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Cell Lines	HEK293T, CHO-K1	**Biosafety**	BSL-2

Purpose & Scope

This protocol establishes standardised procedures for the routine culture, passage, and cryopreservation of HEK293T and CHO-K1 mammalian cell lines used in transfection and recombinant protein production studies at Meridian BioSciences. All personnel working with these cell lines must read this document before initiating any experiments and must complete the mandatory BSL-2 induction training administered by the Cell Biology Core Facility. For lentiviral transduction procedures, refer to SOP–CELL–012.

1 Safety & Biosafety Requirements

Both HEK293T and CHO-K1 cell lines are classified **BSL-2**. HEK293T cells contain stably integrated adenovirus sequences. All work must be performed inside a certified Class II Biosafety Cabinet. Liquid biological waste must be autoclaved at 121 °C / 15 psi for 30 min before disposal.

- Lab coat (fluid-resistant, long sleeve)
- Nitrile gloves – double-glove when handling cryovials
- Safety glasses or face shield
- Closed-toe shoes; no sandals permitted
- Change gloves immediately on contamination or tear

Chemical Hazards

- **DMSO:** penetration enhancer – avoid skin contact; use in fume hood.
- **Trypan Blue:** potential carcinogen – dispose as chemical waste; do not mouth-pipette.
- **Liquid Nitrogen:** cryogenic burns and asphyxiation risk – use insulated cryo-gloves and work in ventilated area; never seal LN_2 in airtight containers.
- **Ethanol (70%):** flammable – keep away from open flame.

2 Materials & Reagents

2.1 Equipment

#	Equipment	Specification	Notes
1	Class II Type A2 Biosafety Cabinet	Apex SafeGuard 1200	UV decontaminate 30 min prior
2	CO_2 Incubator	Vertex ThermoCell 180	37 °C, 5% CO_2, humidified
3	Inverted phase-contrast microscope	Nimbus OptiScope E100	4×, 10×, 20× objectives
4	Centrifuge	Synapse SpinPro 5000	Swing-bucket rotor, $\leq 1\,200 \times g$
5	Haemocytometer + cover slip	Neubauer improved	Clean with 70% ethanol
6	Automated cell counter	Nexa CellVision™	Trypan blue exclusion mode
7	Water bath	Vertex AquaTherm 37W	Maintain at 37 °C
8	Liquid nitrogen storage dewar	Apex CryoStore LN200	-196 °C
9	Pipette controller + serological pipettes	Various (2 mL – 25 mL)	Sterile, individually wrapped
10	T-25, T-75, T-175 flasks	Meridian CellGrade™	Vented caps, TC-treated surface

2.2 Reagents

#	Reagent	Catalogue No.	Storage	Working Conc.
1	DMEM, high glucose (4.5 g/L)	MRD–DMEM-HG	4 °C	Supplemented
2	Ham's F-12K medium	MRD–F12K	4 °C	Supplemented
3	Foetal Bovine Serum (FBS), heat-inactivated	MRD–FBS-HI	−20 °C, aliquots	10% (v/v)
4	Penicillin–Streptomycin (100×)	MRD–PS-100	−20 °C	1× (1%)
5	L-Glutamine, 200 mM	MRD–GLN-200	−20 °C	2 mM final
6	Trypsin–EDTA (0.25%)	MRD–TRYP-025	−20 °C	0.25% (as supplied)
7	Dulbecco's PBS, Ca^{2+}/Mg^{2+}-free	MRD–DPBS-00	RT	Undiluted
8	Trypan Blue, 0.4%	MRD–TB-04	RT	1:1 dilution with cells
9	DMSO (cell-culture grade)	MRD–DMSO-CG	RT, dark	10% (v/v) in freeze media
10	CryoStor CS10 freezing medium	MRD–CS10	4 °C	As supplied

Media Preparation Note

Prepare complete growth media by supplementing basal medium with 10% FBS, 1% Penicillin–Streptomycin, and **2** mM L-Glutamine. Warm to 37 °C before use. Prepared media is stable for 4 weeks at 4 °C. Label every bottle with the preparation date, cell line designation, and operator initials.

3 Procedure A: Cell Passage (Subculture)

Total time: **30–45 min**
Frequency: Every **2–3 days** (HEK293T) or **3–4 days** (CHO-K1)
Best performed: **morning**, to allow equilibration

Subculture at **70–80% confluency** only. Over-confluent cells lose contact inhibition and transfection efficiency.
Never allow HEK293T to exceed 90% confluency.

3.1 Preparation

1 **Warm reagents.** Place complete growth medium, DPBS, and trypsin–EDTA in the 37 °C water bath for at least 20 min before use. Cold trypsin acts slowly and causes cell clumping.

2 **Decontaminate the biosafety cabinet.** Wipe down all interior surfaces with 70% ethanol and allow to evaporate for 2 min. Switch on UV lamp for 15 min if the cabinet has been idle > 1 h. Never expose cells or open media to UV.

3 **Inspect cells by microscopy.** Using an inverted microscope at 10× magnification, confirm confluency (**70–80%**), check for morphological abnormalities, and confirm the absence of contamination (turbid media, unusual granularity, floating debris).

