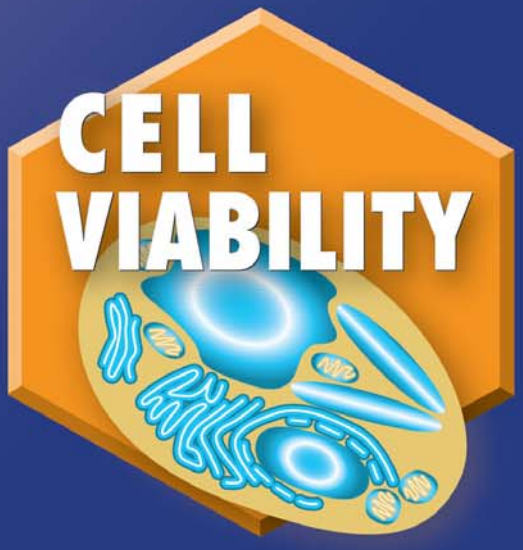
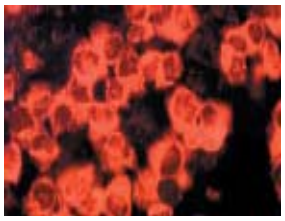
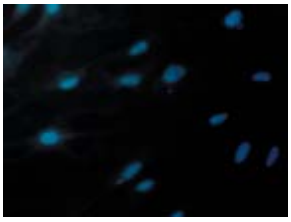
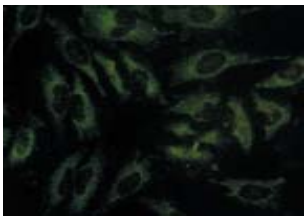
Cell Viability & Cytotoxicity Assay

Protocol



DOJINDO LABORATORIES

Contents

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 well-established methods such as Colony formation method, Crystal violet method, Tritium-labeled thymidine uptake method, MTT, and WST methods are used for counting live cell numbers. A widely used assay for staining dead cells is Trypan Blue. 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 healthy cells and 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 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 the market. However, adenylate kinase and glucose-6-phosphate are not stable; 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

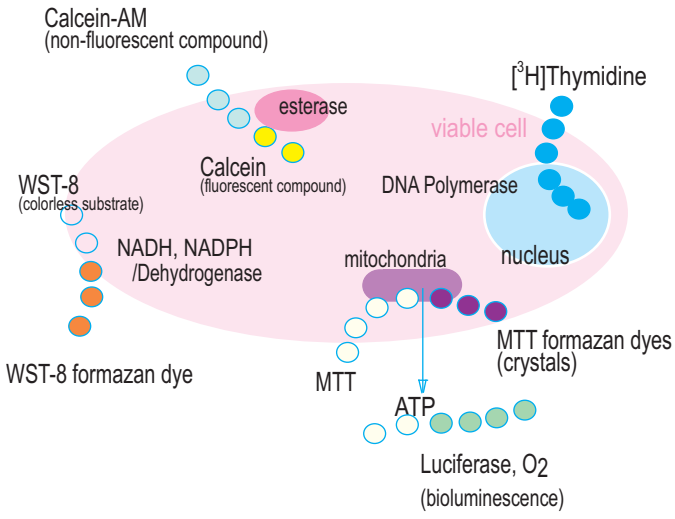


Fig. 1 Reagents for cell viability detection

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 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 living cells. MTT is reduced to a purple formazan by NADH. MTT formazan, however, is insoluble in water, and it forms purple needle-shaped crystals in cells. 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 many 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 receive 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 room temperature and one year at 0-5 °C. Since WST-8, WST-8 formazan, and 1-Methoxy PMS have no cytotoxicity in cell culture media, additional experiments may be carried out using the same assay plate. Dehydrogenase-based assays reflect cell conditions with more sensitivity than the other assays because they depend on several elements including dehydrogenases, 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.

Cell Counting Kit-8 Assay

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 tests. Cell Counting-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 the reagent to a cell culture, 2) incubate, 3) take a colorimetric reading. 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 assaying the cells with Cell Counting kit-8 it is possible to contiune using those cells for further experiments.

Applications: Cell counting, Cell proliferation experiments, Cytotoxicity tests, Drug sensitivity tests

Materials Required for the Assay

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 solution. Stable when stored at 4 °C for 12 months

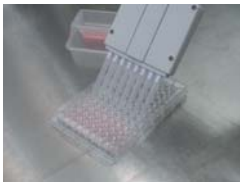
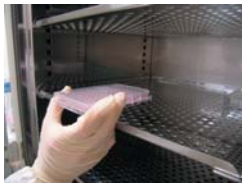
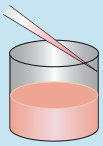
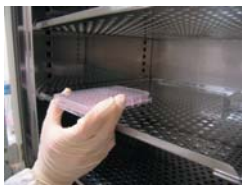
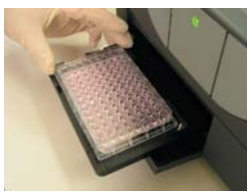
If you use Cell Counting Kit-8 frequently, store in a refrigerator. The Cell Counting Kit-8 solution is stable for one year at 4 °C. The Cell Counting Kit-8 solution is stable even at room temperature for 6 months.

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

Cell Counting Kit-8 Assay

Assay Conditions

When using Cell Counting Kit-8 for proliferation and cytotoxicity assays, it is necessary to have a proportional relationship between absorption and viable cell number. 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.	For adherent cells, recover using trypsin to detach cells, and use a cell scraper if necessary.
Measure the cells and adjust the concentration of the cell suspension. (cell concentration: _____cells/ml)	Use a hematocytometer or a cell counter.
Add a cell suspension of 100 μl to each well in a 96 well microplate using serial dilution. Make a well of only media to measure background. 	Refer to experimental example on the next page for instruction on serial dilution.
Incubate for 24-48 hours in a CO_2 incubator. (start time: _____ end time: _____) 	Be aware that cell number after incubation for 24-48 hours 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.
Add 10 μl of Cell Counting Kit-8 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 touch the of the pipette to the well of the wall when adding the reagent (below picture). 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: _____) 	Since the amount of formazan produced will differ with 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)
Take a colorimetric reading on a microplate reader. filter: 450 - 490 nm 	Bubbles 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.

Cell Counting Kit-8 Assay

Experimental Example

Make serial dilutions of 2.5 x 10^4 , 1.25 x 10^4 , 6.2 x 10^3 ….0 cells/ well 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. 2. Following the previously listed method, use Cell Counting Kit-8 to count the 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 10^5 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.

