

96992 Cell Counting Kit - 8

GENERAL INFORMATION

Cell Counting Kit-8 (CCK-8) allows very convenient assays by utilizing the highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (*Patent No. WO97/38985*) produces a water-soluble formazan dye upon reduction in the presence of an electron carrier, as shown in Figure 1. Cell Counting Kit-8 is a one-bottle solution; no premixing of components is required. Cell Counting Kit-8, being nonradioactive, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give a yellow-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. The detection sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT, XTT, MTS or WST-1.

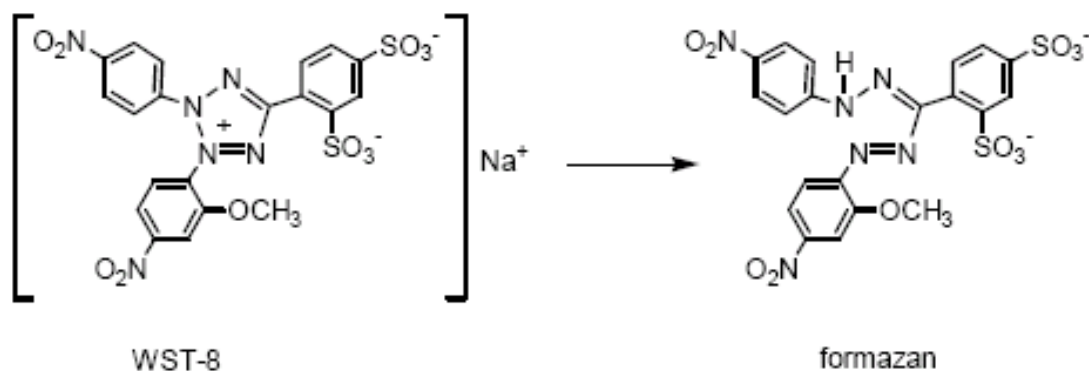


Figure 1. Structures of WST-8 and WST-8 formazan

Figure 2 shows the absorption spectrum of WST-8 formazan. Since the absorbance at 460 nm is proportional to the number of viable cells in the medium, the viable cell number can be determined using the absorbance value of a previously prepared calibration curve.

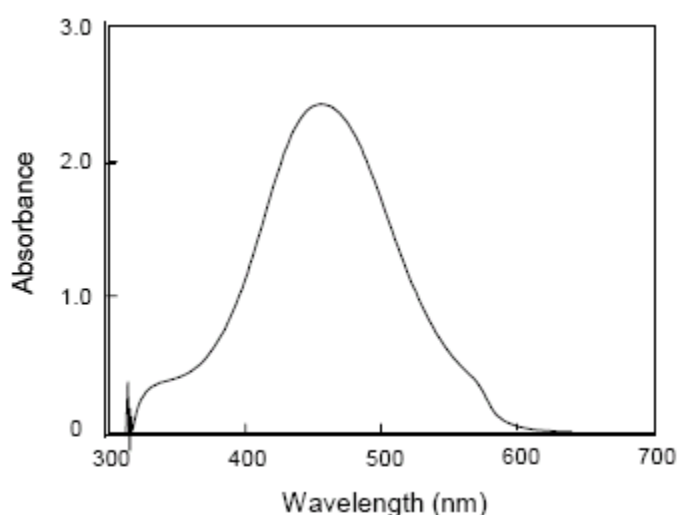


Figure 2. Absorption spectrum of WST-8 formazan



As shown in Figure 3, the absorbance of the CCK-8 assay solution is higher than that of MTT and other tetrazolium salts that produce water-soluble formazan dyes. Figure 4 shows that the cell proliferation assay using CCK-8 correlates well with the [³H]-thymidine incorporation assay. Thus, the CCK-8 assay can also be substituted for the [³H]-thymidine incorporation assay.

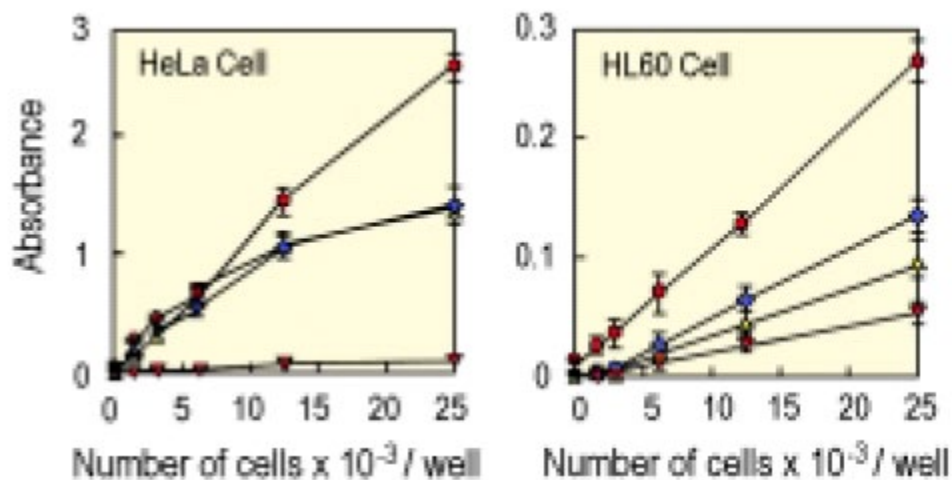


Figure 3: Cell proliferation assay using CCK-8 and other reagents

Culture medium:

MEM, 10% FCS, L-glutamine (HeLa)

RPMI1640, 10% FCS, L-glutamine (HL60)

Incubation:

37 °C, 5% CO₂, 2 hrs (HeLa)

37 °C, 5% CO₂, 3 hrs (HL60)

Detection :

CCK-8 (○): 450 nm, reference: 650 nm

XTT (◇): 450 nm, reference: 650 nm

MTS (▲): 490 nm, reference: 650 nm

MTT (▼): 570 nm, reference: 650 nm

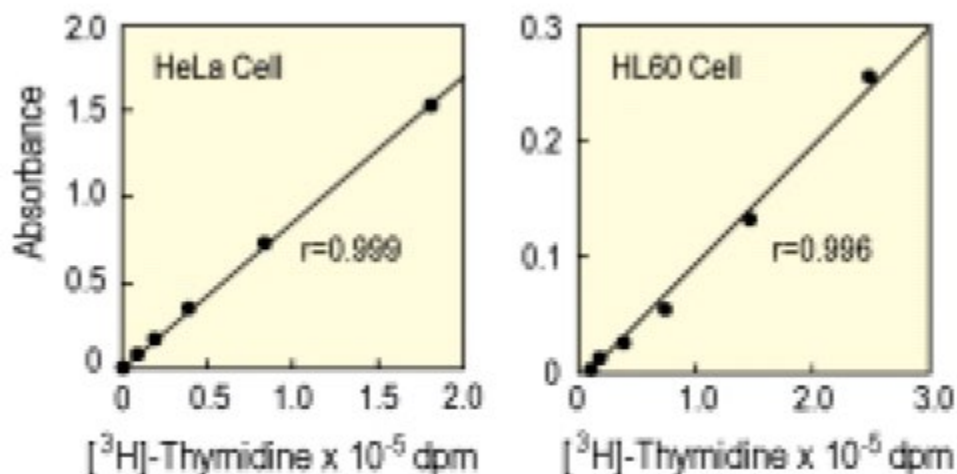


Figure 4: Correlation between [³H]-thymidine incorporation assay and CCK-8 assay.

Cell line:

HeLa, HL60

Culture medium:

MEM, 10% FCS (HeLa)

RPMI1640, 10% FCS (HL60)

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Reagent:

[3H]-thymidine: 37 KBq/well

CCK-8: 10 µl/well

Incubation:

[3H]-thymidine assay: 4 hrs

CCK-8 assay: 3 hrs

ADVANTAGES

- 1 One-bottle, ready-to-use solution
- 2 No organic solvents or isotopes required
- 3 No harvesting, no washing and no solubilization steps
- 4 More sensitive than MTT, XTT, MTS or WST-1

STORAGE

CCK-8 is stable for 3 years at 2-8 °C with protection from light. It can be also stored at -20 °C.

Repeated thawing and freezing causes an increase in the background, which interferes with the assay.

HOW TO USE CELL COUNTING KIT-8

1. Required Equipment and Materials

- plate reader (450 nm filter)
- 96-well plate
- 10 µl, 100-200 µl and multi-channel pipettes
- CO2 incubator

2. Protocol

Cell Proliferation Assay

1) Inoculate cell suspension (100 µl/well) in a 96-well plate. Also prepare wells that contain known numbers of viable cells (to create a calibration curve in step 5). Pre-incubate the plate in a humidified incubator (e.g., at 37 °C, 5% CO₂).

2) Thaw the CCK-8 on the bench top or in a water bath at 37 °C if it is frozen.

Note: It takes about 30 minutes on the bench top at 25 °C or 5 minutes in a water bath at 37 °C.

3) Add 10 µl of the CCK-8 solution to each well of the plate.

Note: Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.

4) Incubate the plate for 1-4 hours in the incubator.

5) Measure the absorbance at 450 nm using a microplate reader. Prepare a calibration curve using the data obtained from the wells that contain known numbers of viable cells.

Note: To measure the absorbance later, add 10 µl of 1% w/v SDS to each well, cover the plate and store it with protection from light at room temperature. No absorbance change should be observed for 48 hours.

