

4.3 Cell Culture

Standard animal cell culture techniques are used with adaptations for fish cell lines when necessary i.e., incubation temperature. Normal appearing cultures composed of rapidly dividing cells will be used for all assay work. Cells will be routinely subcultured to maintain healthy cells, approximately once every two weeks for most cell lines, or split weekly for seeding plates. Aseptic technique is required when working with any cell line. Only one cell line is worked with at a time (Freshney 1983; Jakoby and Pastan 1979; Lannan 1994; Merchant et al., 1964; Rovozzo and Burke 1973; True 2000).

A. Subculture Procedures for Flasks

1. Suggested split ratios and seeding rates are given in Table 4.2.
2. Remove tissue culture medium by decanting fluid.
3. Slowly add trypsin-versene (EDTA) (Section 2, 4.9.E “Trypsin-Versene (EDTA)”) solution and rock the flask gently for one minute. In a 75 cm² flask a volume of 3 to 4 mL is sufficient to coat cells.
4. Decant again.
5. Carefully observe the cell layer and repeat steps 3 and 4 as necessary until cells start detaching from flask. Dislodging of the cells may be completed by sharply striking the edge of the flask against the heel of a hand.
6. Add tissue culture medium to neutralize the trypsin. In a 75 cm² flask, 10 mL is sufficient.
7. Triturate to break up cell clumps and add an appropriate volume of fresh tissue culture medium for transferring to other flasks. Enumeration of cells in the suspension may be done at this time to determine the necessary volume to transfer (Section 2, 4.A2 Cell Enumeration Appendix 2)
8. Aspirate and dispense into new flasks. The sub-cultivation ratio is generally 1:4 to 1:6. Following manufacturers recommendations, bring total volume in each new flask up to the acceptable level with the appropriate tissue culture medium. For a 75 cm² flask, this will usually be about 20 mL. MEM-10/Hepes (Section 2, 4.9.G “MEM-10/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)”) works well in an open system for all cell lines listed in Table 4.2 except SHK-1 and ASK, which do best with L-15 (Section 2, 4.9.H “Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines)”).
9. Incubate flasks at room temperature (20 to 25°C) until they reach confluence and then held at the appropriate temperature for that cell line.
 - a. SHK-1, ASK and CHSE-214 cells should be incubated at 15°C.
 - b. WSS-2 cells should be incubated at 20°C.

- c. FHM and BF-2 cells should be incubated at 25°C (25 to 30°C for LMBV).
- d. EPC cells may be incubated at 15 to 25°C.

Table 4.2. Seeding guidelines for the subculture of fish cell lines.

<u>CELL LINE</u>		Suggested Seeding Rate (per cm ²)	<u>INCUBATION TEMP (°C)</u>	
Common Name	Nominal split Ratio		Suggested	Range
BF-2	1:2 - 3	100,000	25 - 30	20 - 30
CHSE-214	1:3 - 6	100,000	15 - 20	4 - 27
EPC	1:3 - 6	250,000	15 - 25	15 - 30
FHM	1:4 - 6	250,000	25 - 30	10 - 36
WSS-2	1:4 - 8	150,000	20 - 25	20 - 30
SHK-1	1:2 - 3	250,000	15 - 20	15 - 20
ASK	1:2 - 3	250,000	15 - 20	15 - 20

B. Seeding Procedures for Plates

1. Monolayers on plates are prepared approximately 24 to 48 hours prior to inoculation with the sample.
2. A flask of visually healthy cells approximately 7 to 10 days old is selected and trypsinized as described previously. After neutralizing the trypsin with tissue culture medium, the total volume in the flask is adjusted to provide a cell concentration appropriate for the seeding rate listed in Table 4.2 and the area of the wells to be seeded. The appropriate volume of the cell suspension is then pipetted into each well of the plate.

Example: When seeding EPC cells in a 24-well plate, 0.5 mL of a 1×10^6 cells per mL suspension is dispensed per well.

3. At a minimum, control wells are included in each plate set and should be included on each plate whenever possible. Control wells are made by dispensing tissue culture medium into plate wells that contain normal looking cell monolayers that have not been inoculated with a sample. These wells are observed during the incubation along with the sample wells for abnormalities that may arise due to media or cell problems. A plate set refers to the group of plates seeded from a single flask at the same time.
4. Incubate plates overnight at room temperature (approximately 20 to 25°C).