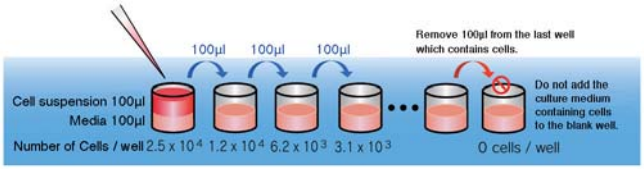


Fig. 2 Serial dilution process

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 experiement, 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, give consideration to the drug's properties such as enhancing cell proliferation, toxicity to cells, and reducing activity, in addition to time of exposure to drugs.

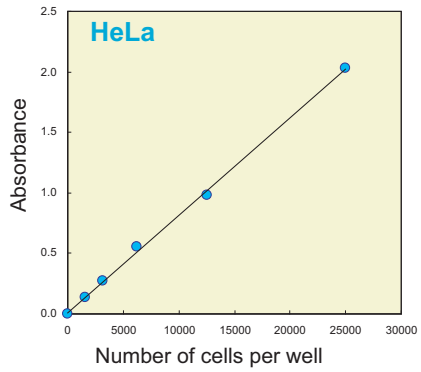


Fig. 3 Color development using HeLa cells
Media: DMEM (10% FBS)
Incubation: 37 °C, 3 hr, 5% CO_2 incubator
Measurement Wavelength: 450nm

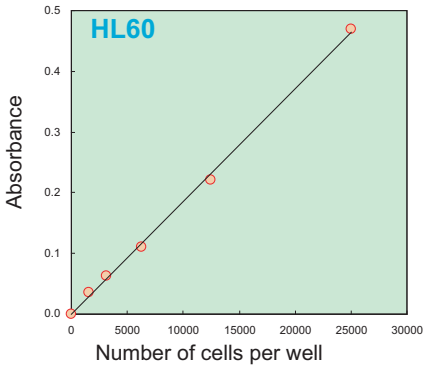
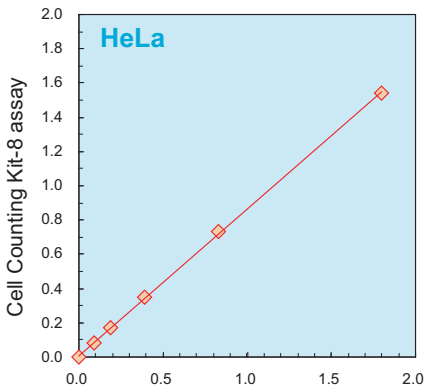


Fig. 4 Color development using HL60 cells
Media: RPMI1640 (10% FBS)
Incubation: 37 °C, 3 hr, 5% CO_2 incubator
Measurement Wavelength: 450nm

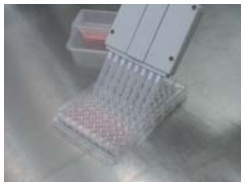
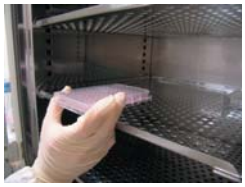
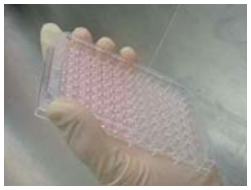
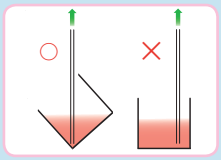
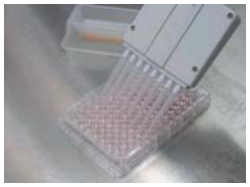
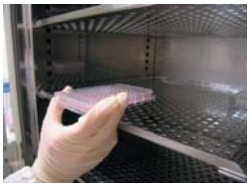


As indicated in Fig. 5, there is a good correlation between the Cell Counting Kit-8 assay and [^{3}H]-Thymidine uptake assay.

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

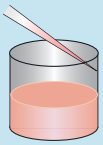
Cell Counting Kit-8 Assay

Cell Proliferation and Cytotoxicity Protocol

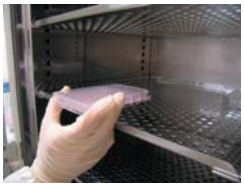
Procedure	Precautions & Tips
Recover the cells to be assayed from flask.	For adhesive cells, recover using trypsin, and use cell scrapers if necessary.
Measure the cells and adjust the concentration of the suspended cells. (cell conc.: _____cells/ml)	Use a hemacytometer or a cell counter.
Add a cell suspension of 100 μl to each well in a 96 well microplate using serial dilution. Make a well of only media for a background measurement. 	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 properties of the tested material (drug) promoting or inhibiting growth, time of coloration, and cell type are all factors that should be used to determine the appropriate cell concentration per well.
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 changing media is necessary, remove media with a micropipet or a Pasteur pipette 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.  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 10 μl of media containing different concentrations of the test substances to each well. 	Add the same amount of test subatnce 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 (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.

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

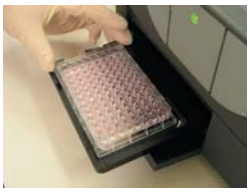
When using a plate or petri dish other than a 96 well plate, please add reagent equal to 1/10 the media volume.
Due to the low volume of reagent added, it is beneficial to touch the tip of the pipette to the well of the wall and when adding the reagent as indicated the figure below. If the reagent sticks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



- Read coloration at 450 nm on a microplate reader



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

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

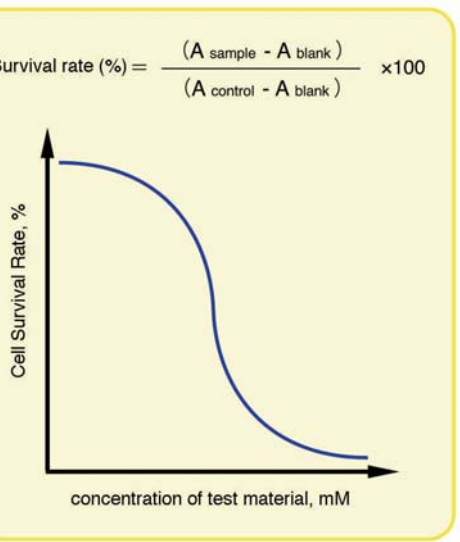


Fig. 6 Typical cell survival curve.

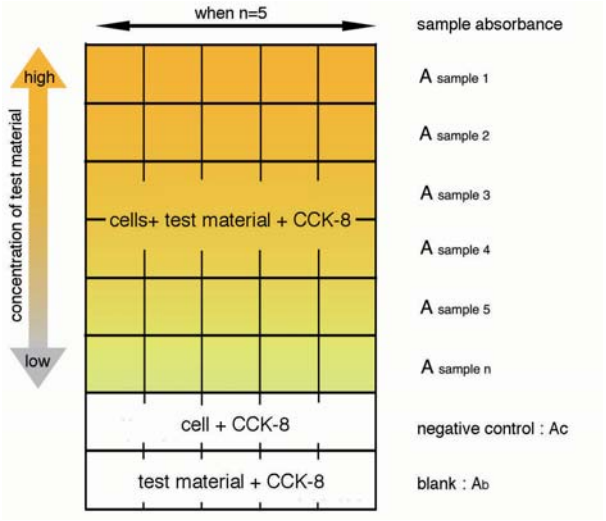


Fig. 7 Example of the plate arrangement and color development

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 have 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 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 the Cell Counting Kit-8.

