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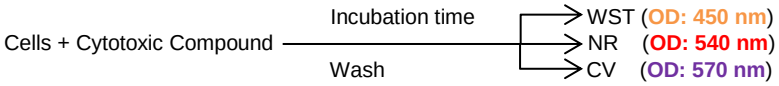
WST-NR-CV Combined Cytotoxicity Assay Kit

(Catalog # K543-1000; 1000 assays, Store kit at -20°C)

I. Introduction:

Cytotoxicity assays are essential tools in drug discovery. *In vitro* single parameter cytotoxicity assays, such as using tetrazolium salt (WST), neutral red (NR) or crystal violet (CV) in cultured cells, are widely used to assess the cytotoxicity of different compounds as these methods are fast, economical and animal-free. However, a major problem of using single parameter assays is determined IC_{50} data using these individual assays sometimes can be quite different due to specific compound inhibition. Consequently, if the cytotoxicity assay is not selected appropriately, false negative compounds that are actually cytotoxic may be easily missed. To partly overcome this problem, multiple parameter cytotoxicity assays are faster and more comprehensive alternatives to determine IC_{50} and cytotoxicity in different pathways. BioVision's WST-NR-CV Combined Cytotoxicity Assay Kit can allow researchers to test the cytotoxicity of their compounds in different pathways such as in cell proliferation (WST), lysosome activity (NR) and total DNA synthesis (CV). The kit is simple and fast, yet it provides a quantitative measurement of the number of viable cells, it offers an excellent and efficient method for *in vitro* cytotoxicity studies as well as high-throughput drug screening.



II. Application:

- *In vitro* cell proliferation cytotoxicity studies
- High-throughput drug screening

III. Sample Type:

- Cell culture: Adherent cells and non-adherent cells

IV. Kit Contents:

Components	K543-1000	Cap Code	Part Number
WST Reagent (lyophilized)	2 vials	Green	K543-1000-1
WST Developing Solution	2 x 5 ml	Amber	K543-1000-2
100X NR Staining Solution	2 ml	Brown	K543-1000-3
2X NR Solubilization Solution	75 ml	WM	K543-1000-4
CV Staining Solution	40 ml	WM	K543-1000-5
10X CV Solubilization Solution	10 ml	NM	K543-1000-6
10X Washing Solution	115 ml	NM	K543-1000-7
20 mM Doxorubicin	100 µl	Red	K543-1000-8

V. User Supplied Reagents and Equipment:

- 96-well clear flat-bottom plate
- Multi-well spectrophotometer
- 100% methanol

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C. The kit components are stable for one year when stored as recommended. WST, NR and CV dyes are light-sensitive and should be protected from light. Briefly centrifuge small vials at low speed prior to opening. Read the entire protocol before performing the experiment. Bring all reagents to room temperature before use.

- **WST Reagent and WST Developing Solution**: Dissolve lyophilized WST reagent (1 vial) by using 5 ml of WST Developing Solution to make WST solution. Aliquot WST solution (1 ml is sufficient for one 96 well plate assays) and store at -20°C. WST solution is stable for 1 year at -20°C. Protect from light. Avoid repeated freeze-thaw.
- **100X NR Staining Solution**: Prepare 1X NR Staining Solution. For one 96 well plate, dilute 0.2 ml of 100X NR Staining Solution by 100 folds to 20 ml by using the culture medium. **Note**: *Do not store 1X NR Staining Solution. Discard unused Staining Solution if not used within 24 hrs*.
- **2X NR Solubilization Solution**: Prepare 1X NR Solubilization Solution. For one 96 well plate, mix 7.5 ml of 2X NR Solubilization Solution with 7.5 ml of 100% methanol (not supplied) to make 1X NR Solubilization Solution. 1X NR Solubilization Solution is stable and can be stored at 4 ^{o}C for 3 months.
- **CV Staining Solution**: Bring it to room temperature before use. Add 11 ml of 100% methanol into the bottle. Shake contents and let it stand for 15 minutes at room temperature. 5 ml of CV Staining Solution is required for one 96 well plate. After use, store it at -20°C.
- **10X CV Solubilization Solution**: Prepare 1X CV Solubilization Solution. For one 96 well plate, mix 1 ml of 10X CV Solubilization Solution with 9 ml of water. After use, store it at room temperature.
- **10X Washing Solution**: Prepare 1X Washing Solution by adding 1 part 10X Washing Solution to 9 parts dH_2O. Store at 4°C.
- **20 mM Doxorubicin**: Ready to use. Store at -20°C.

VII. WST-NR-CV Combined Cytotoxicity Assay Protocol:

1. **Cell Culture**: Grow cells to ~80% confluency. For adherent cells, trypsinize the cells and spin down the cells. For suspension cells, spin down cells before removing any solution (same for detection below). Remove the solution and add culture medium to disperse the cell

pellet. Determine the cell density by using a hemocytometer. Adjust the cell concentration if necessary to 50000 - 250000cells/ml. Add 100 µl of the cells typically containing between 5,000–25,000 cells/well to a 96-well clear flat-bottom plate. Incubate cells overnight at 37 °C, 5% CO_2 controlled incubator.

