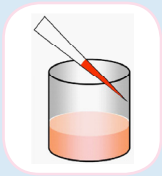


Allow to react at room temperature for 15-30 min.
(start time: _____ end time: _____)

Measure fluorescence intensity on a microplate reader.
Excitation wavelength: 480-500 nm
Emission wavelength: 500-535 nm



When using a plate or petri dish other than a 96 well plate, add reagent equal to 1/10 of the media volume.
Due to the low volume of reagent added, it is recommended to touch the tip of the pipette to the wall of the well when adding the reagent (below picture). If the reagent sticks to the well wall, tap the plate lightly to mix with the media.



Since bubbles can cause error, make sure there are no bubbles in each well. If there are bubbles, use a pipette tip to remove or use a toothpick to pop them.

Calculating Cell Survival Rate

Enter each fluorescence intensity in the equation below to calculate the cell survival rate.

$$\text{Survival rate (\%)} = \frac{F_{sample} - F_b}{F_c - F_b} \times 100$$

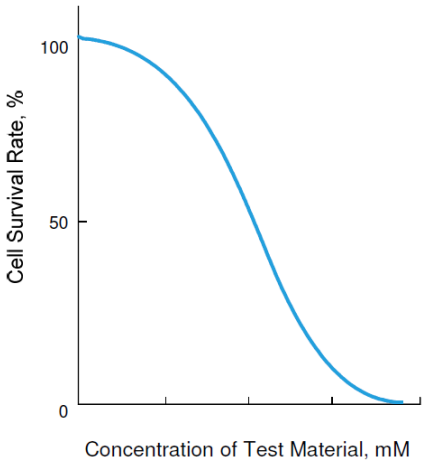


Fig. 16 Typical cell survival curve.

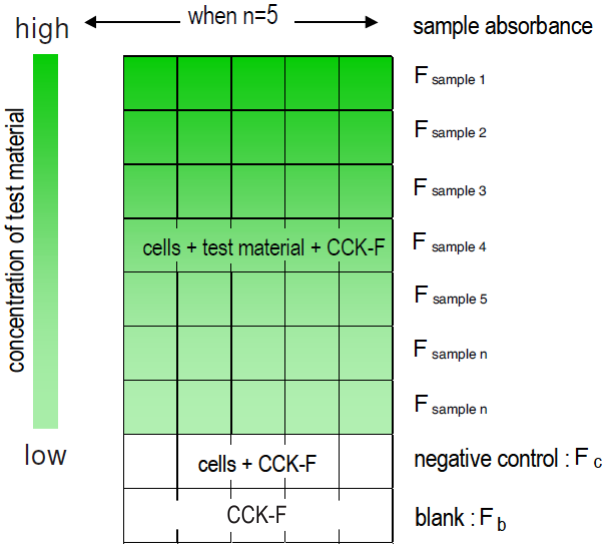


Fig. 17 Example of the plate arrangement and fluorescence development.

Troubleshooting

Problem	Possible Cause	Solution
Low sensitivity (high background)	The cell number is low.	Measure the relationship between the cell number and amount of fluorescence for each cell type. Refer to Assay Conditions. Also, the permeability of Calcein-AM across the cell membrane is different for each cell type.
	The reagent has deteriorated.	Cell Counting Kit-F solution (Calcein-AM) is extremely unstable after diluting with PBS (-). Use the diluted solution soon after preparing.
	The washing is insufficient.	Serum components and phenol red can have an effect on the assay, so be sure to wash the cell sufficiently prior to adding Cell Counting Kit-F solution.
There is a lot of variance in the assay values.	A transparent plate was used.	Please use a black or white plate made for fluorescent measurement.
	There is a change in the reagent concentration due to evaporation of the media.	Evaporation occurs easiest on the outer-most wells, do not use them for the assay. Add only media to the outer-most wells (no cells).
	Cell Counting Kit-F solution was not mixed well with the media.	Lightly tap the outside of the well in order to get the Cell Counting Kit-F that is on the well wall to fall into the media. When tapping the plate, be careful not to splash the media.
	There are bubbles on the surface of the media.	Remove the bubbles using a syringe or needle.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Measuring Cell Viability/Cytotoxicity: Cell Counting Kit-F

Q&A

Q: *Is the Calcein-AM (Calcein) used in Cell Counting Kit-F stable inside and outside the cell?*
A: Calcein is stable both inside and outside of the cell. However, Calcein-AM is not stable. If there is excess Calcein-AM on the outside of the cell, the excess may break-down and fluoresce, which would be a source of error. It is necessary to remove by washing. Fluorescence intensity is affected by a fluctuation in pH, so it is necessary to set the pH conditions.

Q: *Is it possible to do an assay using a regular transparent incubation plate?*
A: Light reflecting off of a transparent plate scatters, making it not possible to get accurate results. It is necessary to use a black or white fluorescent plate for accurate reading.

Q: *Will fluorescence increase as incubation time increases?*
A: Even if the incubation time is increased, the fluorescence intensity will not increase. Rather, there is a possibility that it will decrease. Calcein-AM passes through the cell membrane, and it is hydrolyzed inside the cell, which turns to Calcein and then to fluoresces. Calcein is a foreign object inside the cell, so it is expelled from the cell. Thus, the intensity of the fluorescence gradually reduces.

Q: *Why is it not possible to use Cell Counting Kit-F in assays that contain serum?*
A: Calcein-AM is broken down on the outside of the cell by serum in the media. In order to make it possible to do an assay with small number of cells, remove anything that may cause the background flurescence.

Q: *Can serum and phenol red be in the media during the pre-incubation step?*
A: There is no problem if they are present during the pre incubation step. Just exchange the media prior to adding Cell Counting Kit-F when phenol red is present.

Q: *Are there any other options if the media containing phenol red which can not be exchanged?*
A: Cell Counting Kit-8 is recommended. There is a difference between the fluorometric and colorimetric methods, and the sensitivity will be lower, but it is possible to do an assay with CCK-8 when using media that contains phenol red.

Q: *What is the principle behind the Cell Counting Kit-F assay?*
A: Calcein-AM, which contains an esterol in its structure, is hydrolized by esterase after passing through the cell membrane and form the fluorescent dye Calcein as indicated in Fig. 18. By measuring the amount of fluorescence, it is possible to determine the number of presented cells. In addition, Calcein has low permeability across the cell membrane, so it does not leave the cell easily.

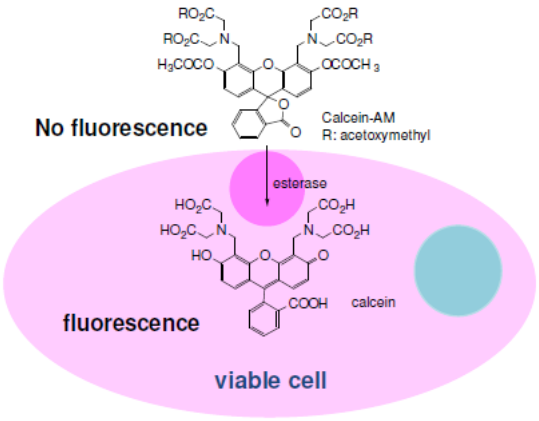


Fig. 18 Cell viability detection mechanism with CCK-F

Introduction

Visualization of a cell with fluorescent compounds provides a wide variety of information for the analysis of cell functions. Various activities and structures of a cell can be targeted for staining with fluorescent compounds (Fig. 19). The most commonly stained cell components are cell membranes, proteins, and nucleotides. Small neutral molecules and positively charged molecules can pass through viable cell membranes and remain inside of cells, depending on their reactivity or hydrophilicity. Negatively charged molecules cannot pass through viable cell membranes. Positively charged molecules are usually cell membrane permeable and accumulate in the mitochondria. Ester is a suitable functional group for staining viable cells because it can pass through viable cell membranes, where it is hydrolyzed by cellular esterases into a negatively charged molecule under physiological conditions. Several fluorescein analogs with ester groups in their structure are available for viable cell staining. Succinimidyl ester compounds can also be used to improve the retention of the fluorescent derivative within the cell. These compounds are neutral molecules that pass through cell membranes and covalently conjugate with cell proteins. Covalently-conjugated molecules can stay in the cell for several weeks. Nucleotide staining with fluorescent intercalators is mostly applied to dead cell detection.

Cell Cytosol Staining

Fluorogenic esterase substrates that can be passively loaded into viable cells, such as Calcein-AM, BCECF-AM, Carboxyfluorescein succinimidyl ester (CFSE), and Fluorescein diacetate (FDA), are converted by intracellular esterases into fluorescein analogs with green fluorescence. Calcein and BCECF are converted into electrically neutral molecules by the addition of acetyl or acetoxymethyl groups to their phenolic OH or carboxylic groups, which allows them to freely permeate into the cell. Once converted into fluorescent products by esterase, these compounds are retained by cells because of their negative charges. These esterase substrates, therefore, can serve as cell viability assay probes.

Fluorescent esterase substrates may also be used in cell viability assays in place of tetrazolium analogs such as MTT or WST. The mechanism of the determination of cell viability is different: though both assays determine cell metabolism, esterase substrates detect esterase activity, and tetrazolium salts detect dehydrogenase activity of viable cells. CFSE is also an ester compound that passes through viable cell membranes. Since it has an amine-reactive succinimidyl group, fluorescein derived from CFSE can covalently bind to proteins or other amino groups in the cell or on the cell membrane. This covalently-attached fluorescein is stable enough to trace the cell over several weeks.