Cytotoxicity Assay

1) Dispense 100 µl of cell suspension (5000 cells/ well) in a 96-well plate.

2) Pre-incubate the plate for 24 hours in a humidified incubator (e.g., at 37 °C, 5% CO₂).

3) Add 10 µl of various concentrations of toxicant into the culture media in the plate.

4) Incubate the plate for an appropriate length of time (e.g., 6, 12, 24 or 48 hours) in the incubator.

5) Thaw the CCK-8 on the bench top or in a water bath at 37 °C if it is frozen.

Note: It takes about 30 minutes on the bench top at 25 °C or 5 minutes in the water bath at 37 °C.

6) Add 10 µl of CCK-8 solution to each well of the plate. *Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.*

7) Incubate the plate for 1-4 hours in the incubator. Measure the absorbance at 450 nm using a microplate reader.

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Note: To measure the absorbance later, add 10 μ l of 1% w/v SDS to each well, cover the plate and store it with protection from light at room temperature. No absorbance change should be observed for 48 hours.

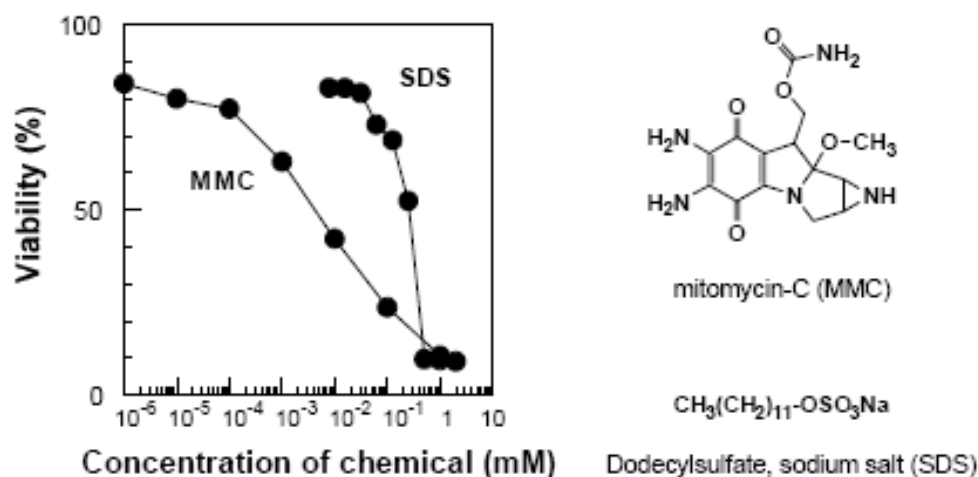


Figure 5. Toxicological test of chemicals using CCK-8

Cell line:

HeLa

Culture medium:

MEM, 10% FCS, L-Glutamine

Chemicals:

Mitomycin-C (MMC)

Dodecylsulfate, sodium salt (SDS)

Incubation:

37 °C, 5% CO₂, 2hrs

Detection:

450 nm, reference: 650 nm

BACKGROUND CONTROL

1. Slight spontaneous absorbance around 460 nm occurs in culture medium incubated with CCK-8. This background absorbance depends on the culture medium, pH, incubation time and length of exposure to light. Typical background absorbance after 2 hours incubation is 0.1 - 0.2 absorbance units. To correct for this, prepare one or more control wells without cells, and subtract the average absorbance of the control wells from that of the other wells.
2. During a 5-hour experiment, the absorbance of the CCK-8 solution does not increase at room temperature.

PRECAUTIONS

1. Since the CCK-8 assay is based on the dehydrogenase activity detection in viable cells, conditions or chemicals that affect dehydrogenase activity in viable cells may cause discrepancy between the actual viable cell number and the cell number determined using the CCK-8 assay.
2. WST-8 may react with reducing agents to generate WST-8 formazan. Please check the background O.D. if reducing agents are used in cytotoxicity assays or cell proliferation assays.
3. Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.
4. Phenol red containing culture media can be used with this kit for cell viability assays.
5. Membrane filtration is recommended for the sterilization of the CCK-8 solution, if necessary.
6. The incubation time varies by the type and number of cells in a well. Generally, leukocytes give weak coloration, thus a long incubation time (up to 4 hours) or a large number of cells (~10⁵ cells/well) may be necessary.
7. Since the cytotoxicity of this kit is very low, further color development is possible after reading the absorbance.

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- Neutral red or crystal violet can be used after the CCK-8 assay.
- Measure the reference wavelength at 600 nm or higher if there is a high turbidity in the cell suspension.