2. **Compound Treatment:** Prepare compounds using DMSO as solvent. Dilute Stock solutions in DMSO appropriately. *Recommended final DMSO concentration in wells should be 0.5% or less*. Add diluted compounds to the wells. Prepare a DMSO vehicle control and a background control (containing only the medium). For inhibitor control, add 1 µl of 20 mM doxorubicin to a well containing the cells. Incubate the plate for 72 hrs at 5% CO_2 and 37 ºC.
3. **WST Detection**: After compound incubation, aspirate culture medium. Add 100 µl of fresh, warm culture medium to the wells. Add 10 µl of WST solution into each well. Incubate the cells in the controlled incubator for 1-3 hrs. Tap the plate gently and do not disturb the cell monolayer. Measure the OD at 450 nm.

   Note: An orange color in vehicle control wells is an indication of proper cell density (5000-25000 cells/wells). Pale coloration may indicate low cell densities, and longer incubation times are required.
4. **NR Detection**: After compound incubation, aspirate culture medium. Add 200 µl of 1X Washing Solution to wash cells once. *Washing must be done as gentle as possible to avoid disturbance of the cell monolayer*. Remove wash solution as much as possible by pipetting. Add 150 µl of 1X NR Staining Solution to each well and stain for 2 hrs. in an incubator. After incubation, remove the solution by pipetting. Add 200 µl of 1X Washing Solution to wash the cells. Repeat twice. Remove wash solution as gentle as possible. Fixation *(eg. by using 4% paraformaldehyde, not included)* sometimes may be required for low adherent cells. Add 150 µl of 1X NR Solubilization Solution to the wells. Incubate the cells in the controlled incubator for 20 min. Tap the plate gently and avoid disturbing cell monolayers. Measure the OD at 540 nm.
5. **CV Detection:** After compound incubation, aspirate culture medium. Add 200 µl of 1X Washing Solution to wash cells once. Remove the solution as gentle as possible. Add 50 µl of CV Staining Solution into each well. Incubate the plate at room temperature for 15 min. Use 250 µl of dH_2O to wash the cells for 4 times. Add 100 µl of 1X CV Solubilization Solution into each well. Shake the plate in a shaker at room temperature for 20 min. Measure the OD at 570 nm.
6. **Calculations:** Correct the background by subtracting the O.D. of the background control from all readings. Calculate the percentage of inhibition using the formula below:

$$\% \text{ Inhibition} = \frac{O.D._{VEHICLE} - O.D._{sample}}{O.D._{VEHICLE}} \times 100\%$$

Where: $O.D._{DMSO}$ is the O.D. of the DMSO control after background correction
$O.D._{sample}$ is the O.D. of the sample after background correction.

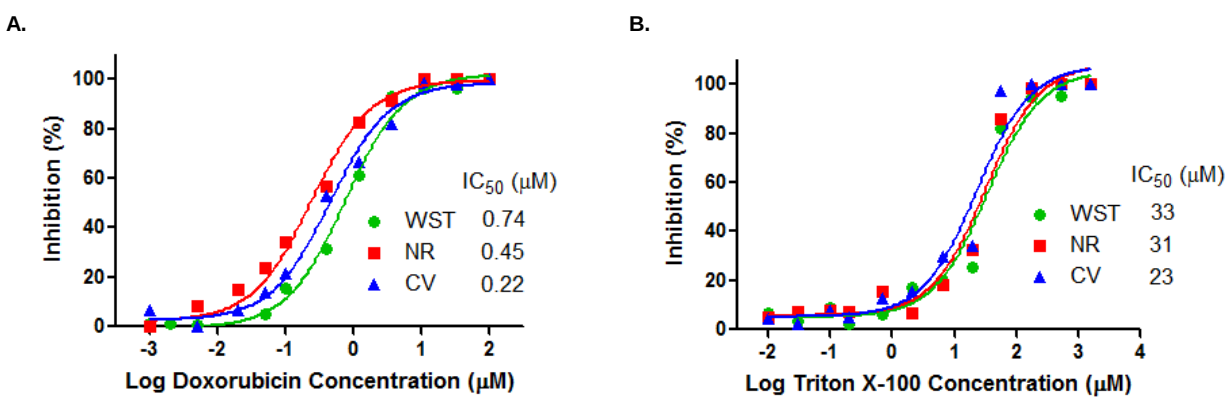


Figure A. Dose-response curves of Doxorubicin detected by the assay kit in the MCF-7 cells. **B**. Dose-response curves of Triton X-100 detected by the assay kit in the MCF-7 cells.

VIII. RELATED PRODUCTS:

Neutral Red Cell Cytotoxicity Assay Kit (K447)
Sulforhodamine B Cell Cytotoxicity Assay Kit (Colorimetric) (K943)
LDH-Cytotoxicity Colorimetric Assay Kit II (K313)
Bioluminescence Cytotoxicity Assay Kit (K312)
PicoProbe™ LDH-Cytotoxicity Fluorometric Assay Kit (K314)
MTT Cell Proliferation Assay Kit (Colorimetric) (K299)
MTS Cell Proliferation Colorimetric Assay Kit (K301)
BrdU Cell Proliferation Assay Kit (K306)
StayBrite™ Highly Stable ATP Bioluminescence Assay kit (K791)
Crystal Violet Cell Cytotoxicity Assay Kit (K329)
Doxorubicin.HCl (1527)
ATP Colorimetric Assay Kit II (K354)
Senescence Detection Kit (K320)
PicoProbe™ Lactate Dehydrogenase Activity Assay Kit (K730)
ADP Colorimetric/Fluorometric Assay Kit (K355)
ApoSENSOR™ ATP Cell Viability Bioluminescence Assay Kit (K254)
ApoSENSOR™ ADP/ATP Ratio Bioluminescence Assay Kit (K255)

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