# DEVELOPMENT OF STABLE TRANSFECTED CELL LINE

## **Materials:**

trypsin-EDTA 1% low melting soft agarose (SeaPlaque® agarose) 2x DMEM + 20% FBS G418 200 mg/ml stock solution Hygromycin B 50 mg/ml stock solution Puromycin 2.5 mg/ml stock solution

## **Procedure:**

## Titrating G418, Hygromycin, and Puromycin (Kill Curves)

Since each cell line has a different sensitivity to G418, hygromycin or puromycin, you should determine the optimal concentration of drug for selection.

- 1. Split confluent cells 1:5 in 10 ml DMEM + 10% FBS media.
- 2. Transfer 0.5 mL cell suspension into 24-well plate containing 500 μl of (media + drug).
- 3. Use the lowest concentration of drug that begins to give massive cell death in 3 days and kills all the cells within two weeks. Clontech recommends 400µg/ml G418 for HeLa cells and 200µg/ml hygromycin for CHO cells. In mammalian cells the optimal level of puromycin is typically around 1 mg/ml. I have found HeLa to be selectable with 500µg/ml G418, 500µg/ml hygromycin, and 2.5 µg/ml puromycin, and SHSY-5Y cells to be selectable with 600µg/ml G418 and 200µg/ml hygromycin.

Volume of Stock Solution Added (µl)		Final Concentration
G418	Hygromycin B	(µg/ml)
0	0	0
0.25	1	50
0.5	2	100
0.75	3	150
1	4	200
1.5	6	300
2	8	400
2.5	10	500
3	12	600
3.5	14	700
4	16	800
5	20	1 mg/ml

Volume of Puromycin Stock Solution Added (µl)	Final Concentration (µg/ml)
0	0
0.2	0.5
0.4	1
0.6	1.5
0.8	2
1	2.5
1.2	3
1.6	4
2	5
3	7.5

# **Transfection and Drug Selection**

- 1. Grow cells to ~80% confluence in complete medium and transfect your plasmid with appropriate method, for example LipofectAmine Plus (Invitrogen) is a good method for HeLa cells.
- After 24-48 hours of transfection, cells are split to 1:10, 1:20 or 1:50 into (2) 15 cm plates containing 25 ml of DMEM + 10% FBS + appropriate concentration of drug. Leftovers of transfected cells can be plated in 10cm plates containing 10 ml media. If you use Tet-OFF system, add 5 µg/ml of tetracycline or 1 µg/ml of doxycycline to shut off the protein expression.
- 3. Observe cell growth in every 2-3 days and change medium with selection drug every week or more if necessary. After 2–4 weeks, isolated colonies should begin to appear.

# **Isolation of Drug Resistant Clones**

- 4. Melt 1% low melting agarose by microwave and incubate in a water bath at 37°C until cool down below 37°C. Mix 10 ml of 1% agarose and 10 ml of 2x DMEM + 20% FBS (and doxycycline if necessary) and pour into 15 cm plate (media removed). Leave the plate at RT for less than 30 min. Put the plate into a CO<sub>2</sub> incubator for refreshment.
- 5. Mark large, healthy and well separated colonies and put colony separator (see figure) directly on surface of soft-agarose. Apply gentle pressure to top of separator to prevent movement.
- 6. Add 100µl of trypsin-EDTA, pipeting several times gently to penetrate the soft-agarose surrounding the isolated colony and incubate at RT.
- 7. Place trypsin treated colony into one well of a 48-well plate containing 1 ml of DMEM + 10% FBS + drug.

- 8. Split clones that reach ~80% confluence into one 12-well plate and one 6-well plate. Use one plate for checking protein expression/induction. Check the protein expression of each clone by its fluorescent intensity under a fluorescent microscope or by Western blotting.
- 9. Split only expression positive clones to 10 cm plate and store in liquid N2.
- 10. For sub-cloning, re-plate about 100 cell per plate. For example, immediately after splitting, take 10-100 μl of culture and re-plate in 10 cm plate.
- 11. Repeat steps 5-11. You may pick 6 colonies to check for protein expression.

#### **Comments:**

Since SeaPlaque agarose starts to polymerize at 26-30°C, mixing and pouring should be done quickly.

It is not good to keep the cells at room temperature longer than 30 min. Polymerization will continue in the incubator so place your cells inside even if agarose is not polymerized completely.

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