Mitochondria Staining

Mitochondria exist in most eukaryotic cells and play a very important role in oxidative metabolism by generating ATP as an energy soruce. The average number of mitochondria per cell is from 100 to 2,000. Though the typical size is about 0.5-2mm, the shape, abundance, and location of mitochondria vary by cell type, cell cycle, and cell viability. Therefore, visualization of mitochondra is important. Since mitochondria have electron transport systems, they can be stained with various redox dyes. MitoRed and Rh123 readily pass through cell membranes and accumulate in mitochondria. The fluorescence intensity of Rh123 reflects the amount of ATP generated in mitochondria.

Nucleus Staining

Fluorescent dyes with aromatic amino or guanidine groups, such as propidium iodide (PI), ethidium bromide (EB), diaminophenylindole (DAPI), acridine orange (AO), and Hoechst dyes, interact with nucleotides to emit fluorescence. EB and PI molecules intercalate inside the DNA double helix. DAPI and Hoechst dye molecules attach at the minor groove of the DNA double helix. On the other hand, AO can form complexes with either double-stranded DNA or single-stranded DNA and RNA. One molecule of AO can intercalate with three base pairs of double-stranded DNA to emit green fluorescence with the maximum wavelength at 526 nm. One molecule of AO can also interact with one phosphate group of single-stranded DNA or RNA to form an aggregated, or stacked, structure that emits red fluorescence with the maximum wavelength at 650 nm. These fluorescent dyes, except for the Hoechst dyes, are impermeable through the cell membranes of viable cells, and can be used as fluorescent indicators of dead cells. Hoechst dyes are positively charged under physiological conditions and can pass through viable cell membranes.

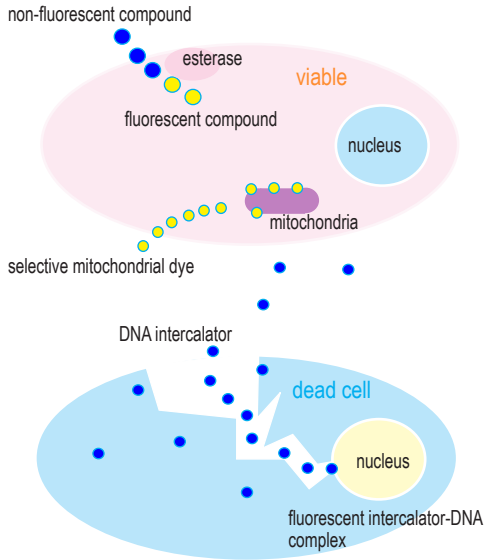


Fig. 19 Cell staining methods.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Cell Staining Reagents

Applications: Fluorescence Microscopy, Flowcytometry, Electrophoresis (Nucleic Acid Screening)

Characteristics of Dye

Target	Dye	Excitation	Emission	Excitation filter	Color	Characteristic
Living cells	BCECF-AM	490 nm	526 nm	B excitation	yellowish green	Fluorescence is produced by hydrolysis inside the cell.
	Calcein-AM	490 nm	515 nm	B excitation	yellowish green	
	CFSE	496 nm	516 nm	B excitation	yellowish green	
	CytoRed	535 nm	590 nm	G excitation	red	
	FDA	488 nm	530 nm	B excitation	yellowish green	
Dead cells	DAPI	360 nm	460 nm	W excitation	blue	Fluorescence is produced by interacting with the nucleus of dead cells.
	PI	530 nm	620 nm	G excitation	red	
Nucleous	AO (dsDNA)	500 nm	526 nm	B excitation	red	Fluorescence is produced by combining with single stranded and double stranded DNA.
	(ssDNA & RNA)	420-460 nm	630-650 nm	B excitation	yellow	
	Hoechst33258	350 nm	461 nm	W excitation	blue	Fluorescence is produced by combining with the nucleus of living and dead cells.
	Hoechst33342	352 nm	461 nm	W excitation	blue	
Mitochondria	MitoRed	560 nm	580 nm	G excitation	red	Fluorescence is produced by accumulation in the mito-chondria.
	Rh123	507 nm	529 nm	B excitation	yellowish green	

Product Information

-Cellstain- Series

Product name	Product code	Unit
BCECF-AM	B262-10	1 mg
Calcein-AM	C326-10	1 mg
Calcein-AM solution	C396-10	1 ml
CFSE	C375-10	1 mg
CytoRed solution	C410-10	1 ml
FDA	F209-10	1 mg
DAPI	D212-10	1 mg
DAPI solution	D523-10	1 ml
PI	P346-10	1 mg
PI solution	P378-10	1 ml
AO	A386-10	1 mg
AO solution	A430-10	1 ml
Hoechst33258 solution	H341-10	1 ml
Hoechst33342 solution	H342-10	1 ml
MitoRed	R237-10	50 mg x 8 vials
Rh123	R233-10	1 mg

Required Materials

Devices, Tools

- CO_2 incubator
- Clean bench
- Fluorescence microscope
- Cytometer or cell counter
- Centrifuge
- Slide glass, cover glass, or chamber slide

Reagents

Living Cell Staining Dyes

-Cellstain- Calcein-AM (product code: C326) *-Cellstain-* Calcein-AM solution(product code: C326)
-Cellstain- CFSE (product code: C375) *-Cellstain-* CytoRed solution (product code:C410)
-Cellstain- FDA (product code: F209) BCECF-AM (product code:B262)

Dead Cell Staining Dyes

-Cellstain- DAPI (product code: D212) *-Cellstain-* DAPI solution (product code: D523)
-Cellstain- PI (product code: P346) *-Cellstain-* PI solution (product code:P378)

Nucleus Staining Dyes

-Cellstain- AO (product code: A386) *-Cellstain-*AO solution (product code: A430)
-Cellstain- Hoechst 33258 (product code: H341) *-Cellstain-*Hoechst 33342 (product code: H342)

Mitochondria Staining Dyes

-Cellstain- MitoRed (product code: R237) *-Cellstain-* Rh123 (product code: R233)

Other Reagents

DMSO, Sterilized Water, PBS(-)

Preparation of Staining Solution

The following is a general protocol for preparing assay solutions. In order to obtain the best results, optimization of staining conditions, such as changing the reagent concentration and staining time will be required.

Some reagents are stable in the solution. However, some reagents are not stable. Please follow the storage conditions for each reagent. Generally, the reagents offered in the solution form are fairly stable. If no microbalance is available to weigh small amounts of the reagent, add an appropriate amount of solvent described in the chart, aliquot, and store it in a freezer.

Dyes for Living Cell Staining

If the reagent is in a solid form, use DMSO to prepare a solution with a certain concentration. Since CFSE has a succinimidyl group, the stability of the prepared DMSO solution is poor. After the preparation of the DMSO solution, aliquot in an appropriate volume and store at -20 °C. The DMSO solution can be used for several months. The working solutions prepared by PBS (-) are not stable enough to store. Discard the remaining working solution after each use.

Product name	Characteristic	Storage	Mol. Weight	Unit size	Stock Solution (DMSO)	Staining solution
-Cellstain- Calcein-AM	white-yellowish solid	avoid light, freeze	994.86	1 mg	0.5 - 1mmol/l	1-20 μmol/l (Storage solution diluted by PBS (-))
-Cellstain- Calcein-AM solution	colorless liquid	avoid light, freeze	994.86	1 ml	1mmol/l	
-Cellstain- CFSE	white-yellowish solid	avoid light, freeze	557.64	1 mg	0.5 - 1mmol/l	
-Cellstain- CytoRed solution	orange-yellow liquid	avoid light, freeze	313.31	1 ml	1mmol/l	
-Cellstain- FDA	white crystal	avoid light, freeze	416.38	1 mg	0.5 - 1mmol/l	
BCECF-AM Special packaging	orange-brown solid	avoid light, freeze	688.59	50 μg x 8	0.5 - 1mmol/l	

Staining solutions are not stable for a storage. Discard the remaining staining solution after each use.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Cell Staining Reagents

Dyes for Dead Cell Staining

If the reagent is in a solid form, use sterilized water to prepare a solution and store according to the indicated condition. Prepare DAPI solution with PBS. Working solutions prepared using PBS(-) are not stable enough to store. Discard the remaining working solution after use.

Product name	Characteristic	Storage	Mol. Weight	Units	Stock Solution (H_2O)	Staining Solution
-*Cellstain*-DAPI	yellow solid	avoid light, freeze	350.25	1 mg	1 mg/ml	1-10 μg/ml (Storage solution diluted by PBS (-))
-*Cellstain*-DAPI soln.	light yellow liquid	avoid light, refrigerate	350.25	1 ml	1 mg/ml*	
-*Cellstain*-PI	red-brown solid	avoid light, refrigerate	668.39	1 mg	1 mg/ml	
-*Cellstain*-PI soln.	red liquid	avoid light, freeze	668.39	1 ml	1 mg/ml	

* Use buffer to prepare a solution

Since the dyes directly stain the nucleus, these dyes are considered mutagens. Gloves, safety goggles, and masks are necessary when handling. If it comes in contact with your skin, immediately wash with a copious amount of water.

When disposing remaining dye solution and solution containing staining dyes, follow handling guidelines and regulations, and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Dyes for Nucleus Staining

If the reagent is in a solid form, use sterilized water to prepare a solution and store according to the indicated condition. Working solutions prepared using PBS(-) are not stable enough to store. Discard the remaining working solution after use.

