

TRIDENT

PENN STATE iGEM 2026

Protocols Handbook

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01 SECTION

EV Production



Yeast EV Production (20 L Batch)

Introduction

Produce one well-characterized, sterile batch of *S. cerevisiae* extracellular vesicles (EVs) from a 20 L culture, clean and concentrated enough to supply every arm of the engineering and cytotoxicity experiments from a single batch. Target late log (OD_{600} 4–6), harvested before the diauxic shift. Deliverable: ≥ 5×10^{12} particles in 0.22 µm-filtered PBS, aliquoted at −80 °C.

Materials

Reagents

- *S. cerevisiae* working stock (fresh colony ≤ 2 weeks old)
- YPD (1% yeast extract, 2% peptone, 2% glucose)
- SD minimal (0.67% YNB w/o amino acids, 2% glucose, + auxotrophic supplements; 0.22 µm-filtered)
- Sterile 0.22 µm-filtered PBS
- Methylene blue (0.01% in citrate) for viability
- Trehalose (cryoprotectant)

Equipment

- 6 L baffled flasks × 8 (or 2.8 L × 20)
- Floor centrifuge, 4×1 L or 6×1 L buckets, 4 °C
- 5 µm / 1.2 µm prefilter + 0.45 µm PES capsules
- TFF system, 100 kDa MWCO hollow-fibre
- Ultracentrifuge, rotor ≥ 100,000×g, polycarbonate tubes
- NTA, micro-BCA, TEM

Procedure

Culture

1. **Day −0** — Streak the working stock onto YPD agar; 30 °C, 48 h (plate ≤ 2 weeks old).
2. **Day −1 AM** — Pick one colony into 5 mL YPD (15 mL tube); 30 °C, 200 rpm, 8–10 h.
3. **Day −1 PM** — Inoculate 250 mL YPD (1 L baffled flask) with 100 µL starter; 30 °C, 200 rpm, 14–16 h.
4. **Day 2 PM** — Measure seed OD_{600}; compute seed volume; inoculate eight 6 L baffled flasks each with 2.5 L pre-warmed YPD; 30 °C, 180–200 rpm. Log the inoculation time.

   CRITICAL Use V_seed = 125 / OD_starter mL per 2.5 L flask to hit OD_{600} 0.05. A 6 L baffled flask is required for 2.5 L (≤40% fill); with 2.8 L flasks run 1 L × 20.
5. **Day 3 AM** — Harvest at late log. OD_{600} check at 8 h then hourly; stop at OD_{600} 4–6.

   CRITICAL Viability by methylene blue must be ≥ 95%. Below that, lysis debris dominates the prep and the batch is not usable downstream.
6. Pellet all 20 L at 4,000×g, 20 min, 4 °C. Discard YPD (Route B) or keep as conditioned medium (Route A).

Conditioning (Route B, recommended)

7. Wash the pooled cell pellet ×2 in sterile PBS (4,000×g, 10 min, 4 °C) to remove carried-over YPD particulate.
8. Resuspend washed cells in 10 L SD minimal (2× original density, half the volume); incubate 30 °C, 180 rpm, 6 h; log start/stop.

   NOTE Re-check viability ≥ 95% at the end — this is the evidence the vesicles are secreted, not lysis-derived.
9. Pellet cells 4,000×g, 20 min, 4 °C. Keep the supernatant = conditioned medium (CM).

Clarification & Filtration

10. Clarify: 4,000×g 20 min → 15,000×g 30 min (debris, cell-wall fragments). Decant carefully, leaving the last ~5%.
11. Prefilter (5 µm → 1.2 µm) → 0.45 µm PES. Record volume in/out; >10% loss = clogging, add membrane area.

    NOTE Use PES/PVDF low-protein-binding membranes. 0.22 µm at bulk scale clips the large-vesicle population — it is used only at the end.

Concentration & Washing

12. TFF: flush/equilibrate the 100 kDa cartridge in 0.22 µm PBS; concentrate 10 L → ~250 mL (~40×); diafilter with 6 diavolumes 0.22 µm PBS (~1.5 L); recover retentate + two 25 mL rinses. Keep TMP low and constant.
13. Ultracentrifuge 100,000×g, 70 min, 4 °C (record rotor & k-factor). Discard SN; resuspend pellet in 10 mL cold PBS (never vortex). Repeat the spin.
14. Resuspend the final pellet in 0.5–1.0 mL 0.22 µm PBS; rest 30 min on ice; pipette gently.

Sterile Filtration, Aliquot, Storage

15. Pass through a 0.22 µm low-protein-binding syringe filter (pre-wet with PBS). Measure particle count before/after and report recovery.
16. Add trehalose to 25 mM; aliquot single-use (~50 µL) labelled with batch code; store −80 °C.

    CRITICAL No aliquot is freeze-thawed more than once — freeze-thaw fuses and lyses vesicles.

Release Gates (MISEV2023)

17. Confirm the batch against every release criterion before use.

EV Release Gates

	A	B	C
1	**Assay**	**Method**	**Release criterion**
2	Particle conc. & size	NTA, ≥5 × 60 s videos	mode 80–200 nm; ≥5×10^{11} total; blank ≤5%
3	Total protein	Micro-BCA	reported in µg
4	Purity	particles / µg protein	≥ 1×10^{10} /µg
5	Morphology	negative-stain TEM	round, membrane-bound, cup-shaped
6	Marker Western	EV vs whole-cell lysate	≥1 positive marker enriched; ER/mito depleted
7	Sterility	streak YPD + LB, 30/37 °C, 48 h	no growth

EV Isolation from Cell-Culture Media (PEG-PPT + UC)

Introduction

Optimized (2024) two-stage isolation of EVs from conditioned cell-culture supernatant: PEG 6000 precipitation to concentrate, then ultracentrifugation to pellet and wash.

Materials

Reagents

- PEG 6000 (50% w/v, autoclaved)
- 3.75 M NaCl (autoclaved)
- 0.9% NaCl (autoclaved)
- 10 mM HEPES / 0.9% NaCl
- Exo-free collection media

Equipment

- Refrigerated centrifuge, 4 °C
- Ultracentrifuge, 100,000×g
- 0.2 µm filters
- Kisker low-retention tubes

Procedure

PEG Precipitation

1. Collect the supernatant (SN) from 50% → 90% confluency every 48 h.

   NOTE Clean tube caps with ethanol/UV; add collection media starting from 50% confluency and leave up to 2 days.

2. Centrifuge the SN at 6,800×g, 20 min, 4 °C.
3. Filter (0.2 µm), transfer to new tubes, store at −20 °C until processing.
4. Thaw SN overnight at 4 °C (or 2 h at RT).
5. Add 10% PEG 6000 and 1% NaCl (3.75 M) to the SN (e.g. 39 mL SN + 10 mL PEG 6000 + 1 mL NaCl). Incubate 4 °C, 8–16 h.
6. Centrifuge 1,500×g, 30 min, 4 °C.

   CRITICAL Do not shake the tubes.

7. Remove the SN and residual medium from the pellet.
8. Resuspend the pellet in 5 mL 0.9% NaCl; combine two pellets (5 + 5 = 10 mL) per ultracentrifuge tube.

Ultracentrifugation

9. Spin at 100,000×g, 4 °C, 4 h (or 2 h).
10. Place UC tubes on ice; remove SN carefully; remove residual medium (pellet as dry as possible).
11. Resuspend each pellet ≥4 min in 1 mL 10 mM HEPES / NaCl.
12. Store EVs in Kisker low-retention tubes at −80 °C.

EV Isolation — Differential Ultracentrifugation

Introduction

Conventional serial-centrifugation method. Removes cells, dead cells and debris at increasing speeds, then pellets exosomes by ultracentrifugation.

Materials

- **Reagents**
  - Culture medium / supernatant
  - PBS
- **Equipment**
  - Refrigerated centrifuge
  - Ultracentrifuge, 100,000×g

Procedure

1. Collect the culture medium / supernatant.
2. Centrifuge 300×g, 10 min → 2,000×g, 10 min → 10,000×g, 30 min (remove cells, dead cells, debris respectively).
3. Obtain exosomes by centrifugation at 100,000×g for 90 min.
4. Wash the pelleted exosomes with PBS; re-centrifuge 100,000×g, 90 min.

   NOTE ~70% of this fraction is 50–150 nm vesicles; the remainder is >150 nm (~20%) or <50 nm (~10%).

EV Isolation — Density-Gradient Centrifugation

Introduction

Ultracentrifugation of the sample with a non-toxic density-gradient medium (sucrose or iodixanol) to enrich exosomes by buoyant density.

Materials

- **Reagents**
  - 0.8 M and 2 M sucrose cushions
  - Iodixanol buffer
- **Equipment**
  - Ultracentrifuge, up to 200,000×g

Procedure

1. After the first ultracentrifugation, add the sample to the 0.8 M and 2 M sucrose cushions.
2. Centrifuge 100,000×g, 2 h; exosomes enrich at density 1.10–1.18 g/mL of the sucrose buffer. Repeat ×2.
3. Take the interface layer, add to the bottom of an iodixanol buffer, centrifuge 200,000×g, 2 h.

EV Isolation — PEG Precipitation

Introduction

Faster and more efficient than centrifugation-only methods: volume-exclusion precipitation with PEG, followed by a low-speed spin.