3.2 Trypsinisation

4 **Aspirate spent medium.** Using a sterile serological pipette connected to the vacuum line, carefully aspirate the conditioned medium from the flask. Tilt the flask at a low angle to collect residual medium without disturbing the cell monolayer.

5 **Wash with DPBS.** Add **5** mL (T-25) or **10** mL (T-75) of Ca^{2+}/Mg^{2+}-free DPBS. Rock gently to cover the entire monolayer, then aspirate completely. This removes residual FBS that would inhibit trypsin activity.

6 **Add trypsin–EDTA.** Pipette **1** mL (T-25) or **3** mL (T-75) of 0.25% trypsin–EDTA evenly over the cell monolayer. Return the flask to the incubator.

HEK293T: 2–3 min at 37 °C (loosely adherent – check every 60 s).
CHO-K1: 4–6 min at 37 °C (more adherent – gentle tap if needed).
Do not exceed 5 min (HEK293T) or 8 min (CHO-K1) to avoid cell damage.

7 **Monitor detachment.** Check under the microscope every 60 s. When > 90% of cells are rounded and floating, remove the flask from the incubator. Gently tap the side of the flask once or twice to dislodge remaining adherent cells. **Do not vortex.**

8 **Neutralise trypsin.** Add **4** mL (T-25) or **10** mL (T-75) of complete growth medium containing 10% FBS immediately. Pipette up and down 3–4 times to create a single-cell suspension. Transfer the entire volume to a 15 mL conical tube.

3.3 Cell Counting & Reseeding

9 **Centrifuge.** Spin at **300** × **g** for 5 min at room temperature. Carefully aspirate the supernatant without disturbing the cell pellet.

10 **Resuspend the pellet.** Add **1** mL of fresh complete medium. Pipette gently to disperse the pellet into a uniform single-cell suspension.

11 **Count viable cells.** Mix **10** μL of cell suspension with **10** μL of 0.4% Trypan Blue. Load **10** μL

into a Neubauer haemocytometer. Count viable (clear) and non-viable (blue) cells in all four outer quadrants. Calculate:

$$\text{Viable cells/mL} = \frac{\text{viable cell count}}{4} \times 2 \times 10^4$$

Target viability: **> 95%**. If viability is < 85%, investigate for contamination or suboptimal culture conditions before continuing.

12 **Reseed into new flasks.** Dilute the cell suspension to the recommended seeding density in pre-warmed complete medium and transfer to a new flask:

Cell Line	Split Ratio	Seeding Density	Flask
HEK293T	1:4 – 1:10	3–5 × 10^4 cells/cm^2	T-75
CHO-K1	1:3 – 1:6	2–4 × 10^4 cells/cm^2	T-75

13 **Label and incubate.** Label the flask with: cell line, passage number, date, operator initials, and complete medium formulation. Place upright in the incubator at 37 °C, 5% CO_2. **Do not stack flasks.**

Passage Record

Record each passage in the Cell Line Logbook (Form MRD-CL-003). Entry must include: date, flask ID, passage number, cell count, viability, split ratio, and operator signature. Maximum recommended passage number: **P30** (HEK293T) / **P25** (CHO-K1). Discard and thaw a new vial from the master cell bank beyond these limits.

4 Procedure B: Cryopreservation & Thawing

4.1 Cryopreservation

Total time: **60 min** active + overnight slow-freeze
Optimal harvest: **log-phase cells at 70% confluency**
Target: **2–5 × 10^6 cells/vial**

Use only **certified cryogenic vials** (1.8 mL, external thread).
Never use standard 1.5 mL Eppendorf tubes. DMSO is cytotoxic at RT – proceed quickly after addition.

1 **Harvest cells.** Perform Steps 1–10 of Procedure A (passage). After centrifugation, aspirate all supernatant and resuspend the pellet in **1** mL of ice-cold CryoStor CS10 freezing medium per 2.5×10^6 cells. Keep on ice throughout.

2 **If preparing DMSO-based freeze medium manually,** dissolve 10% (v/v) DMSO in complete growth medium pre-chilled to 4 °C. Add DMSO *dropwise* to medium – never add medium to neat DMSO. The mixture will warm; chill again before use. **Prepare fresh on the day of use.**

3 **Aliquot into cryovials.** Pipette **1** mL of cell suspension per cryovial. Close caps firmly. Label each vial with: cell line, passage number, date, operator, vial number, and total vials in the batch (e.g. “3 of

8”).

4 **Initiate controlled-rate freezing.** Place vials in a pre-chilled isopropanol Mr Frosty™-style container at -20 °C for 30 min, then transfer to -80 °C overnight ($\approx$16 h). This achieves the optimal -1 °C/min cooling rate.

Transfer vials to liquid nitrogen storage within **24 h** of placing at -80 °C. Vials left at -80 °C for $>$ 2 weeks suffer accelerated ice crystal damage and significantly reduced post-thaw viability.

5 **Transfer to liquid nitrogen.** Using insulated cryo-gloves and face shield, place vials into a labelled cryobox and submerge in the liquid nitrogen dewar. Record vial location (box, row, column) in the cryogenic inventory database (Meridian LIMS – Module: CryoStore).

