Transfection Process for RoosterGEM™ with Mirus Bio TransIT-VirusGEN®

Protocol Summary

RoosterGEM is a complete chemically defined medium that has been engineered for increased efficiency and integration of lentiviral vectors into human mesenchymal stromal cells (hMSCs) and other primary cell types, without the need for additional supplementation. It has also been engineered for increased transfection efficiency of mRNA.

While RoosterGEM functions as a stand-alone medium, it has been optimized for use with RoosterBio hMSCs expanded using RoosterNourish™-MSC-XF (part no. KT-016, protocols are available at www.roosterbio.com). The general process recommendations are outlined below.

Mirus Bio TransIT-VirusGEN® (www.mirusbio.com) is a proprietary transfection reagent originally engineered for efficient delivery of DNA to HEK 293 cell types. The unique combination of Mirus's polymer and lipid transfection technology gives VirusGEN a unique advantage over traditional polymer-based transfection reagents due to its biomimetic behavior *in vitro*. In this example, VirusGEN forms a lipopolyplex or lipid-polymer nanocomplex (LPNC) which has been shown to efficiently deliver mRNA to hMSCs.

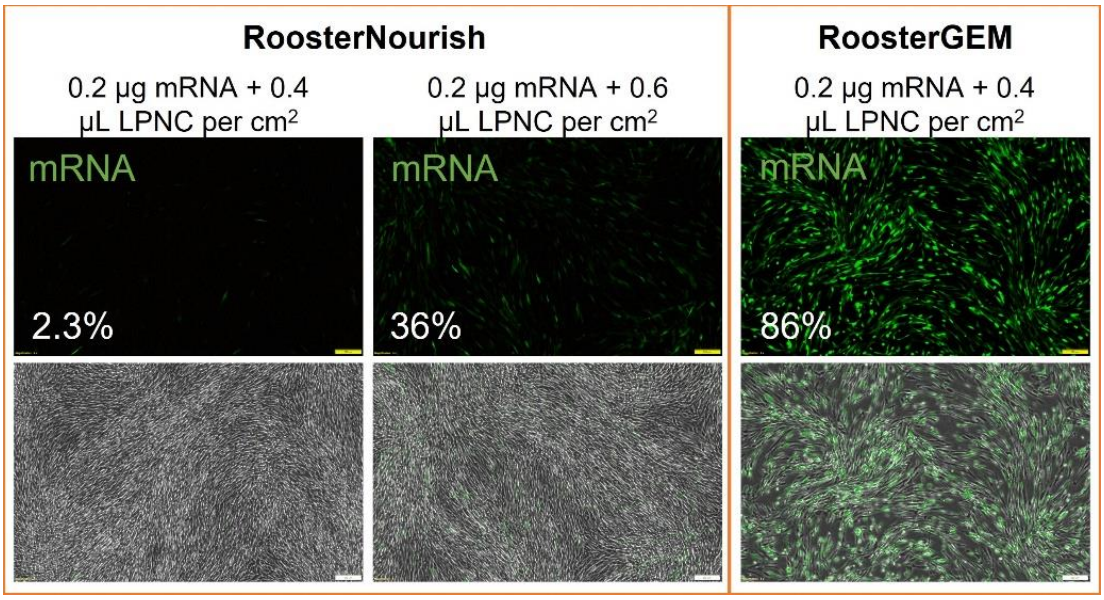


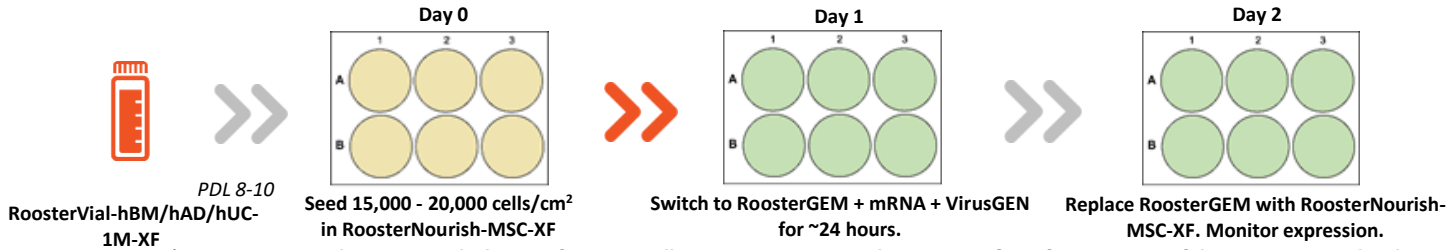
Figure: RoosterGEM increases transfection in hMSCs over other commercially available reagents.

Increased transfection efficiencies were achieved in RoosterBio's Xeno-free (XF) RoosterVial™ hMSCs (part no. MSC-031) from bone marrow-derived tissue sources. mRNA transfection was performed with CleanCap®Enhanced Green Fluorescent Protein mRNA (5-methoxyuridine) (TriLink Biotechnologies).