Product name	Characteristic	Storage	Mol. Weight	Units	Stock Solution (H_2O)	Staining Solution
-Cellstain-AO	red-brown	avoid light, refrigerate	301.81	1 mg	1 mg/ml	1-10 μg/ml (Storage solution diluted by PBS (-))
-Cellstain-AO soln.	orange-yellow liquid	avoid light, freeze	301.81	1 ml	1 mg/ml	
-Cellstain-Hoechst 33258 soln.	yellow liquid	avoid light, refrigerate	533.88	1 ml	1 mg/ml	
-Cellstain-Hoechst 33342 soln.	yellow liquid	avoid light, refrigerate	561.93	1 ml	1 mg/m	

Since the dyes directly stain the nucleus, these dyes are considered mutagens. Gloves, safety goggles, and masks are necessary when handling. if it comes in contact with your skin immediately wash with a copious amount of water.

When disposing remaining dye solution and solution containing staining dyes, follow handling guidelines and regulations and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Dyes for Mitochondria Staining

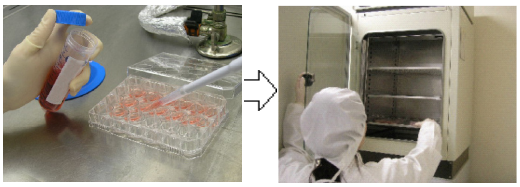
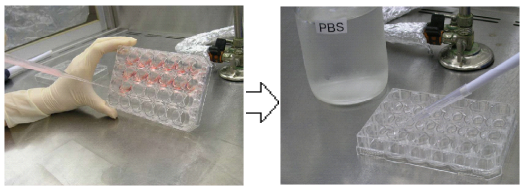
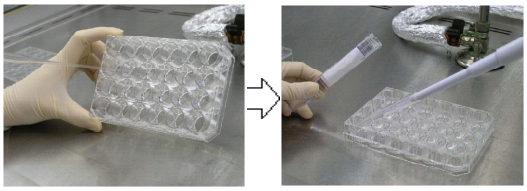
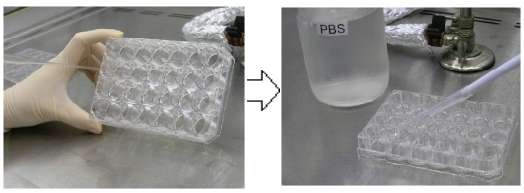
Prepare the stock solution using DMSO.

Product name	Characteristic	Storage	Mol. Weight	Units	Stock Solution (H_2O)	Staining Solution
-Cellstain-MitoRed	purple brown solid	avoid light, refrigerate	637.17	50 μgx8	1 mmol/l*	20 - 200 nmol/l
-Cellstain-Rh123	brown powder	avoid light, refrigerate	380.82	1 mg	1 mg/ml	20 - 100 nmol/l

* Avoid storing in the solution.

Only mitochondria in the living cells will be stained.

Staining Procedure for a Fluorescence Microscopy (for 24-well plate)

Procedure	Precautions & Tips
Add 10^4 -10^5 cells to each well with cell culture medium and incubate overnight. 	Use a cytometer or a cell counter to measure cell number. When using a glass bottom plate, the clear image of cells may be obtained. Gently pipette to avoid damaging the cells.
Remove cell culture medium and wash cells by PBS(-) buffer. 	Gently remove the medium and add PBS buffer without damaging the cells. When using a staining dye for staining living cells, the dye will be hydrolized and emit fluorescence if esterase in the media remains. This is one of the factor for a high background, so it is important to wash the cells several times.
Remove PBS(-) buffer, add 0.2 ml “Staining Solution” to cells and incubate cells at 37°C for 15-30 min. 	Refer to the previous section “Preparation of Assay Solution” and prepare the “Staining Solution”. In order to obtain the best fluorescent image, it is necessary to determine the optimal reagent concentration and staining time.
Remove “Staining Solution” and wash cells by PBS(-) buffer for 1-2 times. 	
Observe the fluorescent image under a fluorescence microscope. 	

If you use suspention cells, it’s necessary to centrifuge the cell suspension at 500 *xg* for 3 min before removing the medium, PBS(-) buffer or “Staining Solution”.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

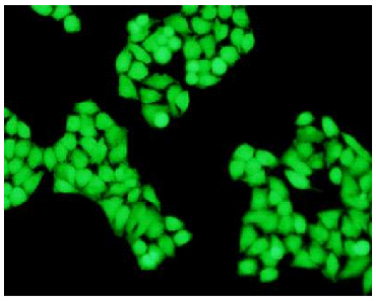
Experimental Example 1

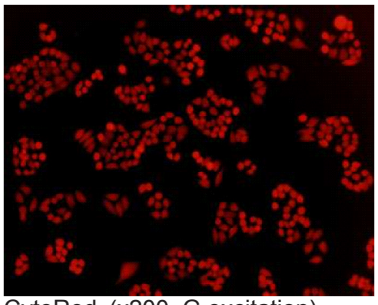
Living HeLa cells were stained with each reagent by following the protocol on page 34.

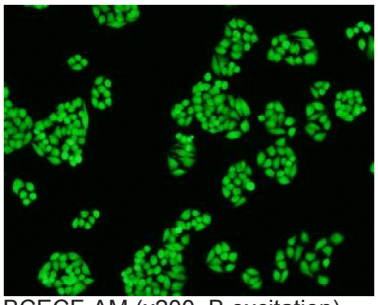
Living Cell Staining Images

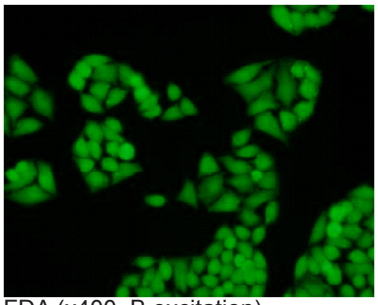
Cellular hydrolysis activity was visualized at strong fluorescence by living cell staining reagents.

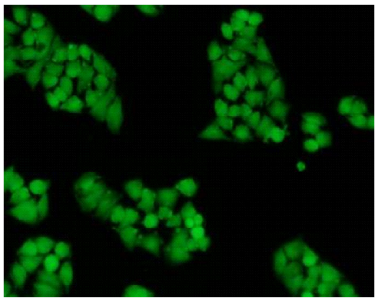
.


Calcein-AM (x400, B excitation)


CytoRed (x200, G excitation)

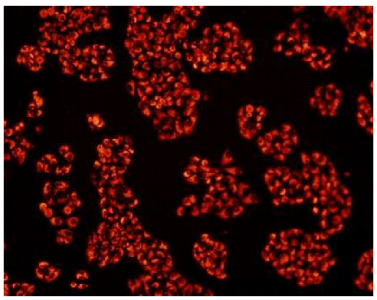

BCECF-AM (x200, B excitation)

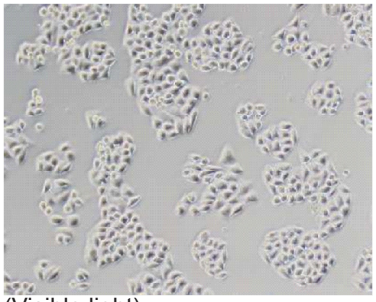

FDA (x400, B excitation)


CFSE (x400, B excitation)

Mitochondria Staining Images

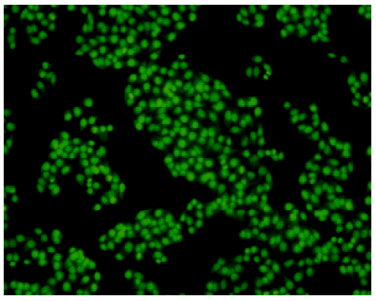
MitoRed accumulated on the mitochondria in the living cell.

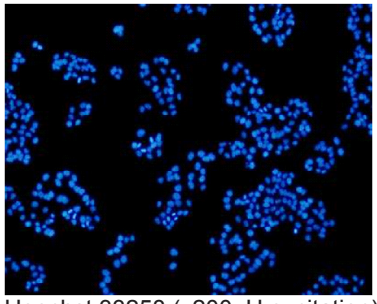

MitoRed (x200, G excitation)


(Visible light)

Nucleus Staining Images

AO and Hochst33258 produced strong fluorescence after binding with nuclear in dead and living cells.


AO (x200, B excitation)

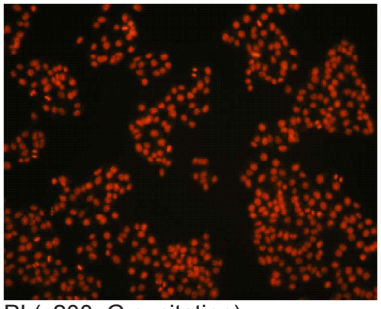

Hoechst 33258 (x200, U excitation)

Experimental Example 2

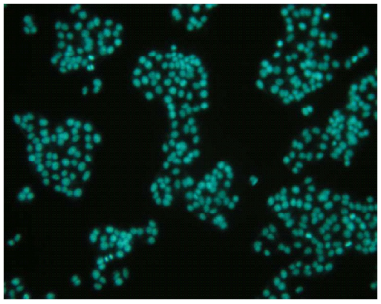
HeLa cells were fixed with 80% ethyl alcohol and stained by each reagent.

Dead Cell Staining Images

The nuclear of all fixed cells was stained with the dead cell staining reagents.