4.2 Thawing

Total time: **20–30 min**. Thawing and dilution must be performed rapidly. Prolonged exposure to DMSO at 37 °C is highly cytotoxic.

1 **Pre-warm media and equipment.** Place **9** mL of complete growth medium in a 15 mL conical tube in the 37 °C water bath. Prepare a T-25 flask with **4** mL of complete medium at 37 °C.

2 **Retrieve cryovial from liquid nitrogen.** Wearing insulated cryo-gloves and face shield, remove the vial from the dewar. Note the vial ID against the inventory. Carry the vial in a dry ice bucket to the biosafety cabinet – do not allow premature thawing in transit.

3 **Rapid thaw.** Place the cryovial in the 37 °C water bath. Agitate gently by hand. Thaw until only a small ice crystal remains (approximately **60–90 s**). Wipe the exterior of the vial with 70% ethanol and transfer to the biosafety cabinet.

4 **Dilute dropwise.** Transfer the cell suspension *dropwise* with a 1 mL pipette into the pre-warmed **9** mL medium in the conical tube. This 1:9 dilution reduces the DMSO concentration from 10% to $\approx$1%, preventing osmotic shock.

5 **Centrifuge and remove DMSO.** Spin at **300** $\times$ **g** for 5 min. Aspirate the supernatant completely. Resuspend the pellet in **1** mL fresh complete medium.

6 **Count viable cells.** Perform trypan blue exclusion count as in Procedure A Step 11. Expected post-thaw viability: **$>$ 85%**. If viability is $<$ 70%, inspect records for incorrect freezing procedure or temperature deviations.

7 **Plate and equilibrate.** Transfer the cell suspension to the pre-warmed T-25 flask. Distribute medium evenly. Place in the incubator. **Do not disturb for 24 h.** Change medium after 24 h to remove any residual DMSO and dead cells.

8 **Record thaw event.** Log the thaw date, operator, vial ID, post-thaw viability, flask ID, and passage

number ($P_{\text{stock}} + 1$) in the Cell Line Logbook and LIMS.

Cell Bank Hierarchy

Master Cell Bank (MCB): Vials from P3–P5, stored in two separate LN_2 locations. Access restricted to Core Facility Manager only.

Working Cell Bank (WCB): Vials from P5–P10, available to authorised users. Replenished from MCB when fewer than 5 vials remain.

Experimental stocks: Vials at P> 10 for daily experiments. Do *not* use for generating new bank stocks.

5 Troubleshooting

Observation	Likely Cause	Corrective Action
Turbid medium; pH drop (yellow) within 24 h	Bacterial or yeast contamination	Discard flask in biohazard bag. Autoclave waste. Investigate media batches and cabinet HEPA filter.
Floating strand-like structures in medium	Fungal contamination	Discard flask. Check water bath for fungal growth. Ensure media caps are not left loose.
Cells detach spontaneously without trypsin	Over-confluency; inadequate CO_2; old medium	Confirm incubator CO_2 set-point (5%). Increase passaging frequency.
Low post-thaw viability (< 70%)	Rapid freeze rate; high passage number; DMSO toxicity	Verify $-1\,^{\circ}$C/min cool rate. Confirm DMSO added on ice. Discard vials > P30.
Poor adherence after thaw	Residual DMSO; damaged surface protein	Ensure DMSO thoroughly removed by centrifugation. Pre-coat flask with 0.1% gelatin if needed.
Cell morphology change (elongated, irregular)	Mycoplasma contamination (most common); passage drift	Submit cells for mycoplasma PCR (Form MRD-QC-011). Do not share reagents between cell lines.
Inconsistent growth rate between passages	Inaccurate cell counts; pipetting errors	Recalibrate counter against haemocytometer. Use wide-bore tips for gentle pipetting.
Trypsin fails to detach cells within 8 min	Over-growth; cold trypsin; FBS not removed	Warm trypsin to 37 °C. Wash twice with DPBS to remove all FBS traces.

Mycoplasma Testing Schedule

All cell lines must be tested for mycoplasma contamination upon receipt, after thawing from any bank, and **at least every 3 months** during active culture. Approved testing method: PCR using the MRD–MYCO-PCR kit (Meridian BioSciences internal). Results must be filed in LIMS and a physical copy retained in the project notebook.

6 Quality Control & Acceptance Criteria

Parameter	Method	Specification	Frequency
Cell viability (fresh culture)	Trypan Blue	$\geq 95\%$	Every passage
Cell viability (post-thaw)	Trypan Blue	$\geq 85\%$	Every thaw
Doubling time – HEK293T	Cell count	22–26 h	Monthly QC
Doubling time – CHO-K1	Cell count	16–22 h	Monthly QC
Mycoplasma status	PCR	Negative	Quarterly
Sterility (media)	Visual + culture	No turbidity / growth	Each new batch
Passage number	Logbook	$\leq$ P30 (HEK293T) / P25 (CHO-K1)	Each passage

Document Approval Signatures

Role	Name	Date
Author (Cell Biology Core)	Dr. Sara Lindqvist	05 Mar 2025
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