Lipid-polymer nanocomplex (LPNC)-mediated transfection of hMSCs on 2D culture surface

Process Overview

Cell Expansion Summary



**RoosterBio strongly recommends the use of Corning CellBIND, Vitronectin, or Fibronectin surfaces for expansion of the Xeno-Free product line.*

- Thaw and seed cells at recommended: 15,000-20,000 cells/cm² in RoosterNourish-MSC-XF and allow for proliferation for up to 24 hours.
- Incubate cultures for 16-24 hours in RoosterGEM + mRNA-complexes (0.4 ml RoosterGEM per 1.9 cm²).
- Replace with RoosterNourish-MSC-XF. Do not rinse wells.
- Monitor for expression of mRNA (~48-72 hours).

Recommended Protocol

1. Shipping and Storage

1.1. RoosterGEM is shipped frozen, and, upon receipt, promptly, store RoosterGEM at -20°C.
1.2. Before use, thaw RoosterGEM at ~2-8°C away from light until no ice remains (typically 24-48 hours).
1.3. After use, return RoosterGEM to ~2-8°C away from light for up to 2 months from initial thaw.

2. Cell Culture

2.1. For 24-well CellBIND plates, seed 15,000 to 20,000 hMSCs/cm² (29k to 38k hMSCs per well) in expansion medium (RoosterNourish-MSC-XF, part no. KT-016).
2.1.1. Note: It is recommended that 24-well plates be utilized for optimization of expression. Once optimal conditions are determined, process can be proportionally scaled accordingly (0.4 ml RoosterGEM per 1.9 cm²).
2.2. If desired, plate additional well to count cells per well prior to transfection step.
2.3. Allow cultures to proliferate for 1 day.

3. Media Preparation

3.1. Aliquot the required volume of RoosterGEM (allow for 0.5 ml per well of 24-well plate) into a separate conical tube and warm to room temperature, protected from light.
3.2. Promptly return unused RoosterGEM bottle to 2-4°C away from light.
3.2.1. Store for use up to 2 months from thaw.

4. Transfection

Note: For initial screening experiments, use 0.8 and 1.2 µL of Mirus Bio TransIT-VirusGEN®.
4.1. Count cells from a single well with a counting device.
4.1.1. Aspirate RoosterNourish-MSC-XF from a single well.
4.1.2. Add 250 µL TrypLE to one well of 24 well plate.
4.1.3. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C incubator.

4.1.4. Check culture every 5 min until cells are detached from surface.
4.1.5. Add equivalent volume of RoosterNourish™-MSC-XF to each vessel to stop the TrypLE activity.
4.1.6. Transfer the cell suspension into an appropriate centrifuge tube.
4.1.7. Count cells with a cell counting device, performing a dilution if required to get within its acceptable range.

Cells (per well)

4.2. Aspirate RoosterNourish-MSC-XF from the remaining wells.
4.3. Replace with 0.4 ml per well RoosterGEM.
4.4. Calculate the amount of reagents needed per N reactions (24-well plate):

Well Count (=N)	**Volume of DPBS for complexes** (40 µL per well of 24-well) **(V=40µl*N)**	**Amount of mRNA** (0.4 µg per well of 24-well plate) **(A=0.4 µg*N)**	**Amount of TransIT-VirusGen®** (1.2 µL per well of 24-well plate) **(B=1.2 µL*N)**

4.5. Dilute calculated amount of mRNA (A) in calculated volume of DPBS (V).
4.6. Add calculated amount of TransIT-VirusGEN (B) to diluted mRNA.
4.7. Incubate 15 minutes at room temperature.
4.8. Add 40 µL of mRNA-LNPC complexes to each well.
4.9. Incubate 16-24 hours in humidified 37°C incubator.
4.10. Aspirate RoosterGEM + mRNA-LNPC complexes and replace with 0.4 ml per well RoosterNourish-MSC-XF. Do not rinse wells.
4.11. Monitor for expression of mRNA [Typical time is 1 to 2 days].

5. Cell Harvest

5.1. Transfer vessel(s) into biosafety cabinet and remove spent medium.
5.2. Add 250 µL TrypLE to each well of 24 well plate.
5.3. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C incubator. Check culture every 5 min until cells are detached from surface. Gently tap to dislodge remaining cells from surface.
5.4. Add equivalent volume of RoosterNourish™-MSC-XF to each vessel to stop the TrypLE activity.
5.5. Transfer the cell suspension into an appropriate centrifuge tube.
5.6. Centrifuge at 350 x g for 5min.
5.7. Aspirate the supernatant and resuspend cells in medium to reach desired cell concentration. Measure the total volume of cell suspension.
5.8. Mix well and transfer 0.5 mL of cells into microcentrifuge tubes for cell counts.
5.9. Count cells with a cell counting device, performing a dilution if required to get within its acceptable range.
5.10. Cells are ready to be used in your application.

Caution to Users: RoosterBio products contain human sourced materials and should be treated as potentially infectious. Employ universal safety precautions and wear protective clothing and eyewear while handling. Practice appropriate disposal techniques per CDC guidelines for biohazardous material.