PI (x200, G excitation)



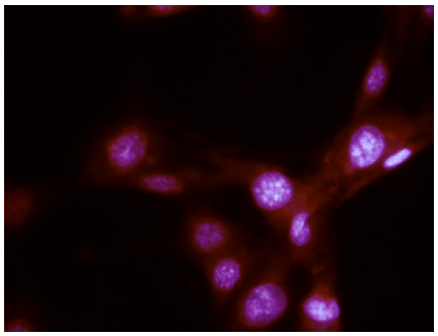
DAPI (x200, U excitation)

Experimental Example 3

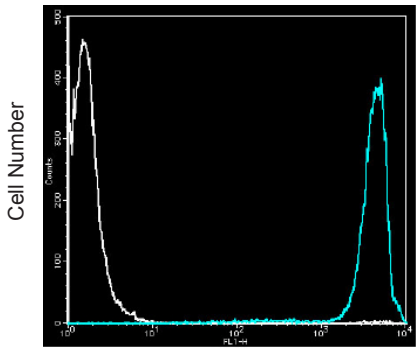
Fluorescent Staining of Fixed Cells

NIH3T3 cells that were fixated with 3% glutaraldehyde were stained with the nuclic acid staining reagent Hoechst 33258. Then, actin filaments were stained with biotin-labeled phalloidin and anti-biotin antibody labeled with HiLyte Fluor™ 555.*

* HiLyte Fluor™ Dyes (patent pending) manufactured by AnaSpec. Inc.



Experimental Example 4



Flowcytometry Example

HL60 cells were stained with Calcein-AM, a reagent used to stain the living cells. The cells were then measured using flowcytometry (excitation: 488 nm). The fluorescence of the stained living cells (blue line) increased dramatically compared to the unstained cells (white line).

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Living and Dead Cells Staining: *-Cellstain-* Double Staining Kit

Introduction

-Cellstain - Double Staining Kit combines Calcein-AM (used for fluorescent staining the living cells) and Propidium Iodide (used for a fluorescent staining of the dead cells) for simultaneous staining of the living and the dead cells.

Product Information

-Cellstain- Double Staining Kit

Product code	Unit	Components
CS01-10	1 set	Solution A(Calcein-AM) x 4 vials, Solution B(PI) x 1 vial

Required Materials

Devices, Tools

- CO_2 incubator
- Clean bench
- Fluorescence microscope
- Hematocytometer or cell counter
- Slide glass, cover glass
- Multi-pipette (8 or 12 channel: 10-100 μl)

Reagents

- *Cellstain* - Double Staining Kit (item code: CS01)

  Kit contents

  Solution A: Calcein-AM stock solution (1 mmol/l) 4 vials

  Solution B: PI stock solution (1.5 mmol/l) 1 vial

⚠ Store at -20 °C and protect from light.

Solution A (Calcein-AM) is easily hydrolized by moisture. Tightly close the cap after the use.

Concentration of Reagent in dye solution: Calcein-AM: 2 mmol/l, PI: 4 mmol/l

- PBS(-)

Preparation

Staining Solution

Bring Solution A and Solution B to room temperature.

Add 10 μl of Solution A and 15 μ of Solution B to 5 ml of PBS (-) and mix.

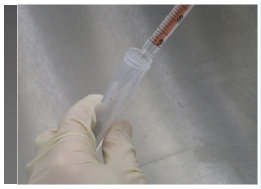
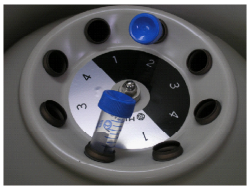
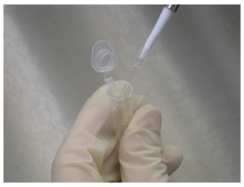
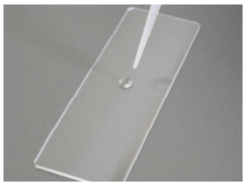
⚠ Prepare the staining solution only prior to each use.

⚠ PI may be mutagenic, so wear gloves, safety goggles, and mask when handling. If it comes in contact with your skin, immediately wash with a copious amount of running water.

⚠ When disposing of remaining dye solution, follow handling guidelines and regulations and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Staining Procedure for a Fluorescence Microscopy

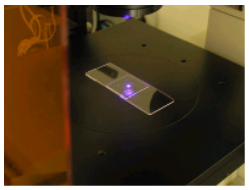
The below procedure is used to stain adherent cells. Please be aware that the staining conditions may vary depending on the cell types and the concentration of the reagent.

Procedure	Precautions & Tips
Recover the cells to be assayed from a culture flask. 	Recover using the trypsin to detach cells, and use a cell scraper if necessary.
Centrifuge the cell suspension (500 xg for 3 min). 	
Remove the supernatant of the media, and add PBS (-). At this step, adjust the cell volume to 10^5 - 10^6 cells/ml.	When using Dye reagents for staining living cells, each ester group of the dye will be hydrolyzed and fluoresce if esterase remains in the media. This is one factor for a high background, so it is important to wash cells several times. Use a hematocytometer or a cell counter.
Add 200 μl of the cell suspension to a microtube. 	Gently pipette to avoid damaging the cells.
Add 100 μl of Staining solution to the same tube.	In order to get the best fluorescent image, it is necessary to determine the optimal reagent concentration and a staining time.
Incubate at 37 °C for 15-30 min with protection from light.	
Place 10 μl of the cell and staining solution on a glass slide and cover with a cover glass. 	

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Living and Dead Cells Staining: *-Cellstain-* Double Staining Kit

View the fluorescent image on a fluorescence microscope.



It is possible to observe yellowish-green stained living cells using a 490 nm excitation filter. In addition, red stained dead cells can be observed simultaneously.

It is possible to observe the fluorescence of dead cells stained red using a 545 nm excitation filter.

How to Determine the Optimum Concentration of Dye

The best concentration for Calcein-AM and PI depends on the cell type, so it is necessary to determine the concetration when staining each cell. The best concentration can be determined using the following protocol.

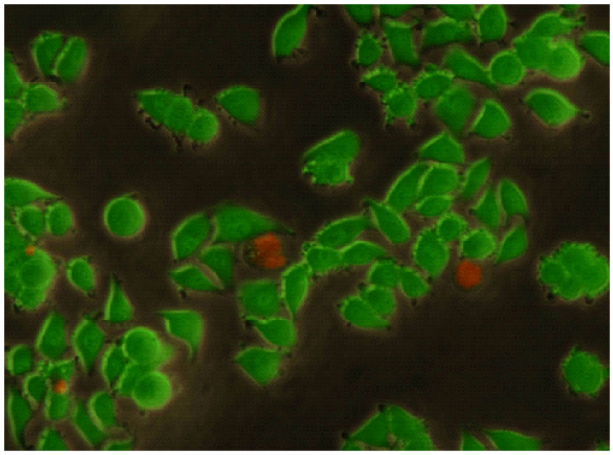
Optimum concetration for PI

Stain the desired cells with 0.1 - 10 μmol/l of PI. Too high concentration of PI will stain not only nuclear but also cytosol, staining concentration should be adjust to appropriate range.
Fix the cells prior to staining using one of the method below if necessary:
-Treat the cells for 10 min with 0.1 % saponin or 0.1 -0.5% digitonin.
-Treat the cells for 30 min with 70% ethanol.

Optimum concentration for Calcein-AM

Using the fixed cells to stain with 0.1 - 10 μmol/l of Calcein-AM solution. Determine the concentration range that will not stain all of the fixed cells. Next, using the living cells, determine if the concentration is enough to stain the cells. If sufficient staining has not been obtained, increase the concentration of Calcein-AM.

Experimental Example



Simultaneous staining using -Cellstain- Double Staining Kit

Green fluorescence indicates the living cells stained by Calcein-AM using B excitation filter.
Red fluorescence indicates the dead cells stained by PI using G excitation filter.

Troubleshooting

Problem	Possible Cause	Solution
The cells are not stained well.	The staining dye was hydrolized or decomposed due to the exceedingly long term storage or incorrect storage conditions.	Check the purchase date of the reagent and the storage conditions. If the reagent has been stored for a year from the purchase date, do not use. The staining dye may not work properly.
	The dye in the working solution was hydrolized or decomposed because the solution was not freshly prepared.	Some of the reagent is unstable in a buffer solution. In particular, viable staining dye is fairly unstable in the buffer solution. Prepare a working solution only prior to each use.
	The dye or the working solution was decomposed by the exposure to light.	Light may accelerate the oxidation process of the dyes. Keep the reagent under the proper storage conditions. Protect the working solution from light during the experiment.
	The concentration of the dye in the working solution is too low.	Increase the concentration of the dye in the working solution. If there is no change, use Pluronic F-127 or another low toxic detergent to improve the dye uptake by the cell if it is allowed
		The dye did not dissolve completely with the solvent. Make sure that the proper solvent was used and the proper concentration was prepared.
The dye seems not to stay inside of the viable cell after staining.	The viable cell expels the dye due to the cell function.	Use the stained cell as quickly as possible for your experiments.
	Not enough reagent was used for the cells.	Probenecid, a transporter inhibitor, may be used to block the leakage of the dye from the cell.
The dye remains insoluble with the solvent.	Since a vacuum centrifuge was used to prepare the dye product, the dye is tightly packed on the bottom of the tube.	Use a vortex mixer or ultra sonic bath to dissolve the dye with the solvent completely.
	The dye was decomposed or hydrolized	Check the purchase date of the reagent & storage conditions. If the reagent has been stored for over a year from the purchase date, do not use. The staining dye may be decomposed or hydrolized.
	The wrong solvent was used to dissolve.	Simultaneuos Staining of living and dead cells Use the proper solvent to prepare a dye solution
High fluorescent background is observed.	Not enough washing and the dye still remained after the washing process.	Repeat washing with PBS(-) or an appropriate buffer to remove excess dye from the cells.
	Too much dye was used for the staining.	Reduce the concentration of the dye in the working solution.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Cell Staining

Q&A

Staining reagents for living cells

Q: *What should the powder-type reagent be dissolved in?*

A: Please dissolve the reagent in DMSO for viable cell staining reagents. Since DMSO easily absorbs moisture, please use fresh DMSO.

