VIII. Cell Viability Assay Protocol:

1. 3D Cell Culture:
   a. Matrix for 3D culture is not provided. Follow the appropriate protocol for matrix preparation. For 3D Cell culture environments, we recommend BioVision Cat. # K518, K519 or K520, 3D Cell Culture Matrices (Alginate, Basement Membrane, or Duo Matrix Kits).
   b. It is recommended that cells are allowed to form spheroids for at least 7+ days before performing any drug screening study.

2. Matrix Dissociation: After cells have formed spheroids in the appropriate matrix, and/or drug screening study is completed, remove all the media using a pipette tip and add 200 μl of Matrix Dissociation Saline Solution. Incubate at RT for 5-10 min and pipette up and down with 1 ml tip until the matrix is dissolved.

   Notes:
   - If matrix doesn't completely dissolve, add an additional 50 μl of Matrix Dissociation Saline Solution and incubate for another 10 min.
   - Matrix Dissociation Saline Solution works best on natural animal-based and plant-based matrices and scaffolds. Synthetic polymers have not been tested with this kit.

3. Neutralization: Add 50 μl of Viability Assay Buffer and centrifuge the plate at 1,000 x g for 5 min at 4°C. Carefully remove all of the liquid from each well without disrupting the cell pellet. Resuspend cells in 150 μl of fresh Viability Assay Buffer.

4. Cell Viability Standard Curve: Obtain a flask actively growing, 70-85% confluent, cells (of the same clone and type, but not from 3D culture or drug screening assay). Harvest the cells and centrifuge at 300 x g for 5 min at 4°C. Resuspend the cell pellet in a small
volume (<1 ml) of Viability Assay Buffer and count the number of cells using a hemocytometer or an automated cell counter. Resuspend the cells in Viability Assay Buffer to a concentration of 2.5 x 10^6 cells/ml. Mix gently by pipetting, then add (in duplicate wells) 0, 5, 10, 20, 30, 50, 100 μl of the cell mixture to seven consecutive wells to get 0, 12.5K, 25K, 50K, 75K, 125K, 250K cells per well. Bring the volume up to 150 μl with Viability Assay Buffer.

**Note:** Each cell type must have a unique Standard curve. It is recommended to prepare a new Standard Curve for each cell type used.

5. **Staining & Detection:** Dilute the WST Concentrate dye at a 1:10 ratio in Viability Assay Buffer to prepare the WST dye working solution (for example, for 100 wells, mix 0.5 ml of WST Concentrate dye and 4.5 ml of Viability assay Buffer). Add 50 μl of WST dye working solution to wells from steps VII-3 (and Standard Curve, step VII-4), making the total volume of 200 μl for each well. Incubate the plate at 37°C. Read the plate at 30, 60, 90, 120, 150, and 180 min by measuring absorbance at 460 nm.

**Note:** Appropriate incubation time depends on the individual cell type and cell concentrations used. Therefore, it is recommended to determine the optimal condition for each experiment by reading every 30 min until the Standard Curve and 3D cultured cells are in the desired absorbance range.

6. **Calculation:** For Standard Curve, subtract 0 Standard reading from all readings and plot the Standard Curve. For assay wells, subtract the 0 standard reading (from step VII-4) from all Sample readings. Apply the absorbance readings to the Standard Curve to determine the number of viable cells in each well.

**Figures:** (a) Standard Curve for viable HeLa cells (0 to 250,000 cells). (b) Standard Curve for viable HepG2 cells (0 to 100,000 cells). (c). Viable cells (HepG2) from 3D culture following 48 hour treatment with Cisplatin. Cells were cultured in 3D Cell Culture BME Matrix (Biovision Cat. #K518) for 18 days to form spheroids. Spheroids were treated as described above and the viable cells determined according to the kit protocol.

**VIII. Related Products:**
- 3D Cell Culture Matrix Alginate (K517)
- 3D Cell Culture Matrix BME (K518)
- 3D Cell Culture Scaffold (K990)
- Trypan Blue (0.4%) (1209)
- 3D Cell Culture Matrix Duo-Matrix (K519)
- Quick Cell Proliferation Colorimetric Assay Kit (K301)
- BrdU Cell Proliferation Assay Kit (K306)
- 3D Cell Culture HTS Cell Viability Complete Assay Kit (K948)

**FOR RESEARCH USE ONLY! Not to be used on humans.**