FREQUENTLY ASKED QUESTIONS

1. How many cells should there be in a well?

For adhesive cells, at least 1000 cells are necessary per well (100 μ l medium) when using the kit's standard 96-well plate. For leukocytes, at least 2500 cells are necessary per well (100 μ l medium) because of low sensitivity. The recommended maximum number of cells per well for the 96-well plate is 25000. If a 24-well or 6-well plate is used for this assay, please calculate the number of cells per well accordingly, and adjust the volume of the CCK-8 solution in a well to 10% of the total volume.

2. Does CCK-8 stain viable cells?

No, it does not stain viable cells because the water-soluble tetrazolium salt (WST-8) is used in the CCK-8 solution. The electron mediator, 1-Methoxy PMS, receives electrons from a viable cell and transfers the electron to WST-8 in the culture medium. Since its formazan dye is also highly water-soluble, CCK-8 cannot be utilized for cell staining purpose.

3. How stable is CCK-8?

CCK-8 is stable for 2 years at -20 $^{\circ}$ C, 1 year at 4 $^{\circ}$ C, and 3 months at room temperature. CCK-8 is stable over 2 days even at 60 $^{\circ}$ C as long as the CCK-8 solution keeps its original red color and does not turn orange.

4. Does phenol red affect the assay?

No. The absorption value of phenol red in a culture medium can be removed by subtracting the absorption value of a blank solution from the absorption value of each well. Therefore, a phenol red containing medium is usable for the CCK-8 assay.

5. Is there a correlation between CCK-8 and the Thymidine incorporation assay?

Yes. For correlation graphs, see page 2. Please note that since CCK-8 uses a different assaying mechanism from that of the Thymidine assay (described on page 2), the CCK-8 and Thymidine assay results may differ.

6. Is CCK-8 toxic to cells?

The toxicity of CCK-8 is so low that, after the CCK-8 assay is completed, the same cells can be used for other cell proliferation assays such as the crystal violet assay, neutral red assay or DNA fluorometric assay.

7. I do not have a 450 nm filter. What other filters can I use?

You can use filters with the absorbance between 450 and 490 nm, even though 450 nm filter gives the best sensitivity.

8. Can I use CCK-8 for 384-well plates?

CCK-8 can be used for 384-well plates. Please dilute the CCK-8 solution using PBS; the required volume of the CCK-8 solution is 5 μ l per well for the 384-well plate.

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APPENDIX

Cytotoxicity Assay Data

Table 1. The LD50 values of anti-tumor agents to human cancer cell lines using CCK-8 and MTT.

Cell lines	Anti-tumor agents	CCK-8 LD50 (µg/ml)	MTT LD50 (µg/ml)
HE49	Bleomycin	154.59	157.34
	Cisplatin	1.55	1.61
	Adriamycin	0.41	0.47
	MMC	0.05	0.03
	Etoposide	13.01	13.18
	5-FU	0.12	0.37
MKN-28	Bleomycin	151.18	139.51
	Cisplatin	0.57	0.60
	Adriamycin	0.11	0.13
	MMC	0.02	0.02
	Etoposide	4.11	4.63
	5-FU	0.05	0.05
IMR-32	Bleomycin	314.15	207.00
	Cisplatin	5.65	5.32
	Adriamycin	2.27	0.33

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	MMC	0.10	0.10
	Etoposide	9.63	9.71
	5-FU	0.04	0.05
H1299	Bleomycin	406.65	1057.12
	Cisplatin	0.51	0.72
	Adriamycin	0.17	0.14
	MMC	0.04	0.06
	Etoposide	14.63	25.96
	5-FU	0.08	0.05
HL60	Bleomycin	280.74	190.84
	Cisplatin	1.54	1.00
	Adriamycin	0.19	0.24
	MMC	0.02	0.03
	Etoposide	12.23	11.32
	5-FU	0.06	0.07
MOLT-4	Bleomycin	218.84	187.26
	Cisplatin	0.94	1.04
	Adriamycin	0.27	0.24
	MMC	0.01	0.01
	Etoposide	3.87	4.27
	5-FU	0.11	0.11
KC12	Bleomycin	739.05	880.56
	Cisplatin	3.59	2.98
	Adriamycin	0.37	0.48
	MMC	0.15	0.11
	Etoposide	52.87	53.31
	5-FU	0.20	0.19
HeLa	Bleomycin	306.89	379.24
	Cisplatin	15.22	11.52
	Adriamycin	1.56	1.98
	MMC	0.18	0.14
	Etoposide	79.16	58.37
	5-FU	0.71	0.78

Cell lines:

HE49:	human normal embryo
MKN-28:	human gastric cancer
IMR-32:	human neuroblastoma
H1299:	human lung cancer
HL60:	human acute promyelonic leukemia
MOLT-4:	human acute lymphoblastic leukemia
KC12:	human renal cancer
HeLa:	human cervical cancer

Anti-tumor agents:

MMC (Mitomycin C)
5-FU (5-Fluoro-uracil)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

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