Q: *Among all the staining reagents used for living cells, which one remains the longest inside cells?*

A: CFSE remains relatively the longest inside the cells. It has been reported in a paper that the fluorescent dye was retained within cells for up to 8 weeks. Also, the fluorescence of Calcein-AM and BCECF-AM have been observed in cells for up to three days. Please refer to the following for more details:

ES.A.Weston, *et.al.*, *J.Immunol.Methods*, **133**, 87-97 (1990)

EH.P.Zhong, *et.al., Hum.Immunol.*, **37**, 264-270 (1993**)**

Q: *Which staining reagents used for living cells have the lowest cytotoxicity?*

A: Calcein-AM and BCECF-AM seem to have the lowest cytotoxicity,
Please refer to the following for more details:

EL.S.D.Clerck, *et.al.*, *J.Immunol.Methods*, **172**, 115-124 (1994)

Q: *What are the characteristics of staining dyes used for the living cells?*

A: Refer to the list below for characteristics of each product:

BCECF-AM : This was originally used to measure pH inside the cell, and is also used as a dye to stain living cells.

Calcein-AM: This has the least effect on cell function.

CFSE: After entering into a cell, it combines with the amino base of protein in the cell membrane on the cytoplasm side. As a result, it leaks out of the cell comparatively less than other dyes.

CytoRed: A compound produced by Dojindo, it posses a higher fluorescence intensity than Calcein-AM.

FDA: The oldest known dye. It leaks out of the cell relatively quickly.

Q: *Are there any papers that report on the toxicity of the dyes?*

A: Refer to the below paper comparing the toxicity of Calcein-AM, BCECF-AM, CFDA, and CFSE.

L. S. D. Clerck, *et al.,J.Immunol. Methods*, **172**,115 (1994)

Q: *Which dye should be used to stain the bacteria?*

A: Since bacterial cells have a cell wall, most cell staining dyes cannot penetrate. For example, Calcein-AM and BCECF-AM will pass through the cell membrane of animal cells, but will not pass through the bacteria cell wall. AO can be used to stain bacteria such as malaria parasites. PI, and DAPI can be used to stain dead bacteria cells. There is a report of using FDA to stain living bacteria. Refer to the paper below for more information:

Appl. Microbiol. Biotechnol., **38**, 268 (1992)

Nucleus staining reagents (dead cells)

Q: *What are the differences between the nuclues staining reagents AO, Hoechst 33258, and Hoechst 33342 other than fluorescent wavelength?*

A: The differences are listed below:

AO : It is possible to distinguish between single stranded DNA and double stranded DNA using the difference in fluorescence wavelength when intercalating with a double stranded DNA and when combining with the phosphoric acid of a single stranded DNA. AO passes through the membrane of living cells.

Hoechst 33258, Hoechst 33342 :

Binds specifically with adenine - thyamine base pairs of DNA. They pass through the cell membrane, and stain the DNA of living cells. Hoechst 33342 has a higher membrane permeability. A better staining is possible when cells are fixed.

Q: *What is the method of disposal after use?*

A: PI is a possible carcinogen. When disposing remaining dye solution and solution containing staining dyes, follow handling guidelines and regulations and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Nucleus staining reagents (living / dead cells)

Q: *What is the difference among dyes used to stain the nucleus?*

A: Some notable differences other than wavelength are listed below;

PI: It does not have base specificity. It binds to all DNA and RNA., but the fluorescence intensity is higher when intercalating and can be used widely among variety of cells.

DAPI:This will bind with the minor groove of a double chain, and has a high affinity for adenine - thimine base pairs.

Mitochondria staining reagents

Q: *Why do MitoRed and Rh123 stain the mitochondria?*

A: Both MitoRed and Rh123 employ the chemical structure Rhodamine. Rhodamine has the characteristic of gathering to mitochondria after entering the cell, so it is used as a mitochondria staining dye. When too much dye is introduced into the cell, other areas are stained also, so it is necessary to determine the best concentration in advance.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Q&A

-Cellstain- Double Staining Kit

Q: *What is the principle behind staining the cells?*

A: Calcein-AM stains living cells, PI stains dead cells. Calcein-AM is a fluorescent dye. The 4 carboxy bases of Calcein are converted to acetoxymethyl (AM) to increase lipid solubility to become cell membrane permiable. Calcein-AM does not fluoresce, but after entering the cell, the AM is hydrolized by estarse to form a strong yellowish-green fluorescence. On the other hand, PI is a nucleic acid staining dye, and intercalates with the double helix structure of DNA to produce a particularly strong red fluorescnce after passing through the damaged cell wall of dead cells. PI does not enter into the living cells. By using two different types of dyes, it is possible to stain living cells with yellowish-green colored fluorescence and stain dead cells with red-colored fluorescence.

Q: *Tell me about the wavelength when viewing the fluorescence.*

A: When viewing at the excitation wavelength at 490±10 nm, it will be possible to view living cells stained with yellowish-green fluorescence and dead cells stained with red-colored fluorescence simultaneously. In addition, it is possible to view only the red colored-fluorescence stained dead cells when using an excitation wavelength of 545 nm.

Q: *Can this kit be applied to any kind of a cell?*

A: Basically, it is for all animal cells that have esterase activity. Plant cells and bacteria cells have a cell wall, so Calcein-AM is unable to enter such cells and therefore can not stain. It is possible to stain the protoplast.

Q: *Is it possible to stain any animal cell using the same concentration of dyes?*

A: It is not the case that the concentration is set same for all of the cells. The optimum concentrations of Calcein-AM and PI differ greatly for each cell type. It is necessary to determine the optimum dye concentration for each cell type. Please refer to page 36 for instructions.

Q: *Is Calcein-AM toxic to cells?*

A: Calcein-AM is considerably less toxic compared to the other staining reagents.
Refer to the below paper for additional information:
L. S. D. Clerck, *et al.,J.Immunol. Methods*, **172**,115 (1994)

Q: *How should the kit be stored?*

A: Keep tightly sealed and store at -20 °C. Calcein-AM becomes hydrolyzed by moisture, so do not open the vial until the temperature of the vial reaches ambient temperature. Also, close the cap tightly after use. Staining solution that has been diluted with buffer or media should be used immediately. PI solution is stable up to one year at -20 °C.

Q: *What is the method of disposal after use?*

A: When disposing remaining dye solution and solution containing staining dyes, follow handling guidelines and regulations and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Antimicrobial Susceptibility Test:
Microbial Viability Assay Kit-WST

Product Description

Microbial Viability Assay Kit-WST is utilized for bacteria and Fungi viability determination. This kit contains WST-8 and electron mediator. WST-8* (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) produces a water-soluble formazan dye upon bioreduction in the presence of an electron mediator. Since the assay solution is stable in a culture medium, it can be added directly to microbial cell culture and incubated for 1-2 hours or overnight if necessary. The microbial cell viability can be determined at 450 nm absorbance.

*WST-8 patent No. 2,251,850(Canada), 6,063,587(US), 0908453(EP), 2757348(JP)

Product Information

Microbial Viability Assay Kit-WST

Product code	Unit	Components
M439-10	500 tests	WST solution : 1 ml x 5 tubes Electron mediator reagent / DMSO : 0.5ml x 1 tube

One test corresponds to one well of the 96-well plate.

Applications: Bacterial and Fungal Viability Monitoring

Required Materials

Devices, tools

- Microplate Reader with a 450 - 490 nm filter and 650 nm filter (reference)
- 96 well microplate, sterilized clear plate for cell assay
- Multi-pipette (8 channel: 10-200 μl)
- Incubator
- Biological safety cabinet
- Spectrophotometer
- Centrifuge rotor

Reagents

- Microbial Viability Assay Kit-WST [product code: M439]
- Culture media
- Material to be tested
- PBS or other buffers for the preparation of material solutions if cell culture medium cannot be used.

Preparation

Microbial Viability Assay Kit-WST

This kit consists of two solutions, one is WST Solution and the other is Electoron Mediator Reagent.
To prepare a coloring reagent, Mix 9 parts of WST solution and 1 part of Electron Mediator Reagent.

⚠ For Gram-positive bacteria, Fungi, and Vibrio parahaemolyticus assay, please make 8-fold dilution of Electron mediator solution with DMSO or sterile water and prepare the coloring reagent.

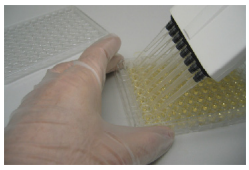
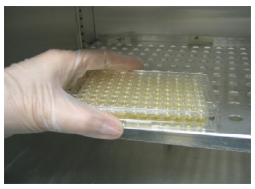
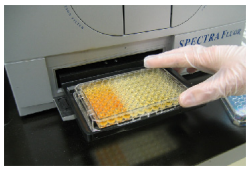
⚠ Coloring reagent is stable when it is stored at 4 °C for 1 month. For a long term storage over a year, store at -20 °C.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Antimicrobial Susceptibility Test:
Microbial Viability Assay Kit-WST

Assay Conditions

When using Microbial Viability Assay Kit-WST for the determination of MIC(Minimum Inhibitory Concentration) of organisms, it is desirable to start with a set number of microbial cells, to develop sufficient color in 2 hours. Below, the method and conditions for using Microbial Viability Assay Kit-WST are described.