Troubleshooting

Problem	Possible Cause	Solution
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 “Assay Conditions” on page 4).
	Too much incubation time	Shorten the incubation time.
Color development occurs even though cells are clearly dead when using the kit for cytotoxicity assays.	WST-8 is being 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: 1) Before adding the Cell Counting Kit-8, change the culture media to remove the test substance or materials in the culture media. 2) Use Cell Counting Kit-F
The absorbance is higher than that of the well with no substance when a toxic substance is added to the cell.	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 high variation in the data.	The assay condition of the outermost 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 for the assay.

Cell Counting Kit-8 Assay

Q&A

Questions about reagents used in the kit

Q *What causes color development according to the viable cell number in Cell Counting Kit-8?*

A WST-8 is reduced to an orange-colored formazan through 1-methoxy PMS by NADH and NADPH which are generated by cellular activities as inducated 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 viabilitiy.

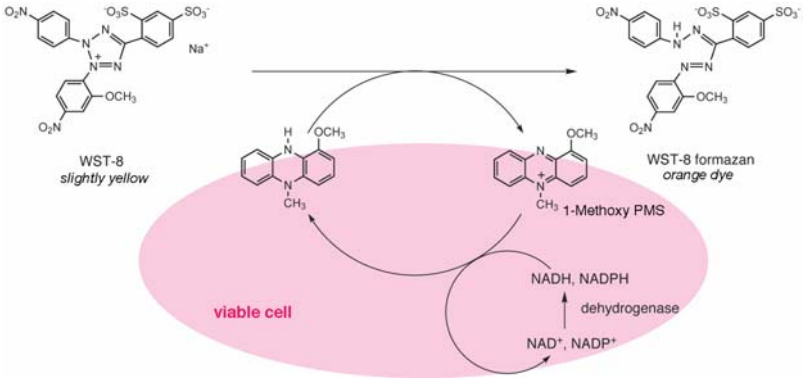


Fig. 8 Cell viability detection mechanism with CCK-8

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 *How is the stability of the Cell Counting Kit-8?*

A The Cell Counting Kit-8 is stable for over 3 months 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 refridgerator and over two years when stored in a freezer.

Q *How 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 incuvation. Because of this, after assaying with Cell Counting Kit-8, those cells can be used for other 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 asayed by Cell Counting Kit-8?*

A Generally, Cell Counting Kit-8 can be used for animal cell lines and primary culture animal cells.

Q *How long of a preincubation time is required prior to assay?*

A It depends on the cell type. The cells for the assay should enter into the logarithmic growth phase. The average incubation time to enter into this phase is from 24 hours to 48 hours. Please check cell databases to estimate the preincubation time.

Cell Counting Kit-8 Assay

Q *Can Cell Counting Kit-8 be used for both adherent cells and non-adherent cells?*

A It can be used for both types of cells. However, the color development for non-adherent cells will be low compared to the coloration for adherent cells, so it may be necessary to increase the time for coloration 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 number of cells depends on the type of cells and the type of experiment. The amount of coloration will differ depending on cell type, even if the cell number per well and coloration 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/well.

Q *Is it necessary to preincubate?*

A It is recommended to preincubate 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 to use for assays. For non-adherent cells, you can skip this step if the same culture medium is used for hervesting 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 It is possible to assay using plates other than a 96 well plate. Please add Cell Counting Kit-8 solution equal to 1/10 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: Be careful not to make bubbles when adding the SDS solution. Bubbles on the surface cause serious error for the measurement of absorbance.

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 from 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 or not. Then, 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 affect the reading. However, absorbance from such dyes can be subtracted as a blank. For example, Phenol Red has an absorbance near the assay wavelength. Such absorbance can be subtracted as a blank and does not affect assay data. For more detailed information, please refer the following Q&A.

Q *The cell culture is not clear. It has some turbidity.*

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.
note: 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.

Cell Counting Kit-8 Assay

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 Cell Counting Kit-8.

Q *What should be done regarding materials that may increase the color development and intefere 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 incubtion, 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 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 containinng 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 Cell Counting Kit-8.

Q *What should be done regarding materials which may inhibit the color development and interefere 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 the Cell Counting Kit-8 assay. 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 compound?*

A Determine whether the reducing material 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.
note: 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.