Materials

- **Reagents**
  - PEG (10 kDa, Sigma)
  - Deionized water
  - PBS
- **Equipment**
  - Centrifuge
  - Ultrasonicator
  - 0.22 µm syringe filter

Procedure

1. Centrifuge the culture media at 10,000×g, 30 min.
2. Dissolve 25 g PEG (10 kDa) in 50 mL deionized water; ultrasonicate 1 h to a homogeneous solution.
3. Centrifuge the PEG solution 5,000×g, 20 min, 4 °C; filter the supernatant through 0.22 µm.
4. Add the PEG mother solution to the conditioned medium to final 10% PEG at 1:1 volume.
5. Incubate 4 °C, 2–16 h.
6. Centrifuge 3,000×g, 10 min; collect the exosome pellet.

02 SECTION

EV Characterization



ZetaView NTA — Startup, Daily Performance & Shutdown

Adapted from: ZetaView Instruction V1 (EN)

Introduction

Operating routine for the ZetaView nanoparticle-tracking analyzer used for the NTA readouts in the EV Characterization Panel: alignment with a 100 nm standard, the Daily Performance check, cell flushing and biofilm inspection, and shutdown.

Materials

- **Reagents**
  - 100 nm polystyrene size standard (Applied Microspheres #10100), 4 °C
  - Distilled water
  - Sample buffer (the same buffer the EVs are suspended in)
  - Warm water + mild soap (cleaning)
- **Equipment**
  - ZetaView instrument + control laptop (ZetaView software)
  - 1 mL syringes
  - 10 mL syringes

Procedure

Startup

1. Switch on the instrument (switch at the back, right side) and the laptop; start the ZetaView program.
2. Prepare the standard: 5 µL 100 nm standard into 5 mL distilled water (1:1,000) — label with date and name, store at 4 °C. Then 188 µL of the 1:1,000 dilution into 50 mL distilled water (1:266,000) — single use, discard afterwards.
3. After the first initialization, fill the cell with water when prompted. After the second initialization, fill the cell with the 1:266,000 standard.

Alignment & QC

4. Adjust the focus by running **Daily Performance**. The result must be 95–105 nm.

   CRITICAL Out of range: flush with water and repeat Daily Performance. If it is out of range again, stop and contact the instrument manager.
5. Flush with the sample buffer from the front and from the back using the pump.
6. Flush 10 mL of the sample buffer and inspect every position for biofilm, dirt or abnormal observations.

NOTE Biofilm: fill the cell with 20 mL warm soap water, 5 min; wash 3× with water. If the problem persists, contact the instrument manager.

7. If everything is clean, start the measurements under **Measurements**.

Shutdown

8. Flush the cell three times with water.
9. Push a 10 mL syringe filled with air through the cell three times.
10. Switch off the instrument and the laptop; empty liquid and pipette waste; clean the work area.

03 SECTION

Cargo Loading (Engineering)



miR-34a Mimic Preparation

Introduction

Resuspension and single-use aliquoting of the synthetic hsa-miR-34a-5p mimic (primary cargo), the scrambled negative control, and the labelled uptake tracer. Dose by moles, not mass.

Materials

Reagents

- hsa-miR-34a-5p mimic (MCE HY-R00714), 20 nmol
- Negative-control mimic (MCE HY-R04602), 20 nmol
- FAM-labelled negative-control mimic (tracer)
- Nuclease-free water or 1× siRNA buffer

Equipment

- Filter tips, dedicated pipettes
- RNase decontaminant
- −80 °C freezer

Procedure

1. Spin the tube briefly before opening — lyophilized mimic migrates to the cap.
2. Resuspend to 100 µM (20 nmol → 200 µL); vortex 30 s, rest 10 min RT, vortex again.
3. Aliquot single-use volumes; label with sequence name, concentration, date; −80 °C.

   CRITICAL Work RNase-free throughout: filter tips, dedicated pipettes, frequent glove changes, bench wiped with RNase decontaminant.
4. Dose mimic and control by moles, not micrograms — the duplexes differ ~5% in MW, so equal mass is not equal copy number.

Endotoxin-Free Plasmid Midiprep

Introduction

Preparation of the pLL3.7_hsa-miR-34a plasmid (secondary cargo arm, CMV-EGFP tracer) to high purity and low endotoxin. Endotoxin carryover would itself alter viability readouts in the cytotoxicity experiment.

Materials

- **Reagents**
  - *E. coli* glycerol stock carrying pLL3.7_hsa-miR-34a
  - LB broth + appropriate antibiotic
  - Endotoxin-free midiprep kit
  - Nuclease-free water or low-TE
- **Equipment**
  - 37 °C shaking incubator
  - Refrigerated centrifuge
  - NanoDrop
  - Agarose gel rig

Procedure

Culture

1. Inoculate LB + antibiotic from the glycerol stock; grow overnight at 37 °C with shaking to stationary phase.

Purification

2. Midiprep with an endotoxin-free kit per the manufacturer.

   CRITICAL Use an endotoxin-free kit specifically — endotoxin carryover confounds the downstream cytotoxicity readout.

3. Elute in nuclease-free water or low-TE, not high-salt buffer.

QC

4. Confirm purity and identity before use.

   A260/280 = 1.8–1.9; A260/230 ≥ 2.0

   Uncut gel ≥ 70% supercoiled

   Diagnostic digest confirming the U6-miR-34a insert

   Sanger sequence across the cassette once; keep the trace

5. Adjust to 1 µg/µL; aliquot; store −20 °C.

Exo-Fect Cargo Loading

Introduction

Load the miR-34a mimic into naive yeast EVs with the Exo-Fect kit. Load cargo first, decorate the surface second — never the reverse (the ExoQuick-TC precipitation would strip a surface conjugate). Run the titration first, then scale the winning condition.

Materials

Reagents

- Naive EVs (from Yeast EV Production)
- hsa-miR-34a-5p mimic
- Exo-Fect reagent + ExoQuick-TC (System Biosciences)
- Sterile PBS

Equipment

- 37 °C heat block
- Ice
- Microcentrifuge, 4 °C
- Low-protein-bind tubes

Procedure

1. Thaw one EV aliquot on ice. Do not vortex. Work on ice except where stated.
2. Per reaction, combine 1×10^{11} naive EVs + mimic (titration 0.25 / 1 / 4 µg) + 10 µL Exo-Fect + PBS to 150 µL.

Exo-Fect Reaction

	A	B
1	**Component**	**Amount**
2	Naive EVs	1×10^{11} particles
3	miR-34a mimic	0.25 / 1 / 4 µg (titrate)
4	Exo-Fect reagent	10 µL
5	Sterile PBS	to 150 µL

3. Mix by gentle inversion. 37 °C, 10 min.
4. Transfer to ice, 3 min.
5. Add 30 µL ExoQuick-TC; invert 6×; ice 30 min.
6. 14,000×g, 3 min, 4 °C. Discard the supernatant completely.

   NOTE Residual ExoQuick interferes downstream — remove it fully.
7. Resuspend the pellet in 200 µL sterile PBS; rest 15 min on ice; pipette gently.

PAUSE Pick the dose that maximizes protected copies/particle without shifting the NTA mode by >20 nm or losing >40% of particles.

RNase Protection Assay

Introduction

A control, not an optional cleanup. Most added mimic ends up stuck to the outside of the vesicles; surface-bound RNA is not cargo. RNase A strips it so loading numbers reflect only internal cargo.

Materials

- **Reagents**
  - Loaded EVs
  - RNase A
  - RNase inhibitor (or proteinase K)
  - 0.1% Triton X-100
  - Heat-labile dsDNase (plasmid arm)
- **Equipment**
  - 37 °C heat block
  - RNA extraction kit
  - Stem-loop RT-qPCR reagents

Procedure

1. Add RNase A to 10 µg/mL in PBS; 37 °C, 20 min.
2. Stop with RNase inhibitor (or proteinase K, 10 min at 37 °C); move straight to RNA extraction.
3. Keep three tubes side by side for QC.

   −RNase — total mimic associated with the prep

   +RNase — mimic protected inside the membrane

   +RNase +0.1% Triton X-100 — membrane lysed first; protected fraction becomes accessible

   CRITICAL The Triton condition must collapse back to ~the −RNase level. If not, the ‘internal’ mimic was never internal.
4. Plasmid arm: identical logic with heat-labile dsDNase (37 °C, 30 min; inactivate 55 °C, 5 min).

04 SECTION

Surface Decoration



Anti-TROP2 VHH – DSPE-PEG(2000)-Maleimide Conjugation

Introduction

Site-specific thiol–maleimide coupling of the C-terminal-Cys anti-TROP2 nanobody (clone SAA2054) to DSPE-PEG(2000)-maleimide, forming micelles for later post-insertion into EVs. Scale: 100 µg nanobody = 6.67 nmol. Run 1 measured values are shown in the tables. Hold pH 7.0 and work under N_2.

Materials

Reagents

- Anti-TROP2 VHH-8His-Cys (AntibodySystem RHC43303), 3.94 mg/mL
- DSPE-PEG(2000)-maleimide (BroadPharm BP-23307), 200 µg film
- TCEP·HCl (10 mM, neutralized)
- Conjugation buffer: 0.1 M NaPi / 0.15 M NaCl / 5 mM EDTA, pH 7.0
- 2-mercaptoethanol (quench); PBS pH 7.4

Equipment

- N_2 glovebox (aqueous approved)
- Amicon Ultra-0.5 3 kDa & 100 kDa
- NanoDrop
- Bath sonicator / vortex

Procedure

Prepare & Degas

1. Pre-rinse 3K and 100 kDa Amicons (450 µL buffer, 14,000×g, 5 min); leave wet at 4 °C.
2. Degas 15 mL conjugation buffer: N_2 through a 20–21 G needle at the bottom, 1–2 bubbles/s, 20 min; cap with gas flowing. Reserve ~3 mL (screw-cap) for the glovebox.

