Triangle Research Labs – Now Part of Lonza

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Using Cryopreserved NoSpin HepaRG™ Protocol

This protocol is suitable for the thawing of NoSpin HepaRG™ hepatic cell line. Please read through this entire protocol before attempting this procedure. The health of the cells is dependent upon following the protocol carefully.

Procedure for Thawing

1. Warm the following media in a 37°C waterbath:
    a. The HepaRG base medium plus supplement (MH100) as well as the thawing and plating additive (MSTAP).
2. Once the media is warm, pipet 7.5 mL's into a sterile 50mL conical tube all while under a laminar flow hood.
3. Take the NoSpin HepaRG (NSHPRG) vial from liquid nitrogen storage and place under the flow hood. Then quickly loosen and then retighten the cap to release any pressure.
4. Once the cap is refastened, place vial in waterbath. Make sure to not fully submerge the vial or to have any water touch the cap surface as to prevent possible contamination.
5. Move the vial slowly around the water bath for roughly 90 seconds or until you see the vial has only a small spindle remaining.
6. Remove vial from waterbath and wipe down with 70% ethyl alcohol. Pipet the contents of the vial into the 50ml conical. Use the 7.5ml's of medium to also rinse the cryovial of any remaining cells.
7. Slowly pipet the new cell stock up and down to break apart clusters of cells and to create a homogenous mixture.
8. The next step will be to add the appropriate amount of thawing and maintenance medium to your cell stock. This will depend on the well format you plan to use. Your COA will detail how much media will need to be added for each well format based upon the batch of HepaRG that you received.

Procedure for Plated Use

*Table 1: Desired Cell Density and Media Volume

Well Format	6-well	12-well	24-well	48-well	96-well
Cell Density (106cells/mL)	2.0	0.8	0.48	0.16	0.072
Media Volume (mL/ Well)	2	1	0.5	0.2	0.1

9. Using a pipette, transfer hepatocytes to a multi-well plate. Use the above table for correct volume of cell stock to add to each well.

Note: For 96-well plates, pre-seed plates with

10. Place plate in a 37°C/5% CO_2 incubator. For all plate formats except 96-well, disperse the cells by moving the plate, with your hand on top of it, parallel to the incubator shelf in a north-south, east-west motion. Note: For 96-well plates, place directly in the incubator without shaking.
11. If using an overlay, proceed to the next section. If not, replace the medium with warm pre-induction and tox medium at 6 hours for induction studies, at 24 hours for tox studies.

Table 2. Medium Volume Per Well

Plate Format	Media Volume Per Well (mL/well) – All Species
6-well	2.0mL/well
12-well	1.0mL/well
24-well	0.50mL/well
48-well	0.20mL/well
96-well	0.10mL/well

Procedure for Overlay

Overlay matrix and the medium used for its dilution should be kept at or below 4°C. Keep everything on ice when preparing and while using the overlay.

12. Calculate the volume of pre-induction and tox medium needed to feed your plate(s). This is generally 12 mL per plate. Add a few milliliters extra for an excess of overlay solution.
13. Find the protein concentration of the overlay matrix on its specification sheet. Use the formula below to determine how much overlay matrix to add to maintenance medium. We recommend a final overlay matrix concentration between 0.25 mg/mL and 0.35 mg/mL.

(Volume of medium needed X 0.3 mg/mL) / Overlay matrix concentration = Volume of overlay matrix to add

(_____ mL X 0.3 mg/mL)/ _____ mg/mL = _____ mL

14. Add the calculated amount of overlay matrix to cold medium on ice. Mix well by pipetting several times.
15. Pipet overlay solution to plated hepatocytes, following the volume/well guidelines from Table 2.

Induction Studies

1A) 72 hours after initial plating, add testing compounds to warm NoSpin HepaRG™ serum free induction medium.

2A) Aspirate pre induction medium and replace with the treated NoSpin HepaRG™ serum free induction medium according to quantities found in table 2.

3A) At 96 hours, remove old medium and replace with treated NoSpin HepaRG™ serum free induction medium.

4A) At 120 hours, remove treated medium and replace with test substrate.

Toxicity Studies

2A) At 96 hours aspirate medium and replace with NoSpin HepaRG™ pre-induction and tox medium per table 2

2B) Repeat step 2A at 144 hours

2C) at 168 hours, renew medium as in the previous two steps and incubate with test compounds specific to your assay.