References

Cell Line	Origin	Reference
293T	human kidney carcinoma	H. Fuda, *et al., J. Lipid Res.*, **48**, 1343 (2007)
3T3-L1	mouse embryonic fibroblast	D. Huang, *et al., FASEB J.*, **19**, 2014 (2005)
3Y1	rat	N. Itano, *et al., PNAS*, **99**, 3609 (2002)
A431	human epithelial carcinoma cell	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
Alexander cell	pancreatic cancer cell	S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
AMO1	multiple myeloma	J. Inoue, *et al., Am. J. Pathol.*, **165**, 71 (2004)
ARO	human anaplastic thyroid carcinoma	F. Furuya, *et al., Endocrinology*, **145**, 2865 (2004)
AsPC-1	pancreatic cancer cell	T. Mori, *et al., Mol. Cancer Ther.*, **3**, 29 (2004) S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
B16F1	murine malignant melanoma	S. Shibata, *et al., J. Immunol.*, **177**, 3564 (2006)
Balb3T3	mouse embryonic cell	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999)
BBMVEC	bovine brain microvascular endothelial	T. Kitamuro, *et al., J. Biol. Chem.*, **278**, 9125 (2003)
BEAS-2B	human bronchial epithelial cell	C. A. Reilly, *et al., Toxicol. Sci.*, **73**, 170 (2003) M. E. Johansen, *et al., Toxicol. Sci.*, **89**, 278 (2006)
BMMSC	bone marrow mesenchymal stem cell	M. Miura, *et al., Stem Cells*, **24**, 1095 (2006)
BxPC-3	pancreatic cancer cell	S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
C33A	human cervical carcinoma	W. Yang, *et al., Mol. Cancer Ther.*, **5**, 1610 (2006)
C8166	T-cell	T. Kasai, *et al., J. Biol. Chem.*, **277**, 5187 (2002)
cardiomyocyte	rat	E. E. Hamm, *et al., PNAS*, **103**, 14176 (2006)
cerebellar granule neuron	rat	X. Wang, *et al., J. Biol. Chem.*, **280**, 16705 (2005)
cortical neurons, primary	mouse	M. Ikonen, *et al., PNAS*, **100**, 13042 (2003)
Daoy	human medulloblastoma	X. Li, *et al., Mol. Cancer Ther.*, **4**, 1912 (2005) S. Kim, *et al., Clin. Cancer Res.*, **12**, 5550 (2006)
Daudi	human burkitt lymphoma	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)
DLD-1	human colorectal adenocarcinoma	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
Gin-1	human gingival fibroblast	R. Takii, *et al., Infect. Immun.*, **73**, 883 (2005)
FRT	rat thyroid cell	H. Shimura, *et al., Cancer res.*, **61**, 3640 (2001)
H1299	human lung cancer cell	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999) S. Semba, *et al., J. Biol. Chem.*, **281**, 28244 (2006)
H441	human pulmonary adenocarcinoma	H. Shimura, *et al., Cancer res.*, **61**, 3640 (2001)
HB1.F3	human neural stem cell	S. Kim, *et al., Clin. Cancer Res.*, **11**, 5965 (2005) S. Kim, *et al., Clin. Cancer Res.*, **12**, 5550 (2006)
HCT116	human colon carcinoma	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
hMSC	human mesenchymal stem cell	D. Huang, *et al., FASEB J.*, **19**, 2014 (2005) L. Song, *et al., Stem Cells*, **24**, 1707 (2006)
HT22	mouse hippocampal cell	H. Sohn, *et al., FASEB J.*, **20**, 1428 (2006).
HTOA	human ovarian adenocarcinoma	M. Furuya, *et al., Cancer Res.*, **65**, 2617 (2005)
HuCCT1	human intrahepatic bile duct carcinoma cell	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
IMR32	human neuroblastoma	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999)
ILT-Hod	T-cell	T. Kasai, *et al., J. Biol. Chem.*, **277**, 5187 (2002)
Jurkat	human T cell	T. Kasai, *et al., J. Biol. Chem.*, **277**, 5187 (2002) L. Lu, *et al., J. Biochem.*, **141**, 157 (2007)
Kasumi-1	acute myeloid leukemia cell	G. Zhou, *et al., Blood*, **109**, 3441 (2007)
KMS-11	multiple myeloma	J. Inoue, *et al., Am. J. Pathol.*, **165**, 71 (2004)
KYSE	esophageal squamous cell carcinoma	I. Imoto, *et al., Cancer Res.*, **61**, 6629 (2001) K. Nakakuki, *et al., Carcinogenesis*, **23**, 19 (2002)
L929	mouse fibroblast	H. Tominaga, *et al., Anal. Commun.*, **36**, 47 (1999)

Cell Counting Kit-8 Assay

Cell Line	Origin	Reference
LCSC#2	non-small-cell lung cancer cell	H. Ishibashi, *et al., Cancer Res.*, **65**, 6450 (2005)
LK87	human lung adenocarcinoma	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
LLC-PK1	porcine renal tubular	T. Yano, *et al., Am. J. Pathol.*, **166**, 1333 (2005)
LNCaP	human prostate carcinoma	D. J. Son, *et al., Mol. Cancer Ther.*, **6**, 675 (2007)
Macrophage	mouse	Y. Miyake, *et al., J. Immunol.*, **178**, 5001 (2007)
Mast cell	human skin mast cell, primary	J. Tessier, *et al., Infect. Immun.*, **75**, 1895 (2007)
MDCK	canine kidney epithelial cell	H. Shimura, *et al., Cancer res.*, **61**, 3640 (2001)
MH134	murine hepatocellular carcinoma	S. Shibata, *et al., J. Immunol.*, **177**, 3564 (2006)
MiaPaCa-2	pancreatic cancer cell	A. Aghdassi, *et al., Cancer Res.*, **67**, 616 (2007)
MIN6	mouse insulinoma	S. Oyadomari, *et al., PNAS*, **98**, 10845 (2001)
MT1	T-cell	T. Kasai, *et al., J. Biol. Chem.*, **277**, 5187 (2002)
Namalwa	human lymphoblastoid	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)
NS/PC	neural stem/progenitor cell	W. Jiang, *et al., J. Clin. Invest.*, **115**, 3104 (2005)
NIH3T3	mouse fibroblast	R. Yu, *et al., Toxicol. Sci.*, **93**, 82 (2006)
NT2N	human embryonal carcinoma	J. Tessier, *et al., Infect. Immun.*, **75**, 1895 (2007)
osteoblast	from calvaria of Wistar rats	E. Hinoi, *et al., FASEB J.*, **17**, 1532 (2003)
OVK18	human ovarial cancer cell	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
PANC-1	pancreatic cancer cell	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)
PBMC	human peripheral blood mononuclear cell	C. Chang, *et al., Stem Cells*, **24**, 2466 (2006) T. Lee, *et al., Mol. Cancer Ther.*, **5**, 2398 (2006)
PFSK	primitive neuroectodermal tumour cell	X. Li, *et al., Mol. Cancer Ther.*, **4**, 1912 (2005).
PSN-1	pancreatic cancer cell	S. Awale, *et al., Cancer Res.*, **66**, 1751 (2006)
Ramos	human burkitt lymphoma	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)
RAW 264	mouse macrophage	M. Shiga, *et al., Anesth. Analg.*, **92**, 128 (2001)
RAW 264.7	mouse macrophage	S. Oyadomari, *et al., PNAS*, **98**, 10845 (2001) D. J. Son, *et al., Mol. Cancer Ther.*, **6**, 675 (2007)
RERF-LC-OK	non-small-cell lung cancer cell	H. Ishibashi, *et al., Cancer Res.*, **65**, 6450 (2005)
RP9	B lymphoblastoid cell	R. A. Dalloul, *et al., Poult. Sci.*, **85**, 446 (2006)
SH10TC	human gastric cancer cell	H. Ohori, *et al., Mol. Cancer Ther.*, **5**, 2563 (2006)
SK-N-SH	human neuroblastoma	Y. Wang, *et al., J. Virol.*, **78**, 7916 (2004)
SMS-KAN	human neuroblastoma	A. Misawa, *et al., Cancer Res.*, **65**, 10233 (2005)
SUIT-2	pancreatic cancer cell	T. Mori, *et al., Mol. Cancer Ther.*, **3**, 29 (2004)
SupT1	human lymphoblast cell	J. Melton, *et al., J. Biol. Chem.*, **279**, 14315 (2004)
T-cell		I. Y. Lee, *et al., J. Immunol.*, **175**, 1658 (2005)
T24	human bladder carcinoma	Y. Shibata, *et al., J. Biol. Chem.*, **277**, 746 (2002)
T98G	human glioblastoma	T. Kitamuro, *et al., J. Biol. Chem.*, **278**, 9125 (2003)
TL-Omi	T-cell	T. Kasai, *et al., J. Biol. Chem.*, **277**, 5187 (2002)
U87MG	human glioblastoma	S. Kim, *et al., Clin. Cancer Res.*, **11**, 5965 (2005) S. Kim, *et al., Clin. Cancer Res.*, **12**, 5550 (2006)
U937	human monoblastic lymphoma	R. Hori, *et al., J. Biol. Chem.*, **277**, 10712 (2002)
WSU-CLL	human B lymphoid	M. Ho, *et al., J. Biol. Chem.*, **280**, 607 (2005)

Cell Counting Kit-F Assay

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 assay has a higher sensitivity than tetrazolium-based assays such as the 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 in assays using non-adherent type cells. A V-bottom plate and a plate rotor for centrifuge are necessary for non-adherent type cells.