Procedure	Precautions and Tips
Culture the microbial cells to be assayed using suitable medium.	
Adjust the absorbance at 550 nm to 0.125 of microbial cell suspension with sterile saline and make an additional 10-fold diluted cell suspension of it with sterile saline. Then, the number of microbial cell is approximately 10^7cfu/ml. (cfu:Colony forming unit)	
Prepare the various concentrations of antibiotic in Mueller -Hinton broth. (e.g. 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03 μg/ml)	
Add 180 μl antibiotic in Mueller-Hinton broth to each well. 	
Inoculate 10 μl organism suspension to each well (final cell density: 10^4 cfu/ml). Make a well of only media to measure background. Add 190 μl of media.	
Incubate the plate for 6 hours at suitable temperature for organisms. 	
Add 10 μl of coloring reagent to each well on the 96 well microplate.	
Place in an incubator for 2 hours to react.	
Take a colorimetric reading on a microplate reader. filter: 450 - 490 nm filter: 650 nm (for background measurement) 	Since bubbles can cause error, make sure there are no bubbles in each well. If there are bubbles, use a pipette tip to remove or use a toothpick to pop them.

Experimental Example

Antimicrobial Susceptibility Test of *Staphylococcus aureus* to Oxacillin

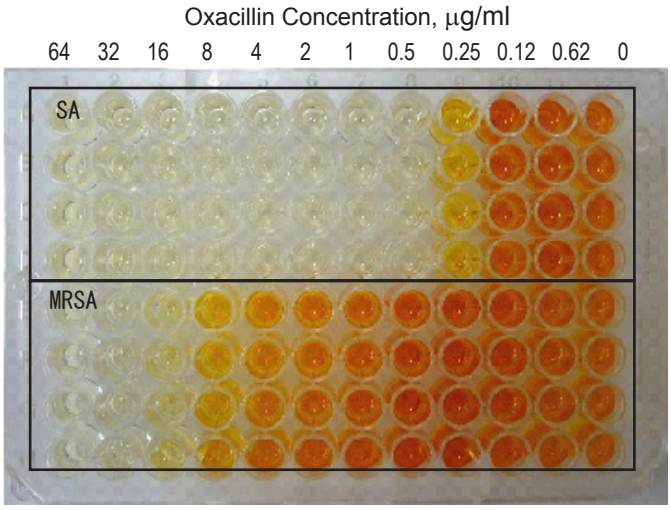
Conditions

Organisms
Methicilin-resistant *Staphylococcus aureus subsp. aureus* (MRSA)
Staphylococcus aureus (SA)

Antimicrobial agent
Oxacillin (beta-lactam antibiotic of the penicillin class)

Procedure

1. Culture MRSA and SA with Mueller-Hinton broth containing various concentrations of Oxacilin for 6 hours at 35°C.
2. Add coloring solution (Microbial Viability Assay Kit-WST) equal to 1/20 volume of the culture medium.
3. Incubate for 2 hours at 35°C.
4. Measure the O.D. at 450 nm to determine the MIC (Minimum Inhibitory Concentration).



The data indicates that MRSA has lower susceptibility than SA.
The MICs of MRSA and SA can be determined as 32 μg/ml and 0.5 μg/ml, respectively. They are close to the MICs determined by CLSI (Clinical Laboratory Standards Institute) method.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

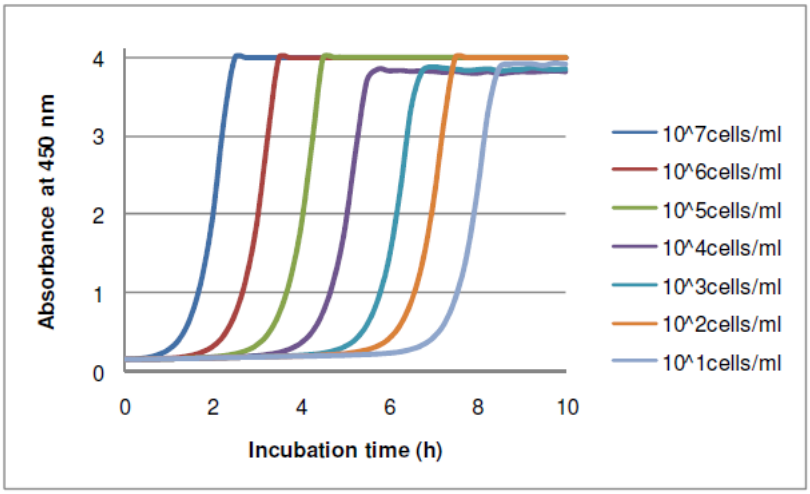
Proliferation Assay (Initial Number of Organisms):
Microbial Viability Assay Kit-WST

Experimental Example

Number of viable organisms can be calculated by following procedure.

Step 1

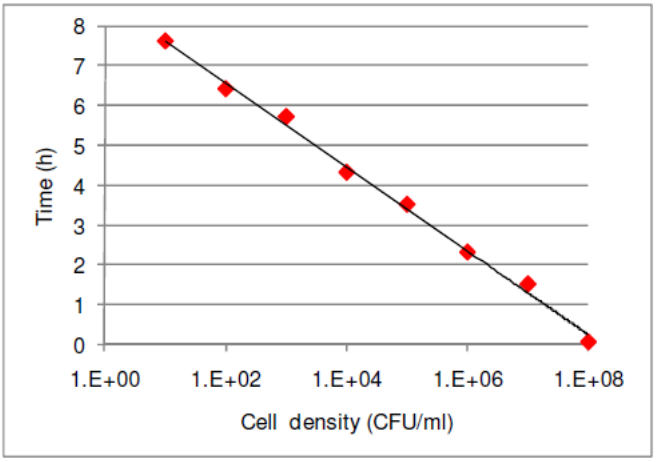
1. Culture microbial cell using suitable culture media.
2. Make serial dilutions of organism suspension (e.g. 10^7, 10^6, 10^5,10^4,10^3, 10^2...0 cfu/ml) and then add dilutions to each well in a 96 well plate.
3. Add Coloring solution with 1/20 volume of the culture medium.
4. Incubate the plate and measure the O.D. every 10 to 15 min at 450 nm.



Step 2

1. Make a calibration curve as following*

   *Each plot represents the time necessary for each cell density to reach 0.5 O.D.



2. Initial number of organisms can be estimated by using calibration curve.

Troubleshooting

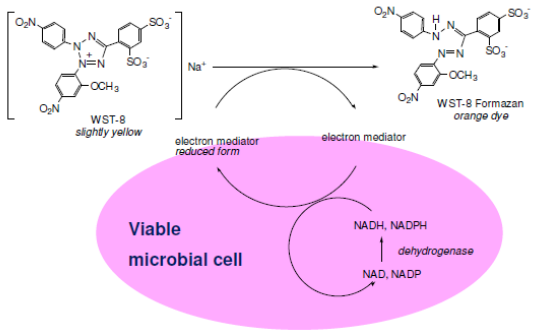
Problem	Possible Cause	Solution
The absorbance reading exceeds the upper limit of the machine.	Too many cells per well.	The number of viable microorganisms may increase during the pre-incubation. Prepare a microplate with lower number of organisms for the assay.
	Too long of a incubation time.	Shorten the incubation time.
The color development occurs even though cells are clearly dead when using the kit for cytotoxicity assays.	WST-8 is reduced by the test substance or materials which are generated in the culture media during the assay.	Mix Microbial Viability Assay Kit-WST solution with the substance to test whether the substance reacts or not. If there is a coloration, follow the following step: 1) Before adding the Microbial Viability Assay Kit-WST solution, centrifuge the plate and replace the culture media to remove the test substance or materials in the culture media.
There is high variation in the data.	The assay condition of outer-most wells has changed due to the edge effect.	Do not use the outer-most wells for the assay. Just add media to the outer-most wells.
	Microbial Viability Assay Kit solutions has not been mixed well with the media.	Lightly tap the outside of the well in order to get thel Microbial Viability Assay Kit-WST that is on the well wall to fall into the media.
	There are bubbles on the surface of the media.	Remove the bubbles using a pipet tip or a toothpick.
No color or less color development even though the number of microbial cells seems to have increased.	Cell viability of each microbial cell is low because of high population of cells per well.	Reduce the number of cells for the assay or shorten the incubation time.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Q&A

Questions concerning the reagent

Q: *What causes color development according to the viable microbila cell number in Microbial Viability Assay Kit-WST?*
A: WST-8 is reduced to an orange-colored formazan through electron mediator by NADH and NADPH activity which are generated by cellular activities. The amount of WST-8 formazen is dependent on the activity of cellular dehydrogenase, so WST-8/ electron mediator system can be used to determine the number of viable microbial cells or microbial cell viabilities.



Q: *Do WST-8 and electron mediator molecules enter into the cell?*
A: There is no clear evidence that these molecules do or do not enter the cell. Generally, a neutrally or positively charged organic molecule such as MTT can enter the cell. Therefore, it is estimated from their charges that electron mediator can enter the cell because it does not have a negative charge, but WST-8 cannot. It is speculated that electron mediator receives an electron from NADH or NADPH at the membrane or inside of the cell and passes the electron to the WST-8 that is around the outer cell membrane.

Q: *What is the stability of the Microbial Viability Assay Kit-WST?*
A: The Microbial Viability Assay Kit-WST is stable for over 1 month at ambient temperature. Therefore, it is possible to ship this kit without dry ice or blue ice. The kit is stable for over one year when stored in a refrigerator.

Q: *What is the toxicity of Microbial Viability Assay Kit-WST?*
A: If you follow the protocol on the technical manual, the cytotoxicity of the assay kit is very low. The kit gives no damage to the microbial cells during the assay.

Questions regarding microbial cells and culture

Q: *What type of culture medium can be used?*
A: Generally, most of the culture medium for organisms culture can be used for the assay without changing the medium.

Q: *How long of a pre-incubation time is required prior to assay?*
A: Pre-incubation is generally not necessary.

Q: *When using Microbial Viability Assay Kit-WST, what number of microbial cell is appropriate?*
A: The appropriate number of cell depends on the type of cell and the type of experiment. The amount of coloration will differ dependent on organism type, even if the cell number per well and coloration times are the same.