Reduce the Cys thiol

3. Spin the vendor vial 3,000×g, 30 s; transfer 25.4 µL + 20 µL buffer rinse = 45.4 µL to a low-bind tube.
4. Assemble the 100 µL reduction and read the protein reference.

   CRITICAL Read A280 on 1.5 µL (blank = buffer + 0.67 mM TCEP) and record as A_ref ≡ 1.00 mg/mL. This mix is the only protein standard for the whole run. Run 1: A_ref = 1.87.

Reduction Mix

	A	B
1	**Component**	**Volume**
2	VHH + rinse	45.4 µL
3	TCEP 10 mM	6.7 µL
4	Conjugation buffer	47.9 µL
5	Total	100 µL

5. Flush headspace N_2 5 s; cap; 45 min RT, dark, no agitation (final TCEP 0.67 mM = 10×).
6. Strip TCEP on the 3K Amicon: R1 100 µL + 400 µL buffer → 14,000×g, 15 min, 4 °C; R2 +460 µL buffer → spin; recover by inversion 1,000×g, 2 min.
7. Read A280 on 1.5 µL retentate = A_ret. Recovery = (A_ret×V_ret) ÷ (A_ref×100 µL).

   NOTE Run 1: 98.5 µL at A280 1.67 → 88.0% recovery = 5.86 nmol = 88.0 µg. The unprotected-thiol clock starts here — into lipid within 4 h.

Couple (inside the glovebox)

8. Hydrate a 200 µg lipid film in 100 µL sparged buffer = 2 mg/mL; vortex/sonicate to completely clear; use within 1 h.
9. Couple lipid into nanobody at 6:1, dropwise; cap, foil, 2–4 h RT.

   CRITICAL Add lipid INTO nanobody, not the reverse. Future runs: lipid µL = recovered nmol × 8.825.

Coupling (Run 1)

	A	B	C
1	**Component**	**Volume**	**Amount**
2	Reduced VHH (all)	98.5 µL	5.86 nmol
3	Lipid 2 mg/mL	51.8 µL	35.19 nmol = 103.5 µg
4	Total	150.3 µL	VHH 39.0 µM / lipid 234 µM

Quench, Purify, Measure

10. Quench: add 2-ME to 2 mM (3.0 µL of 100 mM); 30 min RT (total 153.3 µL).
11. Purify on the 100 kDa Amicon, 3 rounds (R1 +346.7 µL PBS; R2–R3 +460 µL PBS), 14,000×g, 5 min, 4 °C. Keep every filtrate (clears free VHH 1,953×).
12. Pool filtrates, concentrate on the 3K to ~50 µL.
13. Compute conjugation efficiency E from the A280 split across the membrane.

    NOTE E = mass_retentate ÷ (mass_retentate + mass_filtrate). ≥ 0.50 proceed; 0.25–0.50 proceed (favour higher doses); <0.25 reduction failed.

Efficiency (Run 1) ^

	A	B	C	D
1	**Fraction**	**Vol**	**A280**	**Mass**
2	Retentate	75 µL	1.46	58.6 µg = 3.90 nmol
3	Pooled filtrate	95 µL	0.23	11.7 µg = 0.78 nmol
4	E			83.4%

14. Store the conjugate stock at 4 °C, dark. Never freeze.

    CRITICAL Freeze-thaw aggregates the micelles irreversibly. Run 1 stock: 75 µL, 52.0 pmol/µL VHH, 399 pmol/µL PEG-lipid.

Post-Insertion Decoration of EVs

Introduction

Spontaneous insertion of the lipid-anchored nanobody conjugate into pre-formed EV membranes at 37 °C. A 4 cargo × 2 decoration factorial; every tube is 150 µL and gets the same hour and the same cleanup. Characterize the EVs by NTA/DLS/zeta first.

Materials

Reagents

- Conjugate stock (from the conjugation protocol)
- EV batches (plasmid / mimic / scrambled / empty)
- PBS pH 7.4

Equipment

- 37 °C thermomixer (300 rpm) or rotator
- Low-protein-bind tubes
- NTA / DLS / zeta

Procedure

1. Characterize EVs first: NTA (particles/mL — every dose depends on it), DLS (size, PDI), zeta — record as baselines.
2. Make the 1:5 working conjugate fresh: 5 µL stock + 20 µL PBS = 25 µL (single-use).

   NOTE 5 µL of 1:5 delivers the same 52.0 pmol as 1 µL neat but pipettes accurately; do not dilute past ~1:5 (micelles approach their CMC).
3. Set up the eight arms (150 µL each).

The Eight Arms ^

	A	B	C	D	E	F
1	**Arm**	**Cargo**	**Decorated**	**EVs**	**Conj 1:5**	**PBS**
2	1	plasmid	yes	100 µL	5 µL	45 µL
3	2	plasmid	no	100 µL	—	50 µL
4	3	mimic	yes	100 µL	5 µL	45 µL
5	4	mimic	no	100 µL	—	50 µL
6	5	scrambled	yes	100 µL	5 µL	45 µL
7	6	scrambled	no	100 µL	—	50 µL
8	7	empty	yes	100 µL	5 µL	45 µL
9	8	empty	no	100 µL	—	50 µL

4. Mix gently by pipetting (do not vortex). Incubate 37 °C, 1 h, 300 rpm or slow rotation.

   CRITICAL Never 60 °C — that figure is for HSPC liposomes (Tm 52 °C). EV membranes are fluid at 37 °C and 60 °C destroys them.

5. Mock arms (2,4,6,8) get PBS and the same incubation and their own cleanup device — they are mock-decorated, not untouched.

   NOTE At 5 µL per 1.2×10^{11} EVs: 52.0 pmol VHH = 261 copies/EV nominal (~78 after ~30% insertion), 2.00 mol% PEG-lipid (ceiling ~5).

Decoration Cleanup (Nanosep 300K / qEV SEC / UC)

Introduction

Remove free conjugate micelles, which are in large molar excess over the TROP2 receptors in each well and would compete with decorated EVs. Three validated routes, in order of what the lab had available; the Run 1 batch used ultracentrifugation after the Nanosep failed its validation test.

Materials

- **Reagents**
  - Decorated EV arms
  - PBS (+ 25 mM trehalose for UC)
- **Equipment**
  - Nanosep 300K Omega (Pall/Cytiva OD300C33)
  - or qEV original / 70 nm (Izon)
  - or Ultracentrifuge, TLA-55 rotor
  - NTA

Procedure

Route A — Nanosep 300K (if validation passes)

1. Pre-rinse every device (500 µL PBS, 5,000×g, 3 min). Run 4 rounds, 500 → 50 µL at 5,000×g, emptying the filtrate receiver between rounds; recover by pipette.

   CRITICAL Run at 5,000×g not 14,000 — lower flux is gentler and reduces concentration polarization that would retain micelles. If the validation test gave S = 0.25, run 5 rounds.

Route B — qEV SEC

2. Equilibrate a qEV original / 70 nm column in PBS; load 150 µL; discard the 3 mL void; collect 3 × 0.5 mL = 1.5 mL EV pool; discard from fraction 5 on. One column per arm.

   NOTE A 100 kDa filter will NOT work — micelles run ~150–270 kDa apparent and are retained with the EVs. Expect 50–70% recovery, 10× dilution.

Route C — Ultracentrifugation (used in Run 1)

3. Each arm: 150 µL decoration + 1.25 mL PBS + 25 mM trehalose = 1.4 mL; 40,300 rpm = 100,000×g at rmax (TLA-55), 70 min, 4 °C.
4. Aspirate to ~20 µL (do not dry); resuspend in 1.4 mL; repeat the spin; resuspend the final pellet in 100 µL PBS; stand 15–20 min 4 °C; pipette gently (no vortex).

NOTE EVs sediment ~45× faster than 25 nm micelles; 70 min recovers EVs to ~50 nm while micelles stay near the meniscus. The wash is what drops free VHH to ~0.6% of receptors.

5. Verify every retentate by NTA: +5 to +15 nm vs input; count within expectation; no new population >300 nm (= aggregation).

CRITICAL Store cleaned EVs at 4 °C, dark, 24–48 h. Never freeze.

Nanosep Sieving-Coefficient Validation Test

Introduction

One-hour test to decide whether a 300K Omega membrane passes the DSPE-PEG micelles before committing any EVs. Required sieving coefficient S ≥ 0.25 at four rounds. In Run 1 the micelles were retained (S ≈ 0.053) and the method was abandoned in favour of UC.

Materials

- **Reagents**
  - Neat conjugate stock
  - PBS
  - Anti-His-HRP + ECL (dot blot)
- **Equipment**
  - Nanosep 300K Omega
  - 3 kDa Amicon
  - Nitrocellulose

Procedure

1. 2 µL neat conjugate + 498 µL PBS = 500 µL (104 pmol VHH).
2. Pre-rinse a Nanosep 300K (500 µL PBS, 5,000×g, 3 min).
3. Load the 500 µL; spin 5,000×g to ~50 µL (check at 3 min, continue in 1–2 min steps); recover the retentate by pipette.
4. Concentrate the ~450 µL filtrate on a 3K device to ~50 µL.
5. Anti-His dot blot: spot retentate, concentrated filtrate, and an input dilution series (1×, 1:3, 1:10, 1:30) as a standard curve.
6. Read: $S = -\log_{10}(\text{fraction in retentate})$.