Applications: Cell Counting, Cell proliferation experiments, Cytotoxicity experiments, Drug sensitivity tests

Materials Required for the Assay

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

Preparation

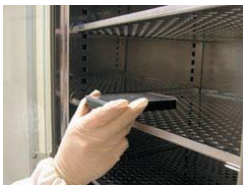
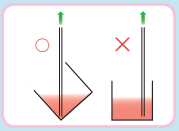
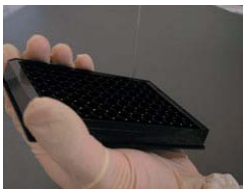
Cell Counting Kit-F solution
Dilute Cell Counting Kit-F by 50 times using PBS (-) to prepare an assay solution

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

Cell Counting Kit-F Assay

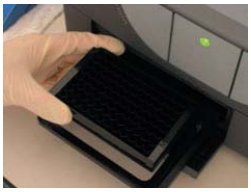
Assay Conditions

When using Cell Counting Kit-F for proliferation and cytotoxicity assays, it is necessary to have a proportional relationship between amount of fluorescence (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.
Measure the cells and adjust the concentration of the cell suspension. (cell concentration: _____cells/ml)	For adherent cells, recover using trypsin to detach cells, and use a cell scraper if necessary.
	Use a hematocytometer or a cell counter.
Add a cell suspension of 100 μl to each well in a 96 well microplate usingserial dilution. Make a well containing only medium for background measurement. 	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 no 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.
	When using a plate or petri dish other than a 96 well plate, add reagent equal to 1/10 the media volume.
Add 10 μl of Cell Counting Kit-F to each well of a the 96 well microplate and allow to react at room temperature for 15-30 min. (start time: _____ end time: _____) 	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 make it go down into the culture medium.

Cell Counting Kit-F Assay

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



Bubbles 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

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. 9. 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 10^5 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.

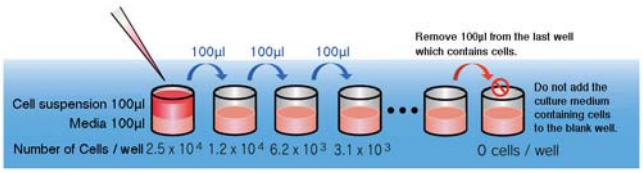


Fig. 9 Serial dilution process

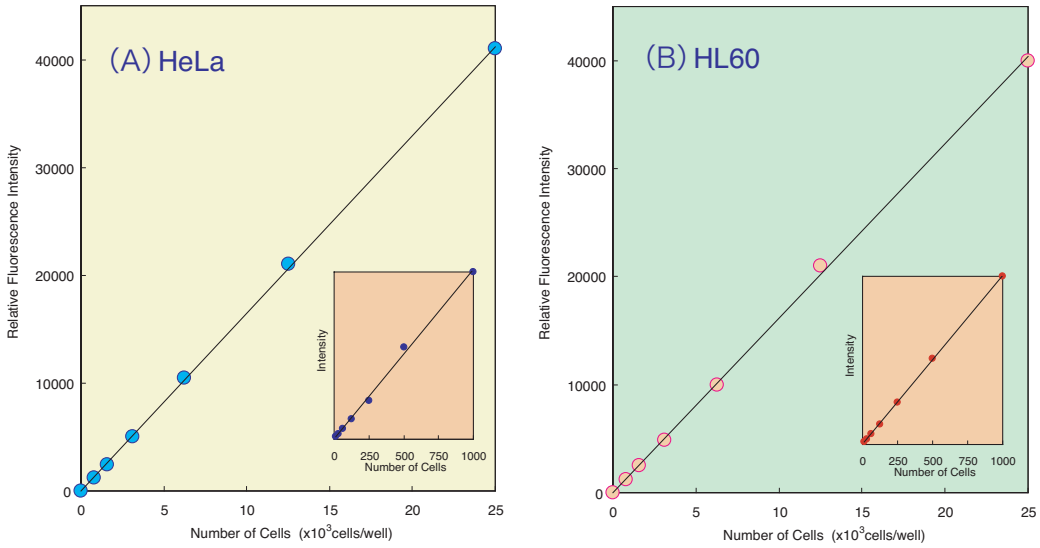
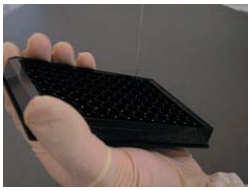
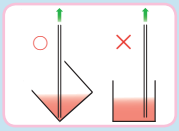
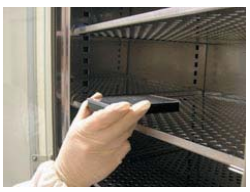


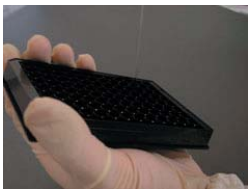
Fig. 10 Assay data of HeLa cells (A) and HL60 cells (B). Sensitivity of the Cell Counting Kit-8 for HL60 (non-adherent type cell) is about ten times lower than that for HeLa (adherent type cell). However, the sensitivity of the Cell Counting Kit-F for HL60 cells (non-adherent type cell) is almost the same as the HeLa cells. The detection range of the Cell Counting Kit-F is about several tens to 25,000 cells per well.

Cell Counting Kit-F Assay

Cell Proliferation and Cytotoxicity Protocol

Procedure	Precautions & Tips
Recover the cells to be assayed from flask.	When incubating for more than 48 hrs, be sure to change the media For adherent cells, recover using trypsin to detach cells, and use a cell scraper if necessary.
Measure the cells and adjust the concentration of the suspended cells. (cell concentration: _____cells/ml)	Use a hematocytometer or a cell counter.
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 suspended 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 mesurement. 	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 the same amount of test subatnce 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.
Add 10 μl of media containing different amounts of the test substance to each well. 	
Incubate for a certain time (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 appropriate.

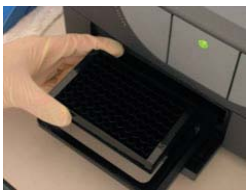
After removing the media from each well, add 100 μl of PBS(-) which does not contain serum and phenol red to each well.



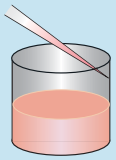
Add 10 μl of Cell Counting Kit-F to each well of the 96 well microplate.

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.



Bubbles 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.

