Protocols for Gene Screening with Single Embryonic Stem (ES) Cell-Derived Embryoid Bodies (EBs)

1. Culture of Undifferentiated ES cells

A. Preparation of Medium and Reagents
   • Cell Lines
     Pluripotent Embryonic Stem Cell: ES-D3 (ATCC, cat no. CRL-1934, Mouse 129/Sv+c/+p)
     Feeder Layer Cell: STO (ATCC, CRL-1503, Mouse SIM Fibroblast)
   • Complete Media for Undifferentiated ES Cells
     KnockOut DMEM (Gibco) containing 10% FBS (HyClone), 1 mM Gluta-Max (Gibco), 100 µM \( \beta \)-mercaptoethanol (Gibco), 1% MEM nonessential amino acid (Gibco), 1 mM sodium pyruvate (Gibco), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco), 12.5 mL of 1 M HEPES buffer (Biosource, cat. no. P305-100).

B. Maintenance of ES Cells in Undifferentiated State
   • STO as feeder layer cells: to block cell proliferation, use Mitomycin-C to treat STO at 0.01 mg/mL for 2.5 h.
   • LIF only (2000 U/mL)
   • STO and LIF combined: monolayer mitomycin-C treated STO cells can be used with 1000 U LIF.

2. Differentiation of ES Cells in Suspension Using a Shaker

A. Preparation of Differentiation Medium
   KnockOut DMEM (Gibco) containing 20% FBS (HyClone), 1 mM Gluta-Max (Gibco), 100 µM \( \beta \)-mercaptoethanol (Gibco), 1% MEM nonessential amino acid (Gibco), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco), 12.5 mL of 1 M HEPES buffer.

B. Formation of Single ES Cell-Derived EBs in Suspension
   Undifferentiated ES cells were completely dissociated, then count the cell number. Single ES cells were then placed at the density of 1000–1500 cells/well of a 6-well ultra low attachment suspension plate. The plates were placed on a titer plate shaker (Lab-Line Instruments) at a speed of approximately 120 rpm or as indicated on the controller (1.25 U). Cells were cultured in this condition for 3 days and carefully observed.

C. Extended EB Culture on Gelatin-Coated Dishes or Plates
   After 3 days culture in a shaker, uniformed cystic-like EBs can be observed. Transfer approximately 200–250 EBs/100-mm 0.2% gelatin-treated dish and allow them attach on the dishes by incubating overnight without disturbance. The following day, you will observe uniformed EBs attached on the dishes. Maintain these EBs by changing fresh media containing 20% FBS LIF-free, every other day. On day 12 (differentiation age, i.e., 3 days in suspension, 9 days in dishes), carefully observe EB size and patterns and perform immunochemical or immunofluorescent staining using specific markers (i.e., PECAM-1 or troponin T).
3. Differentiation of ES Cells Using Shaker and Spinner Flask

A. Procedure for Spinner Flask Preparation
Wash clean spinner flasks with excessive Milli-Q®-Plus water (Millipore) and dry for 1 h at 60°C. Siliconize the spinner flasks by moistening the interior with 10 mL Sigmacoat. Excessive Sigmacoat is removed from the flasks using a 10-mL glass pipet. Dry the silicon coat in an oven for 1 h at 120°C. Rinse spinner flasks three times with 250 mL Milli-Q-Plus water and autoclave subsequently. Moisten the interior of the flasks with 20 mL complete Iscov’s medium.

B. Formation of Single ES Cell-Derived EBs in Suspension with Spinner Flask
Follow the procedure of 2A and 2B. After 3 days culture in a shaker suspension, carefully remove EBs from dishes and put them into a spinner flask (approximately 2500 EBs/250-mL spinner flask). Incubate EBs for 4 more days at the speed of approximately 20–40 rpm. The stirring direction is reversed every 1440°. Fresh media was replaced every day (half of the media was removed and replaced with fresh media).

C. Extended EB Culture on Gelatin-Coated Dishes or Plates
After 3 days cultured in a shaker and 4 days in a flask, transfer approximately 250 EBs/100-mm 0.2% gelatin-treated dish and allow them attach on the dishes by incubating overnight without disturbance. The following day, uniformed EBs should attach to the dishes. Maintain these EBs by changing fresh media containing 20% FBS LIF-free every other day. On day 9 or 10 (differentiation age, i.e., 3 days in suspension, 4 days in spinner, and 2–3 days in gelatin dishes), carefully observe EB size and patterns and perform immunostaining using specific markers (i.e., PECAM-1).

4. Immunofluorescent Staining of ES Cell-Derived EBs Without Fixation
EBs were cultured and differentiated using a standard method as described previously. On the day of staining, wash the EBs once with 1× PBS or complete KnockOut-DMEM containing 20% FBS. Monoclonal antibody was diluted 1:1000 from the original stock with PBS or KnockOut-DMEM containing 20% FBS and directly applied to EBs in the volumes of 250 µL for 48-well and 5 mL for 100-mm dishes. (Note: Do not warm up the media before use—at room temperature or even colder.) Wrap the plate with aluminum foil to avoid light exposure, and incubate the EBs on a shaker placed in a cold (4°C) room for 1.5–2 h, then carefully observe cells under fluorescence microscope. If the staining was appropriately performed, wash cells with media twice and move them into 37°C. Do not keep the EBs in the cold room for more than 2 h.

5. Representation of Lentivirus-Based Gene Trap Vector and Integration