

Title: Cryopreservation of hPSCs using CryoStor CS10

Purpose: To describe standard procedure for maintaining hPSCs

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Media and Supplies

Item	Purpose	Storage	Catalog Number	Supplier
CryoStor CS10	Specialized protein-free cryopreservation medium	2–8°C	07930	STEMCELL
Accutase	Gentle enzymatic dissociation into single cells	-20°C (Aliquots)	10761-312	VWR
mTeSR Plus	Primary culture medium for preparation and recovery	2–8°C	100-0276	STEMCELL
DPBS (-/-)	Ca ²⁺ /Mg ²⁺ free wash buffer	RT	D8537-500ml	Sigma
Advanced DMEM/F12	Washing and quenching dissociation enzymes	2–8°C	12634010	Fisher
Penicillin-Streptomycin	Optional antibiotic	-20°C	15-140-122	Fisher
Trypan Blue (0.4%)	Cell viability counting	RT	15250061	Thermo
Cryovials	2 ml sterile vials for long-term storage	RT	66021-987	VWR
15 ml Conical Tubes	Centrifugation and cell collection	Sterile	21008-936	VWR
50 ml Conical Tubes	Pooling large volumes of cell suspension	Sterile	21008-940	VWR
Serological Pipettes	Sterile liquid handling (5 ml or 10 ml)	RT	76184-746	VWR
Pipette Controller	For use with serological pipettes	RT	13-681-161	Fisher
Controlled Rate Freezer	Isopropanol container (e.g., "Mr. Frosty")	RT	5100-0001	Thermo
Countess Cell Counter	Automated viable cell counting	RT	A50298	Fisher
Inverted Microscope	Culture assessment before harvest (EVOS)	RT	LMI3PH2	Life Tech
Nitrile Exam Gloves	Personal safety	RT	99452683	Fastenal
Permanent Marker	Labeling cryovials with cell line and date.	RT		

Cryopreservation

This protocol is optimized for use with CryoStor CS10, a specialized, protein-free cryopreservation medium designed to maintain high viability during the freezing and thawing process. While CryoStor CS10 is the primary recommendation for this workflow, other options such as STEM-CELLBANKER or a combination of ES-cell qualified FBS with culture medium (e.g., mTeSR Plus) may be suitable alternatives depending on your specific experimental requirements or laboratory situation.

Preparation: Prior to harvesting, confirm that iPSC cultures have reached 70 - 80% confluency and exhibit minimal to no differentiation. Keep CryoStor CS10 in the 4°C refrigerator until just prior to use. Ensure the controlled-rate freezing container is at room temperature and the isopropanol is fresh. Label cryovials with cell line name, passage, date, concentration, and initials. **Note:** If differentiated cells are present, consider delaying cryopreservation and instead perform a "cleanup" passage using a conservative application of ReLeSR (minimal incubation) to selectively detach healthy undifferentiated colonies while leaving differentiated cells behind, to ensure that an optimal population of pluripotent cells are advanced to the cryopreservation stage.

Single cell dissociation: Refer to the Single-Cell Passaging (using Accutase) protocol for technical details. **Briefly:** For one well of a 6-well plate, completely aspirate media from cells, wash 2x with 1 ml DPBS (no Ca²⁺, no Mg²⁺). Add 1 ml pre-warmed Accutase and incubate at 37°C for 5–10 minutes until cells round. Tap plate firmly on each side, to detach cells. Gently pipette up and down 5x to singularize, transfer to a conical tube with 9 ml Advanced DMEM/F12 to dilute the enzyme out. **Note:** If scaling up e.g., freezing down an entire plate, you can pool the 6 ml of cell suspension into a 50 ml falcon containing roughly 44 ml of Advanced DMEM/F12.

Counting and Calculation: Ensure the cell suspension is thoroughly mixed in the quench media. Remove 100 µl of the suspension to a small tube containing 100 µl of Trypan Blue, gently pipette up and down a few times to mix, and load 10 µl of the mixture onto a Countess slide. Using the Countess cell counting SOP, determine the total viable cell count. Finally, calculate the volume of chilled CryoStor required to reach a final concentration of 1.0×10^6 cells/ml.

Pellet and Resuspend: Centrifuge the suspension for 5 minutes at 200 x g (RCF). Aspirate the supernatant completely, taking care not to disturb the cell pellet. Tap the bottom of the tube firmly to flick-loosen the pellet before adding the predetermined volume of CryoStor. Gently pipette up and down several times to ensure the cells are nicely mixed and then distribute 1 ml (1×10^6 cells) to each cryotube. Ensure lids are screwed on tightly, place cryotubes in a controlled rate freezer, and then place the controlled rate freezer and cells in -80°C overnight. The following day, transfer the vials to LN2 for long-term storage.