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

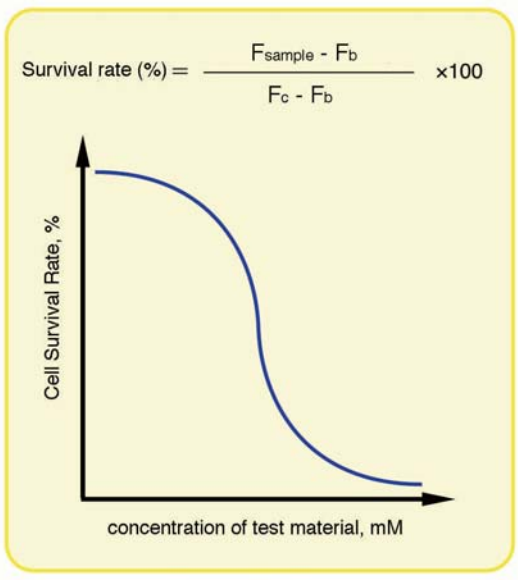


Fig. 11 Typical cell survival curve.

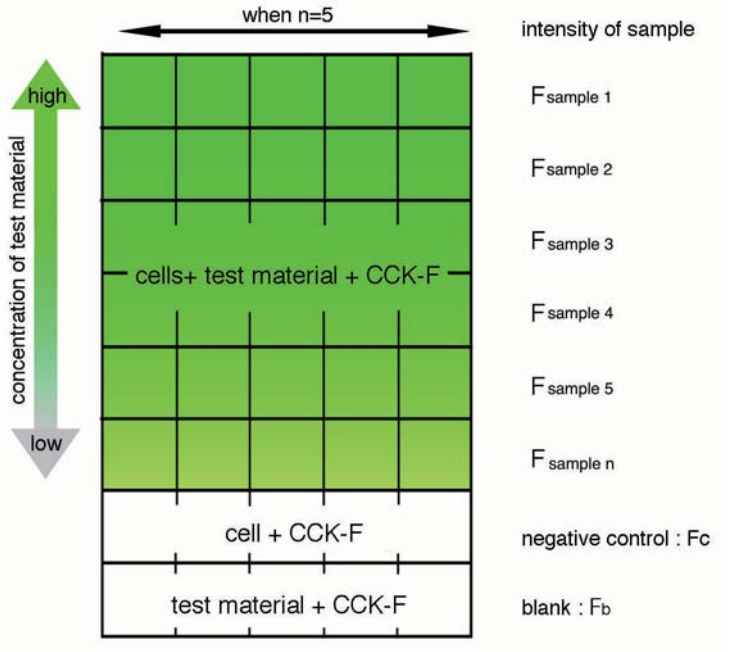


Fig. 12 Example of the plate arrangement and fluorescence development

Troubleshooting

Problem	Possible Cause	Solution
Low sensitivity (high background)	The cell number is low.	Measure the relationship between cell number and amount of fluorescence for each cell type. Refer to (Assay Conditions). Also, the permiability of Calcein-AM across the cell membrane is diferent for each cell type.
	The reagent has deteriorated.	Cell Counting Kit-F solution (Calcein-AM) is extremely unstable after diluting with PBS (-). Please 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 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. Only add media to these wells.
	The 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.

Cell Counting Kit-F Assay

Q&A

Q *Is the Calcein-AM (Calcein) used in Cell Counting Kit-F stable inside and outside the cell?*

A Calcein is stable regardless of inside or 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 get broken-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 Please use a white or black colored plate. Light reflecting off of a trasnparent plate scatters, making it not possible to get accurate results. It is neccesary to use a black or white fluorescent plate for accurate results.

Q *Will fluorescence increase if incubation time is increased?*

A Even if 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, is hydrolyized inside the cell, turns to Calcein and then fluoresces. Calcein is a foreign object inside the cell, so it is expelled from the cell. Therefore, the fluorescence reduces gradually.

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 posible to assay a small number of cells, remove anything that may be background fluorescence.

Q *Can serum and phenol red be in the media during the preincubation step?*

A There is no problem if they are present during the preincubation step. If phenol red is present, then just exchange the media prior to adding Cell Counting Kit-F.

Q *Are there any other options if the media containing phenol red 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 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 to form the fluorescent dye Calcein as indicated in Fig. 13. By measuring the amount of fluorescence, it is possible to determine the number of cells present. In addition, Calcein has low permiability across the cell membrane, so it does not leave the cell easily.

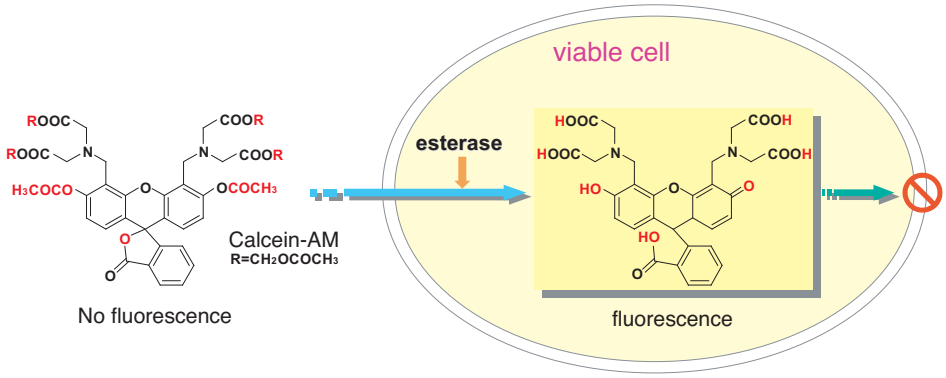


Fig. 13 Cell viability detection mechanism with CCK-F

Cell Staining

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.

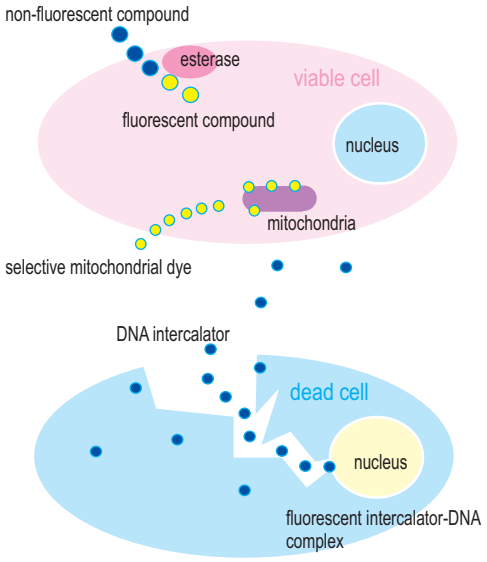


Fig. 19 Cell staining methods.

