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Protocol for Overlaying Monolayer

Procedure for Overlay

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

1. Calculate the volume of maintenance 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.
2. 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

$$\left(\boxed{} \text{ mL} \times 0.3 \text{ mg/mL} \right) / \boxed{} \text{ mg/mL} = \boxed{} \text{ mL}$$

3. Add the calculated amount of overlay matrix to cold maintenance medium on ice. Mix well by pipetting several times.
4. Pipet overlay solution to plated hepatocytes, following the volume/well guidelines from Table 1.
5. Incubate for at least 2 hrs. before use. Replace maintenance medium daily.

Table 1. Cell Volume Per Well

Plate Format	Cell 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	0.125mL/well