Q&A

Q: *Is it possible to do the assay in a 24 or 12 well plate? If so, how much Microbila Viability Assay Kit-WST solution should be used?*
A: Yes, it is possible to assay using plates other than a 96 well plate. Please add Microbial Viability Assay Kit-WST solution with 1/20 volume of the media (if the media is 1 ml, add 50 ul of solution)

Q: *How long of an incubation time is sufficient for the color development?*
A: For antimicrobial susceptibility test, the incubation time is 2 hrs. However, the absorbance will differ between cell types even if the number of cells per well and coloration time are the same. Set an appropriate incubation time to give a proportional relationship between the cell number and the absorbance.

Q: *Are there any materials that can affect the color development?*
A: Reducing agents and materials with reducing activity may react with WST-8 and give a false reading. If the material is considered to have a reducing activity, mix the material solution with Microbial Viability Assay Kit-WST solution and incubate to check whether the material reacts with WST-8 or not. Then, if the material reacts with WST-8, remove the culture medium containing such material from Microbial cells and add new culture medium prior to adding Microbial Viability Assay Kit-WST solution. Dye materials with absorbance around 450-490 nm may affect the reading. However, absorbance from such dyes can be subtracted as a blank.

Q: *How do you subtract the background caused by microbial cells?*
A: Measure the absorbance at 600-650 nm of the well as a reference. Then, the absorbance at 600-650 nm is subtracted from the absorbance of the same well measured at 450 nm to eliminate the background that comes from turbidity.

Q: *The cell culture in the well contains material which has an absorbance around 450 nm.*
A: Use a couple of wells for a background absorbance measurement to subtract the total absorbance of the sample wells. Prepare the well for background measurement which contains all materials except for cells. Measure the background absorbance of the well at 450 nm, and then subtract the background absorbance from the absorbance of the sample well containing all materials and cells.
note: If the background absorbance from the material is too high to subtract, remove the culture medium and wash cells with fresh media and add the same volume of fresh media to the well prior to adding the Microbial Viability Assay Kit-WST solution.

Q: *What should be done regarding materials which may increase the color development and interfere with the Microbial Viability Assay?*
A: Determine whether the material interferes with the assay by adding the Microbial Viability Assay Kit-WST to the material which may interfere with the assay and incubate for a general assay period.
a)If there is no color development during the incubation, the material does not react with the Microbial Viability Assay Kit-WST solution.
b)If there is color development during the incubation, the material does react with the Microbial Viability Assay Kit-WST. Prepare a couple of wells for a background absorbance measurement which contains all material except for cells. Measure the absorbance of the background well at 450 nm and subtract this background from the absorbance of the wells containing all materials and cells.
note: If the color development is too high to subtract, remove the culture media, wash the cells with fresh media, and then add the same volume of fresh media to the well prior to adding the Microbial Viability Assay Kit-WST solution.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Microbial Viability Assay Kit-WST

Applicable Organisms

Bacteria
Acetobacter Pasteurianus
Acinetobacter baumannii
Aerococcus viridans
Aeromonas hydrophila subsp. hydrophila
Alcaligenes faecalis subsp. faecalis
Arthrobacter aurescens
Bacillus cereus
Bacillus circulans
Bacillus coagulans
Bacillus subtilis subsp. subtilis
Bacillus thuringiensis
Bordetella bronchiseptica
Brevibacillus brevis
Cedecea davisae
Chromobacterium violaceum
Citrobacter freundii
Corynebacterium diphtheriae
Corynebacterium glutamicum
Cronobacter sakazakii
Edwardsiella tarda
Empedobacter brevis
Enterobacter aerogenes
Enterobacter cloacae
Enterobacter cloacae subsp. cloacae
Enterococcus casseliflvus
Enterococcus faecalis
Enterococcus faecium
Enterococcus gallinarum
Enterococcus hirae
Erwinia persicina
Escherichia coli
Haemophilus influenzae
Hafnia alvei
Klebsiella pneumoniae
Lactobacillus brevis
Lactobacillus casei
Lactobacillus delbrueckii subsp. lactis
Lactobacillus plantarum
Lactobacillus plantarum subsp. plantarum
Lactobacillus rhamnosus
Lactobacillus sakei subsp. sakei

Bacteria
Lactococcus lactis supsp. cremoris
Lactococcus lactis subsp. lactis
Leuconostoc mesenteroides subsp. mesenteroides
Listeria innocua
Listeria monocytogenes
Microbacterium laevaniformans
Micrococcus luteus
Morganella morganii subsp. morganii
Nocardia asteroides
Pasteurella pneumotropica
Pediococcus pentosaceus *
Providencia alcalifaciens
Proteus mirabilis
Pseudomonas aeruginosa
Pseudomonas fluorescens
Rhodococcus rhodochrous
Salmonella enterica subsp. enterica
Serratia marcescens
Sporosarcina ureae
Staphylococcus aureus subsp. aureus
Staphylococcus epidermidis
*Streptococcus gordonii**
*Streptococcus mitis**
Streptococcus pyogenes
Vibrio alginolyticus
Vibrio fluvialis
Vibrio parahaemolyticus
Yersinia enterocolitica subsp. enterocolitica

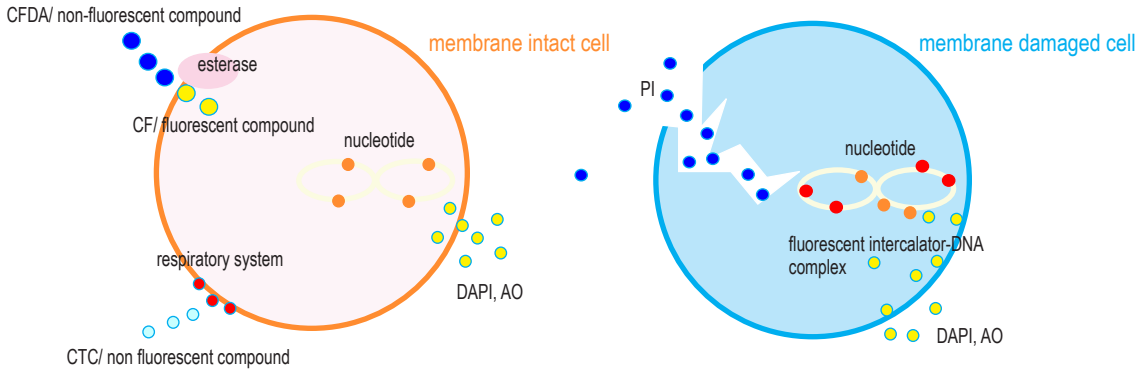
* Under anaerobic condition

Fungus
Aspergillus oryzae
Candida albicans
Candida guilliermondii
Candida krusei
Candida parapsilosis
Candida tropicalis
Candida utilis
Saccharomyces cerevisiae
Zygosaccharomyces rouxii

Bacteria Staining

Product Description

There are several ways to detect bacteria such as from agar plate cultivation to bacteria specific DNA amplification. Fluorescent staining using CTC is one of the methods used to detect viable bacterial cells. The advantage of this method is very quick detection and the possibility of VNC (viable but culturable) bacterial cell detection.
CTC is a tetrazolium salt that is converted to formazan dye by bacterial cell activity. The solid state of the formazan dye emits red fluorescence. Therefore, viable bacterial cells can be stained by CTC and are easily detected by fluorescent microscopy.CFDA also can be used for staining of viable microorganisms. CFDA is bacterial cell wall and cell membrane permeable, and hydrolized by esterase of the cell to stay inside of the cell. DAPI, AO, and EB are used for nucleotide staining and are cell wall permeable except for PI. Therefore, using DAPI and PI , it is possible to stain both membrane intact cells and membrane damaged cells simultaneously. Since PI can stain only membrane damaged cells, membrane intact cells are not stained by this compound. PI is also used for double staining coupled with CFDA.



Applications: Fluorescent microscopy, Flowcytometry

Required Materials

Devices, tools

- Incubator
- Safety cabinet
- Fluorescence microscope
- Flowcytometry
- Centrifuge
- Slide glass, cover glass, or chamber slide

Reagents

Living Bacterial Staining Dyes

-Bacstain- CTC Rapid Staining Kit for Flow cytometry (product code: BS01)
-Bacstain- CTC Rapid Staining Kit for Microscopy (product code: BS02)
-Bacstain- CFDA solution (product code: BS03)

Living and Dead Bacterial Staining Dyes

-Bacstain- DAPI solution (product code: BS04)
-Bacstain- AO solution (product code: BS05)

Dead Bacterial Staining Dye

-Bacstain- PI solution (product code: BS07)

Other Reagents

- Sterilized normal saline
- Formaldehyde
- PBS(-)

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Bacteria Staining

Preparation of Assay Solution

Some reagents are stable in the solution, however, some are not stable. Please follow the storage conditions for each reagent. Generally, the reagents offered in solution form are fairly stable.

Dyes Used For Living Bacteria Staining

Product name	Characteristic	Storage	Unit size
-*Bacstain*- CTC Rapid Staining Kit (for Flow cytometry) Code# BS01-10			100 assays
CTC	white-slightly orange powder	avoid light, refrigerate	
Enhancing Reagent A	slightly-yellowish solution	refrigerate	
-*Bacstain*- CTC Rapid Staining Kit (for Microscopy) Code# BS02-10			100 assays
CTC	white-slightly orange powder	avoid light, refrigerate	
Enhancing Reagent B	dark-purple solution	refrigerate	
-*Bacstain*- CFDA solution Code# BS03-10	colorless solution	avoid moisture, refrigerate	100 assays

Dyes Used for both Living and Dead Bacterial Staining

Product name	Characteristic	Storage	Unit size
-*Bacstain*- DAPI solution Code# BS04-10	pale yellow solution	avoid light, freeze	100 assays
-*Bacstain*- AO solution Code# BS05-10	yellow-orange solution	avoid light, freeze	100 assays

Dyes Used for Dead Bacteria Stain-

Product name	Characteristic	Storage	Unit size
-Bacstain- PI solution Code# BS07-10	orange-red solution	avoid light, freeze	100 assays

Since the DAPI, AO, and PI directly stain nucleus, these dyes are considered mutagens, so gloves, safety goggles, and masks are necessary when handling. If the product comes in contact with the skin, immediately wash with a copious amount of water.