Cell Staining

Applications: Fluorescence microscopy, Flowcytometry, Electrophoresis (nucleic acid screening)

Dye Characteristics

Target	Dye	Exitation	Emission	Exitation 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.
	EB	520-525 nm	615 nm	G excitation	red	
	PI	530 nm	620 nm	G excitation	red	
Nucleous	AO	500 nm	526 nm (dsDNA)	B excitation	red	Fluorescence is produced by combining with single stranded and double stranded DNA
		420-460 nm	630-650 nm (ssDNA and RNA)	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 accumulating in the mitochondria.
	Rh123	507 nm	529 nm	B excitation	yellowish green	

Cell Staining

Materials Required for the Assay

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	*-Cellstain-* Calcein-AM solution
-Cellstain- CFSE	*-Cellstain-* CytoRed solution
-Cellstain- FDA	BCECF-AM

Dead Cell Statining Dyes

-Cellstain- DAPI	*-Cellstain-* DAPI solution
-Cellstain- EB	*-Cellstain-* EB solution
-Cellstain- PI	*-Cellstain-* PI solution

Nucleus Staining Dyes

-Cellstain- AO	*-Cellstain-*AO solution
-Cellstain- Hoechst 33258	*-Cellstain-*Hoechst 33342

Mitochondria Staining Dyes

-Cellstain- MitoRed	*-Cellstain-* Rh123

Other Reagents

- DMSO
- Sterilzed Water
- PBS(-)

Preparation of Assay Solution

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

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

Dyes for Living Cell Stainings

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 DMSO solution is poor. After the preparation of the DMSO solution, aliguot 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 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 packging	orange-brown solid	avoid light, freeze	688.59	50 μg x 8	0.5 - 1mmol/l	

Staining solutions are not stable to store. Discard the remaining staining solution after use.

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_20)	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, refridgerate	350.25	1 ml	1 mg/ml*	
-*Cellstain*-EB	red-brown solid	avoid light, refridgerate	394.31	1 mg	1 mg/ml	
-*Cellstain*-EB soln.	red liquid	avoid light, freeze	394.31	1 ml	1 mg/ml	
-*Cellstain*-PI	red-brown solid	avoid light, refridgerate	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 the product comes in contact with the 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_20)	Staining Solution
-*Cellstain*-AO	yellow solid	avoid light, refrigerate	350.25	1 mg	1 mg/ml	1-10 μg/ml (Storage solution diluted by PBS (-))
-*Cellstain*-AO soln.	yellow liquid	avoid light, freeze	350.25	1 ml	1 mg/ml*	
-*Cellstain*-Hoechst 33258 soln.	yellow liquid	avoid light, refridgerate	394.31	1 mg	1 mg/ml	
-*Cellstain*-Hoechst 33342 soln.	yellow liquid	avoid light, refrigerate	394.31	1 ml	1 mg/ml	

* Avoid storing in solution.

⚠ Since the dyes directly stain the nucleus, these dyes are considered mutagens. 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 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 Stainging

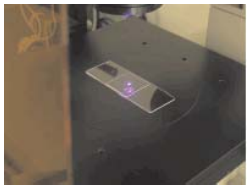
Prepare the stock solution using DMSO

Product name	Characteristic	Storage	Mol. weight	Units	Stock Solution (H_20)	Staining Solution
-*Cellstain*-MitoRed	purple brown solid	avoid light, refridgerate	637.17	50 μgx8	1 mmol/l	20 - 200 nmol/l
-*Cellstain*-Rh123	brown powder	avoid light, refridgerate	380.82	1 mg	1 mg/ml	20 - 100 nmol/l

⚠ Only mitochondria in living cells will be stained.

Cell Staining

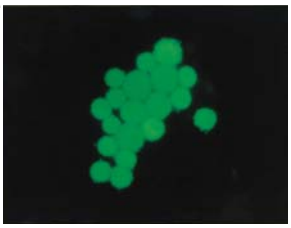
Staining Procedure for a Fluorescence Microscopy

Procedure		Precautions & Tips
Harvest the cells from a culture flask and prepare a cell suspension.		Recover adhesive cells using trypsin and a cell scraper if necessary. $1x10^6$ cells is sufficient for several staining experiments
Centrifuge the cell suspension at 1,000 rpm for 3 min.		
Remove the supernatant, resuspend the cells with 2-5 ml PBS, and then centrifuge again. After removing the supernatant, add PBS(-) and resuspend to adjust the cell number to 10^5 - 10^6 cells/ml.		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 factor for a high background, so it is important to wash several times. Use a cytometer or a cell counter to measure. Gently pipette to avoid damaging the cells.
Add 30 μl of the cell suspension to a microtube, then add 15 μl of Staining solution to the same tube.		In order to obtain the best fluorescent image, it is necessary to determine the optimal reagent concentration and staining time.
Incubate with protection from light at 37 ◦C for 15-30 min.		
Place 10 μl of the cell and staining solution on a glass slide and cover with a cover glass.		
Observe the fluorescent image on a fluorescence microscope.		

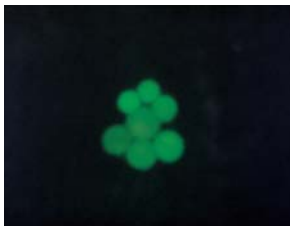
Cell Staining

Experimental Example

Living Cell Staining Images - 1 HeLa cells stained after treatment with trypsin-EDTA.

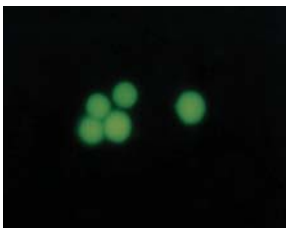


Calcein-AM
(x600, B excitation)



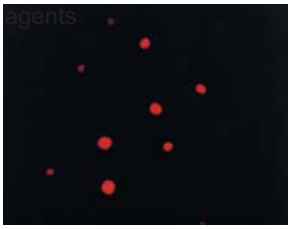
CFSE
(x600, B excitation)

FDA
(x300, B excitation)

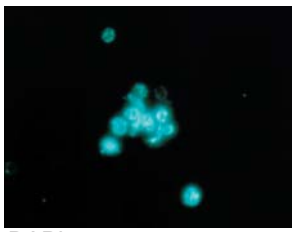


BCECF-AM
(x600, B excitation)

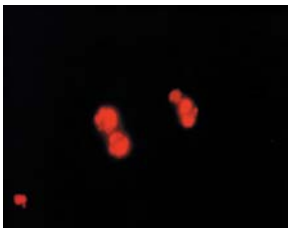
Nucleus Staining re-



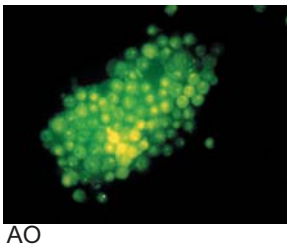
EB
(x300, G excitation)



DAPI
(x400, V excitation)

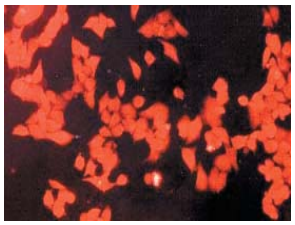


PI
(x600, G excitation)

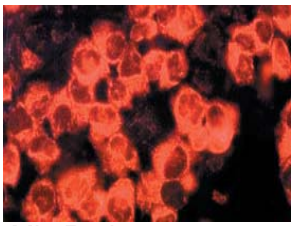


AO
(x300, B excitation)

Culture Cell Staining Images - 2 HeLa cells were incubated on a chamber slide and stained with mitochondria staining dyes.