NOTE Because the conjugate was already 100 kDa-purified, any anti-His in the filtrate is micelle-derived — a clean test.

Decision ^

	A	B	C
1	**Retentate holds**	**S**	**Decision**
2	~10% of input	1.0	excellent — proceed
3	~30%	0.5	good — proceed
4	~55%	0.25	borderline — proceed with 5 rounds
5	>70%	<0.2	stop — use qEV or UC

Glovebox Logistics (Thiol–Maleimide Coupling)

Introduction

Only two operations happen inside the N_2 box — hydrate the lipid film, and combine nanobody + lipid + buffer — so the equipment list is short. Everything enters in one antechamber pass on Day 1, after the reduction. Host lab: Bollinger-Krebs (contact Kallie).

Materials

- **Reagents**
  - C1: reduced anti-TROP2 VHH (~40 µL, screw-cap cryovial)
  - C2: DSPE-PEG-maleimide dried film (200 µg, sealed amber vial, chloroform-free)
  - C3: sparged conjugation buffer (~3 mL, screw-cap conical)
- **Equipment**
  - P200 (pre-set 50.0 µL) & P20 if the box has none
  - 1.5 mL rack, foil, marker
  - 20 P200 + 10 P20 tips loose in a plastic rack
  - Waste tube

Procedure

1. Complete the bench steps first (sparge, reduce, strip TCEP, read A280); move the retentate to a screw-cap cryovial (C1).
2. Assemble the tray: C1, C2, C3, tubes, tips, rack, foil, pipettes if needed. Run the pre-cycle checklist.

   Every liquid capped and in a screw-cap container

   Lipid film held under vacuum/N_2 overnight (chloroform-free)

   No cardboard, paper, or styrofoam; no 2-ME, TCEP, or organic solvent

   A_ref and recovered nmol written down OUTSIDE the box; lipid volume pre-calculated
3. Cycle in: 3 evacuate/refill cycles, 15–20 min. Uncap the buffer only once inside.

   CRITICAL Never bring in 2-mercaptoethanol, TCEP, or any organic solvent — thiols and phosphines poison the copper catalyst bed. This cycle runs inside the 4 h thiol window.
4. Inside: warm the lipid vial to RT before opening, hydrate the film, run the coupling. One item comes out — the capped 150 µL reaction tube, after 2–4 h.

05 SECTION

Cell Culture & Cytotoxicity



Pancreatic Cancer Cell Culture & Seeding

Introduction

Maintenance and seeding of the three cell lines for the cytotoxicity experiment: BxPC-3 (TROP2-high target), PANC-1 (TROP2-low specificity control), and IMR-90 (non-cancer control). Verify TROP2 expression in your own passage before relying on the contrast. Use EV-depleted FBS in all treatment medium.

Materials

Reagents

- BxPC-3 (RPMI-1640 + 10% FBS)
- PANC-1 (DMEM + 10% FBS)
- IMR-90 (EMEM/DMEM + 10% FBS)
- EV-depleted FBS
- PE anti-TROP2 (BioLegend 363803) + isotype
- TrypLE / Accutase (EDTA-free)

Equipment

- 37 °C, 5% CO_2 incubator
- 24-well plates
- Hemocytometer
- Flow cytometer

Procedure

Verify TROP2 (go/no-go)

1. Stain each line with anti-TROP2-PE + isotype; Western vs GAPDH. Gate to pass: BxPC-3 MFI ≥ 5× PANC-1.

   CRITICAL TROP2 drifts with passage — verify in your own cells; re-check every 10 passages; record passage number on every figure.

Seed

2. Seed 24-well plates at 500 µL/well. Target ~20% at seeding, ~80–85% at harvest.

Seeding Densities

	A	B	C
1	**Line**	**Seed / well**	**Role**
2	BxPC-3	$5–6\times10^4$	TROP2-high target
3	PANC-1	4×10^4	TROP2-low control
4	IMR-90	3×10^4	non-cancer control

3. Re-invert the cell reservoir every half plate — uneven seeding is the commonest source of apparent treatment effects that are not real.
4. Use EV-depleted FBS throughout.

   NOTE Standard FBS is full of bovine EVs that compete for uptake and add PS background to the Annexin channel.

5. After treatment, add medium — do not aspirate.

   CRITICAL Aspirating discards detached early-apoptotic cells — the population being measured. Add +250 µL fresh complete medium at 48 h; never aspirate (except transfection wells).

Lipofectamine Transfection (Ceiling Control)

Introduction

Direct transfection of the miR-34a mimic (and the plasmid, optionally) into the cell lines — the positive-control ceiling that answers whether miR-34a can kill these cells at all, independent of EV delivery. Build master mixes; per-well pipetting is impossible at these doses.

Materials

› Reagents

- hsa-miR-34a-5p mimic (100 µM) & scrambled (25 µM)
- pLL3.7 plasmid (1,530 ng/µL) + P3000 (plasmid only)
- Lipofectamine 2000/3000
- Opti-MEM

› Equipment

- 37 °C, 5% CO_2 incubator
- Filter tips

Procedure

1. Per group start each tube with 200 µL Opti-MEM; build the master mixes (8 wells each, serving both plates).

   NOTE P3000 is used in the plasmid tube only. Reagent 1.0 µL/well RNA, 1.5 µL/well DNA — the low end, because the readout is apoptosis and lipid toxicity would compress the window.

   Master Mixes ^

	A	B	C
1	**Group**	**Tube A**	**Tube B**
2	T1 mimic (50 nM)	2.0 µL mimic (200 pmol)	8.0 µL Lipofectamine
3	T2 scrambled (50 nM)	8.0 µL scrambled (200 pmol)	8.0 µL Lipofectamine
4	T3 plasmid (500 ng)	2.6 µL plasmid (4,000 ng) + 8.0 µL P3000	12.0 µL Lipofectamine

2. Spin stock tubes 3,000×g, 15 s before opening. Build Tube A set, flick; build Tube B set, flick, stand 5 min.
3. Add A into B (matched), flick; stand 20 min (Lipo 2000) or 15 min (3000).
4. Add 50 µL/well dropwise; rock front-back then side-to-side (do not swirl). Final 550 µL; no antibiotics during the window.
5. At 6 h post-transfection, aspirate all transfected wells and replace with 500 µL fresh complete medium.

   CRITICAL This is the one exception to the no-aspiration rule — at 6 h nothing has detached yet, and Lipofectamine is harsh over the long exposure. Applied identically to T1/T2/T3.

6. Interpretation: lipofection delivers ~100–350× more miR-34a than the EV arms — this is a ceiling, not a matched comparison.

EV Treatment & Dosing

Introduction

Dose cleaned, decorated EV arms onto the seeded cells by matched particle number (never by protein — engineered arms carry added lipid-protein conjugate). Arms carrying nanobody are also matched on display MFI.

Materials

- **Reagents**
  - Cleaned EV arms (NTA-quantified)
  - PBS
  - EV-depleted complete medium
- **Equipment**
  - 24-well seeded plates
  - Filter tips

Procedure

1. Treat 24 h after seeding. Dose at 1×10^{4} particles/cell.

EV Dose per Well

	A	B	C
1	**Line**	**Cells at treatment**	**Particles / well**
2	PANC-1	~5.2×10^{4}	5.2×10^{8}
3	BxPC-3	~6.5×10^{4}	6.5×10^{8}

2. Dilute each ~50 µL retentate to 1 mL in PBS (~8.4×10^{7}/µL) → ~6–8 µL/well.

   NOTE Volume/well = particles required ÷ NTA particles/µL. Recompute from the actual NTA, not the estimate.

3. Match Nb-bearing arms (F, G, H, P) on bead-flow display MFI to within ~20%, not just on particle count.

   CRITICAL Coupling efficiency is lot-variable, so equal micrograms in ≠ equal density out. If H carries 3× the density of G, H−G measures density, not targeting.

4. Dose-response for the main experiment: 1×10^{9}, 5×10^{9}, 2.5×10^{10}, 5×10^{10} particles/mL in 0.5 mL/well; read at 24/48/72 h (primary 48 h).

Annexin V-APC / PI Apoptosis Flow Cytometry

Introduction

Apoptosis readout for every arm. Use Annexin V-APC (not FITC — the plasmid arms express EGFP and the tracer uses FAM/Cy3). Pool supernatant + wash + detached cells for every well, or every arm looks healthier than it is.

Materials

- **Reagents**
  - Annexin V-APC; PI (or SYTOX Blue)
  - 1× Annexin V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$)
  - Accutase (EDTA-free); PBS
  - Staurosporine 1 µM (positive control)
- **Equipment**
  - Flow cytometer (405/488/640)
  - 5 mL FACS tubes
  - Refrigerated centrifuge

Procedure

1. Harvest everything: collect conditioned medium into a labelled tube; wash the well with PBS into the same tube.

   CRITICAL Apoptotic cells detach — the medium + wash carry the population you are counting.

2. Detach with Accutase, 3–5 min, 37 °C (EDTA-free); stop as soon as cells round up; add to the same tube.

   NOTE Over-trypsinization causes false Annexin positives and strips surface-bound EVs, which carry their own PS.

3. 300×g, 5 min, 4 °C; wash once in cold PBS.

