Time: 2024.08.25-2024.08.31

1. **Experiment:** Human hepatoma organoid lysis detection
2. **Time:** 2024.08.25-2024.08.31
3. **Member:** Xiaoyuan Chen, Hanyue Liu
4. **Material:**
   (1) Organoids: human hepatoma organoids
   (2) Reagent: LDH detection reagent, 200 mM Tris-HCl (pH 7.3), 10% Glycerol, 1% BSA, 10% Triton® X-100
5. **Method:**
   (1) Before You Begin
   ① Preparation of LDH assay working solution
   Immediately before use, prepare LDH Detection Reagent by combining the LDH Detection Enzyme Mix and Reductase Substrate as shown in the table below. The volumes given are for a 96-well plate format using 50 µL of sample and 50 µL of LDH Detection Reagent per well.

Component	Per Reaction	Per 96-Well Plate
LDH Detection Enzyme Mix	50 µL	5 mL
Reductase Substract	0.25 µL	25 µL

   ② Mix the LDH Detection Reagent by gently inverting five times.
   Note: Store unused LDH Detection Enzyme Mix below –65 °C or at –30 °C to –10 °C. Store unused Reductase Substrate below –65 °C protected from light.
   ③ Sample Dilution for LDH Assay Set Up
   Lower dilutions will increase sensitivity while higher dilutions are required to extend linearity when working with high cell numbers or in the presence of serum (contains significant amount of LDH). For the above reasons, the experiment followed the dilution principle in the following table.

Media Composition	Cells/100µL	Fold Dilution
Without Serum	<1,000	5×
	1,000-10,000	25×
	10,000-50,000	100×
With 10% Serum	1,000-25,000	100×
	25,000-50,000	300×

   ④ Preparation of LDH Storage Buffer
   Prepare LDH Storage Buffer from stock solutions to a final concentration of 200 mM Tris-HCl (pH 7.3), 10% Glycerol, 1% BSA. Store at 4 °C. LDH Storage Buffer is used for diluting and freezing samples. Samples frozen in medium, or PBS will have significantly decreased LDH activity.
   (2) Protocol
   ① Set Controls
   - No-Cell Control: Set up triplicate wells without cells to serve as a negative control to determine culture medium background.
   - Vehicle-Only Cells Control: Set up triplicate wells with untreated cells to serve as a vehicle control. Add the same solvent used for test compounds to the vehicle control wells.
   - Maximum LDH Release Control (Optional): Set up triplicate wells to determine the Maximum LDH Release. Add 2 µL of 10% Triton® X-100 per 100 µL to Vehicle-Only Cells for 10–15 minutes or

longer before collecting the samples for LDH detection. Note: This control is only required if calculating percent cytotoxicity.

② Set up a 96-well assay plate containing cells in culture medium. Include wells without cells as negative control to determine background luminescence from LDH in the medium.

③ Add test compounds and vehicle-only controls to the appropriate wells. Return treated assay plates to the cell culture incubator for the duration of the treatment time.

④ Collect samples of the culture medium at the desired experimental time points by removing 2–5 µL into 48–95 µL LDH Storage Buffer. Mix well by pipetting up and down 2–3 times (without touching or disturbing cells) to ensure the sample is homogenous.

⑤ After collecting and diluting all samples, proceed to Step ⑥ or store at or below –20 °C for future assay.

⑥ On the day of the assay, thaw frozen samples and further dilute in LDH Storage Buffer (if needed) to fit the linear range of the assay. General recommendation for sample dilution is given in Step ③. Ensure that samples are equilibrated to room temperature before proceeding to Step ⑦.

⑦ Transfer 50 µL of diluted sample into a 96-well opaque-walled, non-transparent assay plate (with clear or opaque bottom).

⑧ Add 50µL of LDH Detection Reagent prepared as described in 1. (1) and 1. (2) to each well.

⑨ Incubate for 60 minutes at room temperature.

⑩ Record luminescence.

⑪ Calculate Percent cytotoxicity.

$$\text{Percent Cytotoxicity} = 100\times\frac{(\text{Experimental LDH Release} - \text{Medium Background})}{(\text{Maximum LDH Release Control} - \text{Medium Background})}$$

(3) LDH Positive Control

① Reconstitute Lactate Dehydrogenase with 275 µL of LDH Storage Buffer to make a 1,000 U/mL LDH Standard. Gently mix to dissolve and place on ice. Prepare aliquots to avoid multiple freeze-thaw cycles and store below –20 °C.

② Dilute the 1,000 U/mL LDH Standard to 3.2 U/mL by adding 10 µL LDH Standard to 3.115 mL LDH Storage Buffer.

③ Using a 12-channel reagent reservoir, further dilute the 3.2 U/mL LDH to 32 mU/mL as described in the table below, then perform serial dilutions.

Dilution #	Volume LDH (µL)	Volume LDH Storage Buffer (µL)	mU/mL
1	10µL of 3.2U/mL (Section 2.3.2)	990	32
2	500µL from Dilution #1	500	16
3	500µL from Dilution #2	500	8
4	500µL from Dilution #3	500	4
5	500µL from Dilution #4	500	2
6	500µL from Dilution #5	500	1
7	500µL from Dilution #6	500	0.5
8	0	500	0

④ Transfer 50 µL of each LDH Standard dilution (dilutions #1–8) to a 96-well assay plate in triplicate. The remainder of the plate can be used for samples.

⑤ Prepare LDH Detection Reagent as described in Section 1. (1) and 1. (2) and transfer to a reagent reservoir.

⑥ Using a multi-channel pipette, add 50 µL prepared LDH Detection Reagent to each well of a 96-well assay plate containing LDH standards.

⑦ Incubate for 60 minutes at room temperature.

⑧ Record luminescence.