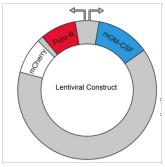

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Generation and utilization of a HEK-293T murine GM-CSF expressing cell line

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bpkkmkuw

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Extended Methods

Comprehensive Methods in the Creation of an mGM-CSF-Producing HEK293T Cell Line, Collection of mGM-CSF-rich Supernatant, and Using Cell Line Supernatant to Generate Dendritic Cells From Myeloid Progenitors

Creating Cell Line

1. Acquire mGM-CSF gene (Addgene: Plasmid #74465)
2. Sequence to check for correct composition
3. PCR mGM-CSF gene
4. Create and acquire primers with required restriction sites (NotI-HF and PspXI)
5. PCR (F:TCCGCTCGAGCCACCATGTGGCTGCAGAATTTACTTTTCC, R:GACGCGGCCGCTCATTTTTGGCCTGGTTTTTTGC) using 2X Phusion High-Fidelity PCR Master Mix (Thermo Scientific)
6. Purify/isolate mGM-CSF gene (450bp)
7. Ligation of the mGM-CSF gene with 681 bidirectional vector plasmid
8. Restriction digest 681 bidirectional vector with Not1-HF and PspXI and purify
9. Restriction digest modified mGM-CSF gene with Not1-HF and PspXI and purify
10. Ligate vector with thegene
11. Transform E. coli. with 681 + GM-CSF plasmid construct
12. Extract and isolate 681 + GM-CSF plasmid construct
13. Incubate E. coli in liquid LB
14. Miniprep
15. Colony PCR and run through the gel to determine sample with GM-CSF gene inserted in 681 vector
16. Create lentiviral constructs containing plasmid

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17. Infect HEK293 cells using lentiviral constructs
18. Select for cells with 681 + GM-CSF plasmid construct
19. Allow cells to recuperate from infection and grow to confluence
20. Puro-select
21. FACS to determine concentration of mCherry+ cells (>90% required)

Collecting mGM-CSF-rich supernatant

1. Allow for HEK293 cells with plasmid construct to grow to confluency
2. (To be redone in T-175) Plate 2 million cells per 10 cm plate
3. Incubate for 3 days
4. Collect supernatant
5. If needed to confirm the concentration of mGM-CSF in the supernatant, perform ELISA (~200 ng/ml)

****In a T-175 flask***

1. Allow for HEK293 cells with plasmid construct to grow to confluency
2. Plate 9 million cells in 50 ml DMEM per T-175 flask
3. Incubate for 3 days
4. Collect supernatant
5. If needed to confirm the concentration of mGM-CSF in the supernatant, perform ELISA (~200 ng/ml)

Generation of Bone-Marrow-Derived DCs

Day 0

1. Sacrifice one or two mice and reserve femur and tibia of mice
2. Extract bone marrow cells from femur and tibia
3. In 6 well plate, plate equal amounts of cells into each well
4. Total media should be equal to 2 ml per well (cells + DMEM + cytokine)
5. Incubate for three days

Day 3

1. On the third day of differentiation, wash off dead cells
2. Aspirate media
3. Perform PBS wash
4. Aspirate PBS
5. Replace media

Day 4 (if cells reach confluency)

1. On the fourth day of differentiation, move cells onto a 10cm plate
2. Save conditioned media
3. Perform PBS wash
4. Aspirate PBS
5. Using cell scraper, gently scrape cells off theplate
6. Transfer cells onto 10 cm plate
7. Replace media
8. Total media should be 10 ml (cells + DMEM + cytokine)
9. Incubate for 2-3 days

Day 5 or 6

1. Replace media
2. Save conditioned media
3. Perform PBS wash
4. Aspirate PBS
5. Replace media (10 ml total; 6.750 ml DMEM + 2 ml conditioned media + ~1.250 ml supernatant)
6. Incubate for 1-2 days

Day 7 or until usage before day 14

1. Replace media every 2-3 days

Cells finished differentiation. Ready for use

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