4. Resuspend in 100 µL 1× binding buffer + 5 µL Annexin V-APC + 5 µL PI (or 1 µg/mL); RT, 15 min, dark.

   CRITICAL Annexin V binding is Ca^{2+}-dependent — the buffer must contain 2.5 mM $CaCl_2$ and no EDTA may touch the cells after staining.

5. Add 300–400 µL binding buffer; keep on ice; acquire ≥10,000 singlet events within 1 h. Do not fix.

   NOTE Gate FSC/SSC loosely — apoptotic cells shrink and a tight live gate deletes the signal. Set the boundary with the staurosporine control.

6. Report total Annexin V+ (early + late). Compensation controls: unstained, AnnV-APC single (staurosporine), PI single, GFP single (plasmid arm), labelled-mimic single (tracer arm).

Harvest & Flow Collection (Plate Pooling)

Introduction

End-of-run collection scheme pooling three wells per group into one tube (n = 1 per group; statistics come from repeating on separate days). Everything collected and on ice within 40 minutes.

Materials

Reagents

- Binding buffer (BioLegend 10× diluted 1:10)
- SYTOX Blue (Thermo C10491) + Annexin V-APC (BioLegend)
- TrypLE/Accutase; complete medium; PBS

Equipment

- 28 × 15 mL conicals (P1–P14, B1–B14)
- FACS tubes
- Centrifuge 4 °C
- Ice

Procedure

1. Pool each group (half a row, odd or even columns) into one labelled 15 mL conical. Never aspirate to waste.

   CRITICAL The PBS rinse is the step people skip — a large part of the early-apoptotic population is loosely attached and comes off there.

Pooling (per group → one conical)

	A	B
1	**Transfer**	**Volume**
2	Medium from all 3 wells	~2.25 mL
3	PBS rinse 250 µL/well (swirl)	0.75 mL
4	TrypLE 150 µL/well, 37 °C 3–5 min	—
5	Neutralize 250 µL medium/well	0.75 mL

2. Work one plate at a time (PANC-1 A → PANC-1 B → BxPC-3 A → BxPC-3 B); all four plates on ice within 40 min. Never scrape.
3. Spin all 28 conicals 400×g, 5 min, 4 °C.

   NOTE 400×g not 300 — apoptotic cells shrink and a real fraction stays in the supernatant at 300×g.

4. Aspirate leaving ~100 µL; resuspend in 100 µL binding buffer → FACS tube (~4.8×10^5 cells).
5. Stain in two batches of 14: +5 µL Annexin V-APC, 15 min RT dark; +400 µL binding buffer; +5 µL 100 µM SYTOX (1 µM final). Do not wash, do not fix; acquire within 30–60 min.

6. Record: export every FCS file; events/tube; harvest start/end time and plate order. Repeat on two further days for n = 3 biological replicates.

06 SECTION

Protein Quantification & Western Blot



Cell Lysis & BCA Protein Assay (Microplate)

Adapted from: BCA Protein Assay V2 (translated from German)

Introduction

Lysis of cultured cells in RIPA or NP-40 buffer and total-protein determination with the BCA kit in a 96-well plate: duplicate wells against a BSA standard curve from 25 to 2,000 µg/mL. Buffer recipes: Recipes & Buffers — Lysis Buffers.

Materials

Reagents

- BCA Protein Assay Kit (Thermo #23225) — reagents A and B, BSA standard 2 mg/mL
- 0.9% NaCl (B. Braun #3570130) — diluent
- 4× RIPA or NP-40 lysis buffer
- cOmplete Mini protease inhibitor (Roche #11836170001), 10× stock

Equipment

- 96-well flat-bottom clear microplate (Greiner #655182)
- Microcentrifuge, 4 °C
- 37 °C incubator
- Microplate reader, 562 nm

Procedure

Cell Lysis

1. Prepare lysis buffer fresh: 900 µL lysis buffer + 100 µL 10× protease inhibitor.
2. Resuspend 1×10^6 cells in 100 µL lysis buffer.
3. Incubate 30 min on ice, vortexing briefly every 10 min.
4. Centrifuge 14,000 rpm, 20 min, 4 °C. Transfer the clear supernatant to a new tube.

Standards & Working Reagent

5. Dilute the BSA standard in 0.9% NaCl.

BSA Standard Series ^

	A	B	C	D	E
1	**Vial**	**NaCl (µL)**	**BSA (µL)**	**BSA source**	**Final (µg/mL)**
2	A	0	300	stock	2,000
3	B	125	375	stock	1,500
4	C	325	325	stock	1,000

	A	B	C	D	E
1	**Vial**	**NaCl (µL)**	**BSA (µL)**	**BSA source**	**Final (µg/mL)**
5	D	175	175	vial B	750
6	E	325	325	vial C	500
7	F	325	325	vial E	250
8	G	325	325	vial F	125
9	H	400	100	vial G	25
10	I	400	0	—	0 (blank)

6. Working reagent (WR) = 1 part reagent B + 50 parts reagent A. Make (standards + samples) × 2 (duplicates) × 200 µL plus extra, e.g. 6 mL for 30 samples.
7. Dilute samples of unknown concentration, usually 1:5 (12 µL sample + 48 µL NaCl) and 1:10 (6 µL sample + 54 µL NaCl).

Plate Assay (at least duplicates)

8. Load 25 µL of each standard and each diluted sample per well, vortexing well before loading. Add 200 µL WR per well.
9. Incubate 30 min at 37 °C. Let the plate cool to RT before reading.
10. Read absorbance at 562 nm (BCA protocol; adjust the plate layout to the loading). Export the data to Excel and calculate from the standard curve.

BCA & Bradford Protein Quantification (Microtube)

Adapted from: Bradford and BCA Protein Quantification protocol

Introduction

Quick total-protein estimates on small sample volumes against a single BSA standard. The sample vehicle is the buffer the protein is dissolved in (e.g. urea buffer), so the blank and standard carry the same matrix as the sample.

Materials

- **Reagents**
  - BSA standard
  - BCA working solution (reagents A + B)
  - Bradford reagent
  - MilliQ water
  - Sample vehicle (sample buffer)
- **Equipment**
  - 60 °C heat block
  - Spectrophotometer or plate reader (562 nm / 595 nm)

Procedure

A. BCA Quantification

1. Set up the tubes.

BCA Set-Up

	A	B	C	D	E	F
1	**Tube**	**BSA (µL)**	**Sample (µL)**	**Sample vehicle (µL)**	**MilliQ (µL)**	**BCA working solution (µL)**
2	Blank	0	0	8	12	400
3	Standard (1.25 µg/ml)	8	0	8	4	400
4	Sample	0	8	0	12	400

2. Incubate at 60 °C for 30 min.
3. Cool down for 10 min; read A_{562}.

B. Bradford Quantification

4. Set up the tubes.

Bradford Set-Up ^

	A	B	C
1	**Tube**	**Volume of sample (µL)**	**Volume of Bradford (µL)**
2	Blank	8.5 (from the vehicle)	250
3	Standard (1 µg/ ml)	8.5	250
4	Sample	8.5	250

5. Read A_{595}.

SDS-PAGE (12% Resolving / 4.5% Stacking Gel)

Adapted from: SDS-PAGE protocol

Introduction

Hand-cast mini gel for protein separation ahead of Coomassie or silver staining and in-gel digestion. Volumes make ~10 mL resolving gel and ~5 mL stacking gel.

Materials

- **Reagents**
  - Acrylamide/bis-acrylamide solution (30%)
  - 1.5 M Tris pH 8.8; 1 M Tris pH 6.8
  - 10% SDS; 50% glycerol
  - 10% APS and TEMED (add last)
  - Isopropanol
  - 1× SDS running buffer
- **Equipment**
  - Casting frame, glass plates, comb
  - Mini gel running chamber + power supply
  - 95 °C heat block
  - Filter paper

Procedure

Gel Casting

1. Assemble the gel plates in the casting frame.
2. Prepare the 12% resolving gel.

12% Resolving Gel

	A	B
1	**Component**	**Volume**
2	Distilled water	1600 µL
3	Acrylamide/bisacrylamide solution (30%)	4200 µL
4	1.5 M Tris pH 8.8	2500 µL
5	10% SDS	100 µL
6	50% glycerol	1700 µL
7	10% APS*	100 µL
8	TEMED*	10 µL

NOTE * Add APS and TEMED immediately before pouring between the plates.

3. Pour the resolving gel between the plates, leaving sufficient space for the stacking gel. Add a few drops of isopropanol.
4. After the resolving gel solidifies, remove the isopropanol with filter paper.
5. Prepare the 4.5% stacking gel.

4.5% Stacking Gel

	A	B
1	**Component**	**Volume**
2	Distilled water	2950 µL
3	Acrylamide/bisacrylamide solution (30%)	750 µL
4	1 M Tris pH 6.8	1250 µL
5	10% SDS	50 µL
6	10% APS*	40 µL
7	TEMED*	5 µL

6. Pour the stacking gel and insert the comb.

Running

7. After the stacking gel solidifies, remove the gel from the casting frame and assemble the running chamber.
8. Add running buffer and remove the comb.
9. Boil the samples at 95 °C for 5 min; load.
10. Run the gel at 125 V for 80 min.
11. Stain the gel (Coomassie Staining or Silver Staining for Mass Spectrometry).