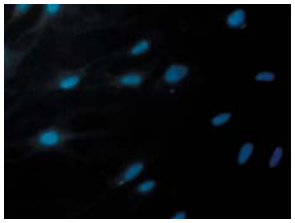


CytoRed
(G excitation)

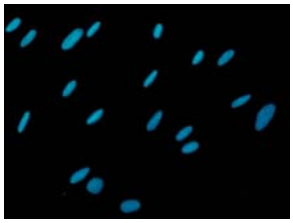


MitoRed
(G excitation)

Culture Cell Staining Images - 3 Normal human fetus-derived cells stained with Hoechest dyes after being fixated with 1% glutaraldehyde / PBS (-).



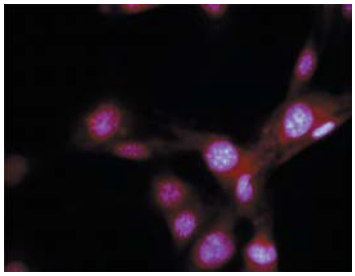
Hoechst 33342
(WU excitation)



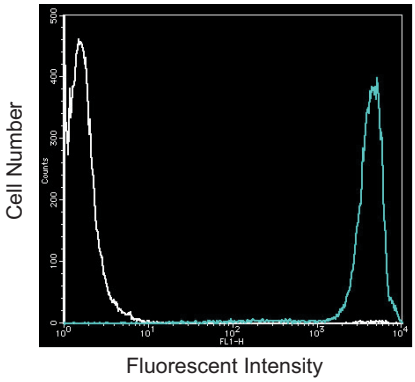
Hoechst 33258
(WU excitation)

Cell Staining

Fluorescent Staining of Fixed Cells - 4 NIH3T3 cells that were fixated with 3% glutaraldehyde were stained with the nuclear staining reagents Hoechst 33258. Then, actin filaments were stained with biotin-labeled phalloidin and anti-biotin antibody labeled with HiLyte Fluor™ 555.*



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



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

Simultaneous Staining of Living and Dead Cells

Introduction

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

Materials Required for the Assay

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	1 vial

⚠ Store at -20 °C and protect from light.
Solution A (Calcein-AM) is easily hydrolized by moisture. Tightly close the cap after use.

- PBS(-)

Preparation

Staining solution

Warm 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.

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

⚠ Prepare staining solution only prior to 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 copiuos 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.

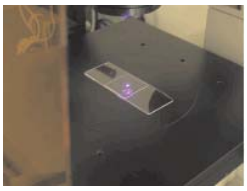
Simultaneous Staining of Living and Dead Cells

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 cell types and reagent concentration.

Procedure	Precautions & Tips
Recover the cells to be assayed from a culture flask. 	Recover using trypsin to detach cells, and use a cell scraper if necessary.
Centrifuge the cell suspension (1,000 rpm 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 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. 	

View the fluorescent image on a fluorescence microscope.



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

It is possible to observe the fluorescence of only 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 below protocal.

Optimum concetration for PI

Stain the target cells with 0.1 - 10 μmol/l of PI. This is used as a rough concentration range for staining the nucleus rather than the whole cell.

Fix the cells prior to staining using one of the methods below if necessary:

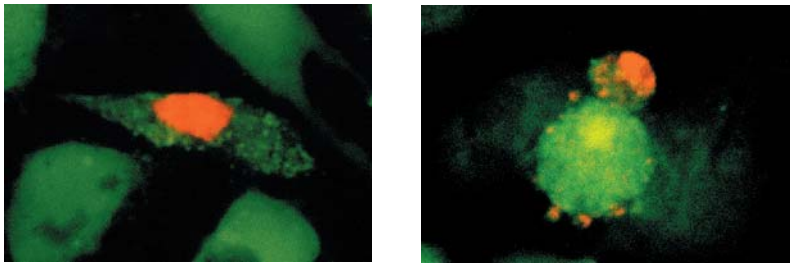
-Treat the cells for 10 min with 0.1 % saponin or 0.1 -0.5% digitonin.

-Treat the cells for 30 min with 70% ethanol.

Optimum concentration for Calcein-AM

Using the dead cells to stain with 0.1 - 10 μmol/l of Calcein-AM solution. Determine the concentration range that will not stain the whole dead cell. 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



Double staining using the Cellstain-Double Staining Kit

MHD-1 cell double staining image (480 nm excitation filter was used)
[Photograph provided by Hiroshima University Med, Dr. Yamamoto]

Simultaneous Staining of Living and Dead Cells

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 & storage conditions. If the reagent was stored over one 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 buffer solution. In particular, viable staining dye is fairly unstable in the buffer solution. Prepare a working solution only prior to use.
	The dye or the working solution was decomposed by the exposure to light.	Light may accelarate 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.
	Enough reagent was not 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 was stored over one 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 was observed	Extra dye still remianed 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.

Cell Staining

Q&A

Staining reagents for living cells

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

A Please dissolve in DMSO for viable cell staining reagents. Please use fresh DMSO since DMSO easily absorbs moisture.

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

A CFSE remains relatively the longest inside 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*, **1990**, *133*, 87-97

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

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*, **1994**, *172*, 115-124

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

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

BCECF-AM : This was orginially 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*, **1994**. *172*,115.

Q *What dye should be used to stain 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, EB, and DAPI can be use 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., **1992**, *38*,268.

Cell Staining

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 of dead cell staining dyes are listed below:

AO : It is possible to distinguish bewteen 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 permiability. 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 reguations 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 wave length are listed below

EB: It doesn't have base specificity. It binds to all DNA and RNA.

PI: Same as EB, but the fluroescence intensity is higher than EB when intercalating and can be more widely used.

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.

Simultaneous Staining of Living and Dead Cells

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 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 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 set concentration of dye?*

A It is not the case that the concentration is set the same for all 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 insturctions.

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*, **1994**. *172*,115.

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 temparature. 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 reguations 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.

Dojindo Laboratories
Kumamoto Techno Research Park
2025-5 Tabaru, Mashiki-machi,
Kamimashiki-gun, Kumamoto, 861-2202, JAPAN
Phone: +81-96-286-1515, Fax: +81-96-286-1525
Web: www.dojindo.com