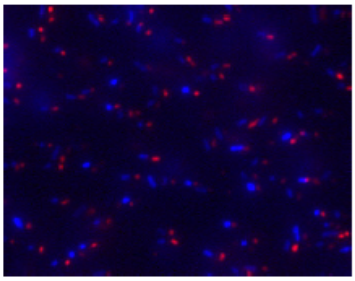
When disposing remaining dye solution and solution contains staining dyes, follow the handling guidelines and the regulations at your institution and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute are allowed, use a paper towel to adsorb and mix it in the plastic tubes used for the preparation of the staining dye solution to incinerate.

Living & Dead Bacteria Staining:

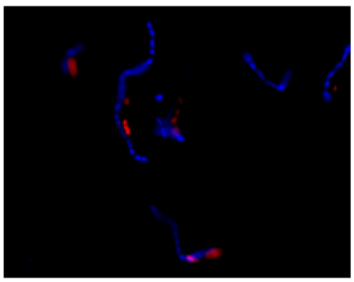
-Bacstain- CTC Rapid Staining Kit, *-Bacstain-* DAPI solution

Introduction

-Bacstain- CTC Rapid Staining Kit (used for fluorescent staining living cells) and *-Bacstain-* DAPI solution(used for fluorescent staining of living and dead cells) for simultaneous staining of living and dead cells.



CTC/DAPI Double-staining (*E.coli*)



CTC/DAPI Double-staining (*L.casei*)

Required Materials

Devices, tools

- Incubator
- Safety cabinet
- Fluorescent microsco (UV exitation light for DAPI, Blue or Green exitation light for CTC)
- Slide glass, cover glass
- Micropipette (1-10 μl, 100-1000 μl)

Reagents

- *-Bacstain-* CTC Rapid Staining Kit (for Microscopy) (product code: BS02)
  Kit contents
  CTC: 10 mg/ tube 3 vials
  Enhancing reagent B: 1 vial

- *-Bacstain-* DAPI solution (product code: BS04)
  ⚠ Store at 5°C and protect from light.

- PBS(-)

⚠ DAPI may be mutagenic, so wear gloves, safety goggles, and mask when handling.
If it comes in contact with your skin, immediately wash with a copious amount of running water.

When disposing remaining dye solution and solution contains staining dyes, follow the handling guidelines and the regulations at your institution and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute are allowed, use a paper towel to adsorb and mix it in the plastic tubes used for the preparation of the staining dye solution to incinerate.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

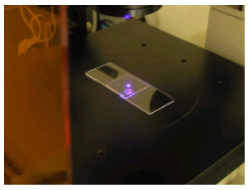
Living & Dead Bacteria Staining:

-Bacstain- **CTC Rapid Staining Kit,** *-Bacstain-* **DAPI solution**

Staining Procedure (preparing samples for use with a fluorescent microscope)

Procedure	Precautions & Tips
Add 750 μl of distilled water to one CTC tube and vortex gently to dissolve (final concentration: 50 mM) [a)] .	a) This solution is stable at -20°C for 2 weeks.
Resuspend the organisms with PBS(-) or saline and adjust the number of cells to 10^6 cells/ml (flow cytometry) or 10^8-10^9 cells/ml(microscopy) [b)] .	b) Since remaining culture medium in the sample undergoes unspecific col ored-reaction, it should duly be re-moved.
Add each reagent into the 1 ml of microbial cell suspension and vortex gently to mix[c)] . *Refer to the conditions in the following table	c)When CTC-staining is insufficient, add extra CTC solution or increase the incu-bation time. In this case, CTC solu tion should be limited less or equal to 100 ml /sample.
Incubate the microbial cells at 37°C for 30 min [d)].	d)Formaldehyde fixation (1–4% final concentration) is not required for the DAPI staining. However, if fixation is necessary for the downstream experi-ment or if the formaldehyde fixation is a standard protocol to prepare samples, it can be done in between CTC-staining and DAPI staining.
Add 1ml of DAPI solution to CTC-stained cell suspension and incu-bate at room temperature for 5-10 min.	
Analyze the stained cells under a microscope.	

	CTC solution	Enhancing reagent B
Microscopy	20 μL	5 μL

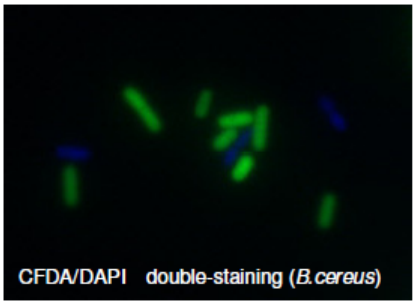


Living & Dead Bacteria Staining:

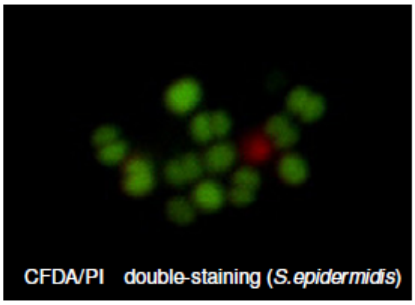
-Bacstain- **CFDA solution,** *-Bacstain-* **DAPI solution,** *-Bacstain-* **PI solution**

Introduction

-Bacstain- CFDA solution (used for fluorescent staining living bacteria) and *-Bacstain-* DAPI solution (used for fluorescent staining of living and dead bacteria) or *-Bacstain-* PI solution (used for fluorescent staining of dead bacteria) can be utilized for simultaneous staining of living and dead cells.



CFDA/DAPI double-staining (*B.cereus*)



CFDA/PI double-staining (*S.epidermidis*)

Required Materials

Devices, tools

- Incubator
- Safety cabinet
- Fluorescent microscope (UV exitation for DAPI, Blue exitation for CFDA, Green exitation for PI)
- Slide glass, cover glass
- Micropipette (1-20 μl, 100-1000 μl)

Reagents

- *-Bacstain-* CFDA solution (product code: BS03)
- *-Bacstain-* DAPI solution (product code: BS04)

⚠ Store at 5°C and protect from light.
CFDA solution is easily hydrolized by moisture. Tightly close the cap after each use.

- *-Bacstain-* PI solution

⚠ Store at -20°C

- PBS(-)

⚠ DAPI and PI may be mutagenic, so wear gloves, safety goggles, and mask when handling. If it comes in contact with your skin, immediately wash with a copious amount of running water.

When disposing remaining dye solution and solution contains staining dyes, follow the handling guidelines and the regulations at your institution and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute are allowed, use a paper towel to adsorb and mix it in the plastic tubes used for the preparation of the staining dye solution to incinerate.

301.987.2667
1.877.987.2667
info@dojindo.com
www.dojindo.com

Living & Dead Bacteria Staining:

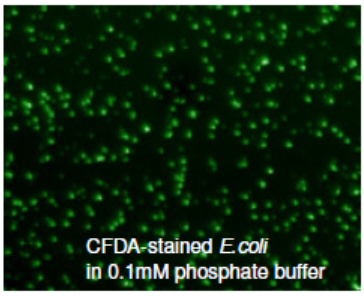
-Bacstain- **CFDA solution,** *-Bacstain-* **DAPI solution,** *-Bacstain-* **PI solution**

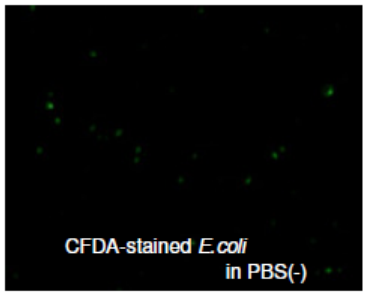
Staining Procedure (preparing samples for fluorescent microscope)

Procedure	Precautions & Tips
Allow CFDA solution to stand at room temperature for 30 min for thawing.	Solution should be protected from moisture.
Resuspend the organism with an appropriate buffer (phosphate buffer, saline,etc) and adjust the number of cells to 10^6cells/ml (flow cytometry) or 10^8-10^9cells/ml (microscopy).	
Add CFDA solution into the 1 mL of microbial cell suspension and vortex gently to mix. * Refer to the conditions in the following table.	
Incubate the sample at 37°C for 5 min .	If CFDA staining is insufficient, increase the incubation time.
Fix the microbial cells by addition of formaldehyde (1-4 % final concentration).	
Remove the buffer by filtration or centrifugation, and resuspend the cells with the buffer.	
Analyze the stained cells under a microscope or by flow cytometer.	

	Microscopy	Flow cytometry
CFDA solution	15 µL	5 µL

Gram-negative bacteria tend to exhibit lower fluorescence intensity than Gram-positive bacteria, because of their cell structure (outer membrane impedes penetration of CFDA). Thus, the following buffer can be recommended. 0.1 mM-Phosphate buffer (pH 8.5, 5%(w/v)-NaCl, 0.5 mM-EDTA disodium salt)





CFDA staining efficiency is increased by using 0.1 mM-Phasphate buffer.

NOTES

301.987.2667
1.877.987.2667

info@dojindo.com
www.dojindo.com