Western Blot (Wet Mini, Criterion & Semi-Dry Transfer)

Consolidated from: Western Blot V4 and Western Blot – Large Format V1 (translated from German) · Western Blotting protocol

Introduction

Sample preparation, SDS-PAGE, transfer and chemiluminescent detection, with three transfer routes for the available hardware: A — wet tank, Mini Trans-Blot; B — wet tank, Criterion (large format); C — semi-dry. Blocking, antibodies and detection are shared. Buffers: Recipes & Buffers — Western-Blot Buffers and 4× Laemmli Sample Buffer. Cell lysates: see Cell Lysis & BCA Protein Assay.

Materials

Reagents

- 30% acrylamide (Bio-Rad #161-0156), TEMED (Invitrogen #15524-010), 10% APS, 10% SDS
- 1.5 M Tris pH 8.8 (resolving) and 1 M Tris pH 6.8 (stacking)
- 4× Laemmli buffer (Bio-Rad #1610747) ± DTT (Applichem #A2948) or β-ME (Sigma #M3148)
- PageRuler Prestained ladder (Thermo #26616)
- PVDF membrane 0.45 µm (Millipore #IPVH00010); methanol
- 1× running buffer, 1× transfer buffer (pre-chilled), TBST
- Blocking: 5% non-fat milk or 5% BSA in TBST; Ponceau S (optional)
- SuperSignal West Pico Plus (#34580) and West Femto (#34095) ECL substrates

Equipment

- Hand-cast gel system (Hoefer, Invitrogen or Bio-Rad Criterion)
- Wet blotter (Mini Trans-Blot or Criterion) or semi-dry blotter
- Filter paper, sponges, roller, ice + cooling pack
- Rocker; plastic incubation boxes
- Chemiluminescence imager (e.g. Fusion FX7 or ChemiDoc)

Procedure

Gel Casting

1. Clean every surface that may touch the gel or membrane with 70% ethanol and lint-free wipes. Assemble the casting chamber (Hoefer: ceramic plate → 1.5 mm T-spacer → glass plate; tighten the screws diagonally). Check for leaks with dH_2O, then dry with filter paper. The Invitrogen system needs no assembly.
2. Mix the resolving gel. Add acrylamide and TEMED in the fume hood only, and TEMED last, immediately before pouring.

SDS Gels — 12% Resolving / 4% Stacking

	A	B	C	D	E
1	**Component**	**2 gels: resolving**	**2 gels: stacking**	**4 gels: resolving**	**4 gels: stacking**
2	dH_2O	6.6 mL	7.2 mL	13.2 mL	14.4 mL
3	Tris	5 mL (1.5 M, pH 8.8)	1.25 mL (1 M, pH 6.8)	10 mL (1.5 M, pH 8.8)	2.5 mL (1 M, pH 6.8)
4	10% SDS	0.2 mL	0.1 mL	0.4 mL	0.2 mL
5	10% APS	0.2 mL	0.1 mL	0.4 mL	0.2 mL
6	30% acrylamide	8 mL	1.34 mL	16 mL	2.68 mL
7	TEMED	8 µL	10 µL	16 µL	20 µL

CRITICAL Acrylamide and TEMED are toxic until polymerized — fume hood only. Optional: add 30 µL bromophenol blue.

3. Insert the comb once to mark the pour height (~0.5 cm below the comb teeth). Pour the resolving gel with a stripette to the mark and overlay with isopropanol (straight edge, no bubbles). Let the leftover gel polymerize in its tube as a control.

4. After polymerization, pour off the isopropanol and dry with filter paper. Add TEMED to the stacking gel, vortex, pour to the top edge and insert the comb, avoiding air bubbles.

   NOTE Cast only as many gels as needed. Spare gels keep wrapped in water-soaked paper in a plastic bag at 4 °C for 1 week (up to 2–3 weeks for mini gels); label with date, name and acrylamide %.

Sample Preparation & Run

5. Adjust samples to equal protein and equal volume with their own buffer (EVs: HEPES/NaCl; cell lysates: RIPA or NP-40), e.g. 10–30 µg MSC-EVs. Add 4× Laemmli to ¼ of the final volume (e.g. 18 µL sample + 6 µL 4× Laemmli).

   Reducing: 4× Laemmli with SDS + DTT (or β-ME) — heat 95 °C, 5 min

   Non-reducing: 4× Laemmli with SDS only — heat 70 °C, 10 min

   NOTE Maximum well volume: mini 10-well 40 µL; Criterion 12+2-well 45 µL; Criterion 20-well 25 µL. Choose reducing or non-reducing per antibody datasheet.

6. Load samples and record the order. Load 7 µL ladder in the first (and last) lane (5 µL with a 15-well comb). Optionally fill empty lanes with 1× Laemmli.

7. Fill the chamber with cold, freshly diluted 1× SDS running buffer. Check that the connectors are dry. Run until the bromophenol-blue front has run out.

Run Conditions

	A	B
1	**System**	**Conditions**
2	Mini (Hoefer / Invitrogen), 1–2 gels	200 V constant, ~70 min
3	Criterion, 1–4 gels	130 V constant, ~130 min
4	Hand-cast mini gel (SDS-PAGE protocol)	125 V, 80 min

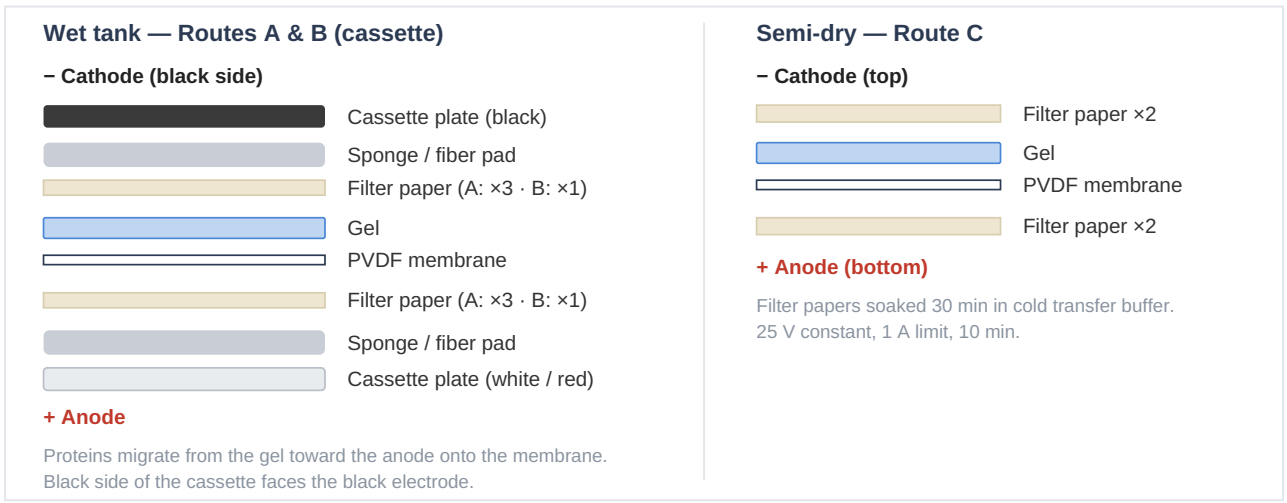
Transfer

8. Disassemble the gel cassette in a tray of 1× transfer buffer and remove the gel carefully. Trim excess gel; cut off the small top-left corner for orientation.
9. Activate the PVDF membrane in methanol (30 s – 3 min), then wash out the methanol in cold 1× transfer buffer; mark the membrane's top corner. Soak filter papers and sponges in transfer buffer until saturated.

   NOTE Route C: soak 4 filter papers in cold transfer buffer for 30 min; equilibrate the membrane 30 min with shaking, changing the buffer once to wash out methanol. Keep the gel in running buffer until assembly.
10. Assemble the sandwich and roll out all air bubbles. Black side of the cassette faces the black electrode (cathode).

Transfer Routes

	A	B	C
1	**Route**	**Hardware**	**Conditions**
2	A	Wet — Mini Trans-Blot (central core + cooling pack, on ice, pre-chilled buffer)	200 mA constant per gel (400 mA for two), 150 min — swap front/ back cassettes halfway; or overnight 100 mA per gel (200 mA for two), 8 h at 4 °C
3	B	Wet — Criterion blotter (cooling pack, on ice, pre-chilled buffer)	100 V constant, 35 min for one gel (50 min for two)
4	C	Semi-dry blotter (Thermo / Bio-Rad)	25 V constant, 1 A limit, 10 min



Transfer sandwich assembly for wet-tank (Routes A, B) and semi-dry (Route C) transfer.

11. Open the blot carefully. If transfer is incomplete, reassemble and run longer (A: +30 min; B: +5–10 min).

Blocking & Antibodies

12. Optional transfer check: Ponceau S, 10 min; discard and rinse with MilliQ (re-stain while shaking if bands are faint). The gel can be Coomassie-stained (~20 min) to confirm transfer.

    NOTE Ponceau staining can weaken subsequent band detection.

13. Trace the gel outline on the membrane in pencil and trim. Wash 10 min in TBST, then block 60 min at RT in 5% milk in TBST (or 5% BSA) with gentle shaking: 10 mL per mini membrane, 15–20 mL per Criterion membrane.

14. Primary antibody in 5% milk/TBST (or 5% BSA, per datasheet): 2 h at RT or overnight at 4 °C on a shaker. Small box 6–10 mL; 50 mL tube 5 mL; Criterion box ≥ 15 mL.

    NOTE Primary antibody can be reused 2–3× — keep it, store at −20 °C, with PenStrep 1:100.

15. Wash: 3× brief rinses + 1× 10–15 min in TBST (or 4 × 15 min).

16. HRP-conjugated secondary antibody in 5% milk/TBST (dilution per datasheet): 60 min at RT on a shaker. Use once.

17. Wash: 3× brief rinses + 1× 10–15 min in TBST (or 3 × 5 min).

Detection

18. Switch on the imager, set autofocus and check the distance. Place the membrane with as little buffer as possible and take a marker image.

19. Mix ECL solutions A + B (1:1) in a tube, apply to the membrane and incubate ~5 min at RT.

ECL Volumes ^

	A	B	C
1	**Membrane**	**First substrate**	**If bands are weak**
2	Mini	West Pico, 2 mL (1 mL A + 1 mL B)	add West Femto, 200 µL (100 µL A + 100 µL B)
3	Criterion	West Femto, ~800 µL (400 µL A + 400 µL B)	optional West Pico, 2 mL (1 mL A + 1 mL B)

20. Save every image as JPEG and TIFF, named with marker, exposure time and condition (red. / non-red.).

21. Keep the membrane in TBST at 4 °C for further imaging (long term: labelled 50 mL tube with name, antibody, samples, date). Proteins of clearly different MW can be probed sequentially without stripping: wash 30 min in TBST, re-block in 5% milk, incubate the next primary. Otherwise strip (Western-Blot Buffers — stripping solutions).

Silver Staining for Mass Spectrometry

Adapted from: Silver Staining for Mass Spectrometry protocol

Introduction

MS-compatible silver stain (kit-based), followed by band excision and silver destaining so the gel pieces can go straight into in-gel trypsin digestion.

Materials

Reagents

- MS-compatible silver stain kit: fixing solution, sensitizer, stain, enhancer, developer, stop solution
- Silver Destain Reagents A and B; Wash Solution
- 10% ethanol
- Ultrapure water

Equipment

- Staining tray, rocker
- Light box; clean scalpel or spot picker
- 0.5 mL microcentrifuge tubes

Procedure

Staining

1. Wash the gel in ultrapure water, 2 × 5 min.
2. Discard the water; incubate in fixing solution 2 × 15 min at RT.
3. Wash with 10% ethanol 2 × 5 min, then ultrapure water 2 × 5 min.
4. Just before use, prepare sensitizer working solution (25 µL sensitizer + 12.5 mL water). Incubate the gel in it for exactly 1 min, then wash 2 × 1 min with ultrapure water.
5. Prepare stain working solution (0.125 mL enhancer + 12.5 mL stain), add immediately and incubate 5 min.
6. Prepare developer working solution (0.125 mL enhancer + 12.5 mL developer).
7. Wash the gel quickly with ultrapure water (20 s).
8. Immediately add the developer working solution; incubate until bands appear (2–3 min).

   NOTE Bands begin to appear within 30 s and keep developing. Band-to-background is best at 2–3 min; after 3 min the lane background can rise to undesirable levels.
9. At the desired band intensity, replace the developer with Stop Solution. Wash briefly, replace the acetic acid and incubate 10 min.
10. Proceed immediately to band excision and destaining.

Band Excision & Destaining

11. Wash the gel in ultrapure water, 2 × 10 min.
12. On a light box, excise the protein bands with a clean scalpel or spot picker. From a blank region, excise a piece of the same size as a control. Place the pieces in clean 0.5 mL tubes.
13. Prepare destain solution: 37 µL Silver Destain Reagent A + 122.5 µL Reagent B + 2 mL ultrapure water (enough for 10 gel pieces per lane).

    NOTE Use the same day; do not store.
14. Add 100 µL destain to the pieces, mix gently, 15 min at RT. Remove; incubate in another 100 µL destain for 15 min.

    NOTE To continue the next day, hold the pieces in fixing solution.
15. Same day: remove the destain and wash 3 × 15 min with 0.2 mL Wash Solution.
16. Proceed with In-Gel Digestion. Alternatively store the gel pieces overnight at −20 °C — do not exceed overnight.

07 SECTION

Proteomics Sample Prep



Urea Protein Extraction (Cells)

Adapted from: Urea Protein Extraction protocol

Introduction

Denaturing lysis of cultured cells for LC-MS/MS proteomics, ahead of in-solution digestion or dimethyl labeling.

Materials

- **Reagents**
  - Urea buffer: 8 M urea, 500 mM Tris-HCl pH 8.5
  - Protease inhibitor
- **Equipment**
  - Refrigerated centrifuge
  - Shaker

Procedure

1. Wash the cells off with their existing medium and transfer them to a centrifuge tube.
2. Centrifuge 4,000×g, 5 min, 4 °C.
3. Discard the supernatant. Depending on pellet size, add 50–100 µL urea buffer (8 M urea, 500 mM Tris-HCl pH 8.5) and 1 µL protease inhibitor.
4. Shake vigorously at room temperature for 1 h.
5. Centrifuge 10,000 rpm, 30 min, 4 °C to remove cell debris.
6. Transfer the supernatant to a new tube.

Urea Step-Gradient Extraction (Bacterial Pellet)

Adapted from: Urea Protein Extraction (bacterial pellet) protocol

Introduction

Sequential extraction of poorly soluble (liposoluble) proteins from a previously extracted bacterial pellet with increasing urea concentrations, to find the concentration that solubilizes the protein best.

Materials

- **Reagents**
  - Urea
  - HEPES-NaCl buffer
- **Equipment**
  - Tube rotator
  - Centrifuge (5,000 rcf)
  - SDS-PAGE set-up

Procedure

1. Prepare 8 M urea by dissolving it in HEPES-NaCl buffer.
2. From the 8 M solution, prepare 1 M, 2 M, 4 M and 6 M dilutions.
3. Add 15 mL of the 1 M solution to the (previously extracted) bacterial pellet. Incubate 20 min on a tube rotator.
4. Centrifuge 5,000 rcf, 20 min.
5. Collect the liquid phase in a labelled tube.
6. Add 15 mL of the 2 M solution and repeat steps 3–5.
7. Continue in ascending order until the 8 M solution.
8. Run SDS-PAGE of all fractions to see at which urea concentration the protein is extracted best.

   NOTE Once the ideal urea concentration is defined, extract with that concentration only.

Acetone Protein Precipitation

Adapted from: Protein Precipitation protocol

Introduction

Concentrates protein and removes interfering salts and detergents before reconstitution in urea buffer for digestion.

Materials

- **Reagents**
  - Acetone, chilled
  - Urea buffer (8 M urea, 500 mM Tris-HCl pH 8.5)
- **Equipment**
  - −80 °C and −20 °C freezers
  - Centrifuge

Procedure

1. Transfer the sample volume to a new tube and add 4 volumes of chilled acetone.
2. Incubate at −80 °C for 30 min.
3. Incubate at −20 °C overnight.
4. Centrifuge 10,000 rpm, 30 min.
5. Discard the supernatant and leave the tube at room temperature to evaporate the remaining acetone.
6. Reconstitute the pellet in 100 µL urea buffer.

Flow Cytometry Surface Staining (General)

Adapted from: Staining of Cells for Flow Cytometry Analysis V1 (EN)

Introduction

General surface-marker staining of MSCs, HSPCs or MNCs with the lab's pre-defined antibody panels; refer to the panel sheets for antibody preparation.

Materials

- **Reagents**
  - DPBS 1× (Gibco #14190-094)
  - Isotone (Beckman Coulter #8546859)
  - Antibody panel(s)
- **Equipment**
  - FACS tubes (Sarstedt #551579)
  - Flow cytometer (e.g. FACSAria III, ImageStream, CytoFLEX)
  - Refrigerated centrifuge

Procedure

1. Check the availability of the cytometer; prepare the cells (from culture or a functional assay).
2. Label the tubes according to the panel list.
3. Add the antibodies to the bottom of the tube.
4. Add the cells on top of the antibodies in 50–100 µL (MSCs ≥ 1×10^5 per tube; HSPCs or MNCs 50 µL).
5. Vortex carefully; incubate 20–30 min at 4 °C.
6. Fill the tube with PBS (or Isotone); centrifuge 900×g, 5 min, 4 °C.
7. Discard the supernatant and resuspend the pellet; measure directly or store briefly at 4 °C.

11 SECTION

Recipes & Buffers



Bacterial & Yeast Culture — Starters & Glycerol Stocks

Introduction

Routine liquid-culture and stock-keeping for the *E. coli* (plasmid) and *S. cerevisiae* (EV production) strains used in the project.

Materials

- **Reagents**
  - YPD or LB broth (+ antibiotic for plasmid strains)
  - Agar plates
  - 50% glycerol (sterile)
- **Equipment**
  - 30 °C (yeast) / 37 °C (E. coli) shaking incubators
  - Baffled / culture tubes
  - Cryovials, −80 °C

Procedure

Starters

1. *E. coli*: inoculate 3–5 mL LB + antibiotic from a single colony or glycerol stock; 37 °C, 200 rpm, overnight.
2. *S. cerevisiae*: inoculate 5 mL YPD from a fresh colony; 30 °C, 200 rpm, 8–10 h; scale up into a 250 mL YPD seed overnight.

   NOTE Keep yeast plates ≤ 2 weeks old — older plates give lagging, heterogeneous cultures.

Glycerol Stocks

3. Mix 500 µL overnight culture + 500 µL 50% glycerol in a cryovial (25% final); snap-freeze and store −80 °C.

YPD Medium & SD Minimal Medium

Introduction

Growth and conditioning media for *S. cerevisiae* EV production. SD minimal is 0.22 µm-filtered, never autoclaved with the glucose.

Materials

- **Reagents**
  - Yeast extract, peptone, glucose (YPD)
  - YNB without amino acids, glucose, auxotrophic supplements (SD)
  - Agar (for plates)

Procedure

1. Assemble the following reagents in the given proportions.

Media

	A	B
1	**Medium**	**Composition**
2	YPD	1% yeast extract, 2% peptone, 2% glucose
3	YPD agar	YPD + 2% agar
4	SD minimal	0.67% YNB (no amino acids), 2% glucose, + auxotrophic supplements; 0.22 µm-filtered

2. Mix thoroughly, label, and store as noted.

Conjugation Buffer

Introduction

0.1 M sodium phosphate / 0.15 M NaCl / 5 mM EDTA, pH 7.00 — for the thiol–maleimide coupling. pH 7.0 (not 7.4) keeps maleimide from hydrolysing; EDTA chelates trace metals that oxidize thiols. Dissolve in 45 mL water, adjust to pH 7.00, bring to 50 mL, 0.22 µm filter, store 4 °C; degas before use.

Materials

- **Reagents**
  - $NaH_2PO_4·H_2O$
  - Na_2HPO_4 (anhydrous)
  - NaCl
  - $EDTA·2Na·2H_2O$
  - MilliQ water

Procedure

1. Assemble the following reagents in the given proportions.

Conjugation Buffer (50 mL) ^

	A	B
1	**Component**	**Mass**
2	$NaH_2PO_4·H_2O$	0.276 g
3	Na_2HPO_4 (anhydrous)	0.426 g
4	NaCl	0.438 g
5	$EDTA·2Na·2H_2O$	0.093 g

2. Mix thoroughly, label, and store as noted.

TCEP Stock (10 mM)

Introduction

Neutralized reducing agent for the nanobody Cys thiol. Equilibrate the TCEP·HCl bottle to RT before opening; titrate to pH 7.0; aliquot single-use and freeze. TCEP·HCl is strongly acidic — neutralize it or it drags the reaction below pH 6.

Materials

- **Reagents**
  - TCEP·HCl
  - 1 M NaOH
  - MilliQ water

Procedure

1. Assemble the following reagents in the given proportions.

TCEP 10 mM (5 mL) ^

	A	B
1	**Step**	**Detail**
2	Weigh	14.3 mg TCEP·HCl
3	Dissolve	4 mL water
4	Neutralize	1 M NaOH dropwise to pH 7.0 (~200 µL)
5	Bring to	5 mL
6	Aliquot	20 µL / PCR tube, −20 °C, single use

2. Mix thoroughly, label, and store as noted.

EV Buffers (PEG 6000, NaCl, HEPES/NaCl)

Introduction

Stock solutions for PEG-based EV isolation. Autoclave each solution.

Materials

- **Reagents**
  - PEG 6000
  - NaCl
  - HEPES (1 M)
  - Distilled water

Procedure

1. Assemble the following reagents in the given proportions.

EV Isolation Buffers

	A	B
1	**Buffer**	**Preparation**
2	PEG 6000 (50% w/v)	100 g PEG 6000 → 200 mL dist. water (stir, optional 60 °C); autoclave
3	3.75 M NaCl	43.82 g NaCl → 200 mL dist. water; autoclave
4	0.9% NaCl	1.8 g NaCl → 200 mL dist. water; autoclave
5	10 mM HEPES / 0.9% NaCl	49.5 mL 0.9% NaCl + 500 µL 1 M HEPES

2. Mix thoroughly, label, and store as noted.

Western-Blot Buffers

Introduction

Buffer and reagent set for SDS-PAGE and Western transfer. Make 10% APS fresh daily.

Materials

- **Reagents**
  - Tris base
  - 6 N HCl
  - Ammonium persulfate
  - NaCl
  - Tween-20
  - Non-fat milk
  - Power Blotter 5× transfer buffer
  - MilliQ water
  - Glycine
  - SDS
  - Methanol
  - BSA
  - Ponceau S
  - Acetic acid
  - β-Mercaptoethanol

Procedure

1. Assemble the following reagents in the given proportions.

Western-Blot Buffers

	A	B
1	**Buffer**	**Preparation**
2	1.5 M Tris-HCl pH 8.8 (50 mL)	9.07 g Tris base, 40 mL water, pH 8.8 w/ 6 N HCl, to 50 mL
3	0.5 M Tris-HCl pH 6.8 (50 mL)	3 g Tris base, 40 mL water, pH 6.8 w/ 6 N HCl, to 50 mL
4	10% APS (fresh daily)	0.10 g ammonium persulfate in 1 mL water
5	10× TBS	12 g Tris base + 44 g NaCl in 400 mL MilliQ, pH 7.6, to 500 mL
6	1× TBST (0.1% Tween)	100 mL 10× TBS + 900 mL MilliQ + 1 mL Tween-20
7	Blocking (5% milk)	2.5 g non-fat milk to 50 mL TBST
8	1× transfer buffer (300 mL)	60 mL Power Blotter 5× to 300 mL

2. Additional buffers for the Western Blot protocol (wet and semi-dry transfer, stripping). Where a buffer above also appears in a source SOP, the values above are kept.

Additional Western-Blot Buffers

	A	B
1	**Buffer**	**Preparation**
2	1 M Tris-HCl pH 6.8 (500 mL) — stacking gel	60.5 g Tris in ~300 mL dH_2O, pH 6.8 with HCl, to 500 mL; 12 months
3	10% SDS (500 mL)	50 g SDS pellets in ~400 mL dH_2O, to 500 mL; 12 months
4	10× SDS running buffer, pH 8.3 (1 L)	30 g Tris (25 mM) + 144 g glycine (192 mM) + 10 g SDS (0.1% at 1×), to 1 L dH_2O; RT, 12 months; remake if flocculent
5	10× wet transfer buffer, pH 8.3 (1 L)	30.3 g Tris (25 mM) + 144 g glycine (192 mM), to 1 L dH_2O; do not adjust pH; RT
6	1× wet transfer buffer (1 L)	100 mL 10× transfer buffer + 200 mL methanol, to 1 L dH_2O; pre-chill at 4 °C
7	Semi-dry transfer buffer (1 L)	3.03 g Tris + 14.4 g glycine + 1 g SDS + 200 mL methanol + 500 mL MilliQ; pH 8.3–8.5; to 1 L MilliQ
8	Membrane wash solution (25 mM Tris, 5% methanol)	0.3 g Tris + 5 mL methanol + 100 mL MilliQ
9	Blocking (5% BSA)	5% BSA in TBST
10	Ponceau S stain	1% Ponceau S, 1% acetic acid
11	Medium stripping solution	15 g glycine, 1 g SDS, 10 mL Tween 20
12	High stripping solution (100 mL)	20 mL 10% SDS + 12.5 mL Tris-HCl pH 6.8 + 67.5 mL MilliQ + 0.8 mL β-ME

NOTE Corrected from source: one SOP lists 5 g SDS per litre of 10× running buffer, which gives 0.05% at 1×; 10 g gives the stated 0.1%. Its 10× TBS amounts were swapped — the 10× TBS above is correct.

3. Mix thoroughly, label, and store as noted.

Annexin V Binding Buffer

Introduction

Calcium-containing buffer for Annexin V staining. Binding is strictly Ca^{2+}-dependent — no EDTA may contact the cells after staining.

Materials

- **Reagents**
  - HEPES (pH 7.4)
  - NaCl
  - $CaCl_2$
  - MilliQ water

Procedure

1. Assemble the following reagents in the given proportions.

1× Annexin V Binding Buffer ^

	A	B
1	**Component**	**Final concentration**
2	HEPES pH 7.4	10 mM
3	NaCl	140 mM
4	$CaCl_2$	2.5 mM

2. Mix thoroughly, label, and store as noted.

4× Laemmli Sample Buffer (Reducing)

Adapted from: 4× Laemmli reducing buffer recipe

Introduction

Reducing SDS sample buffer for Western blots — 8% SDS, 40% glycerol, 200 mM DTT, pH 6.8. For non-reducing samples use the ready-made 4× Laemmli (Bio-Rad #1610747) without reductant. Add ¼ of the final sample volume.

Materials

- **Reagents**
  - Tris-HCl
  - SDS
  - Glycerol
  - DTT
  - Bromophenol blue
  - Distilled water

Procedure

1. Assemble the following reagents in the given proportions.

4× Laemmli Reducing Buffer (30 mL)

	A	B	C
1	**Component**	**Amount**	**Final (4×)**
2	Tris-HCl	1.182 g	—
3	SDS	2.4 g	8% SDS
4	Glycerol	12 mL	40% glycerol
5	DTT	0.9255 g	200 mM DTT
6	Distilled water	to 25 mL	
7	Adjust pH	pH 6.8	before adding bromophenol blue
8	Distilled water	to 30 mL	
9	Bromophenol blue	~6 mg	

CRITICAL Adjust the pH before adding bromophenol blue.

NOTE Corrected from source: the large-format Western blot SOP lists “0.9255 mg DTT”; 0.9255 g gives the stated 200 mM in 30 mL (as in the stand-alone recipe).

2. Prepare 1 mL aliquots and freeze at −20 °C.

Lysis Buffers (RIPA, NP-40, Protease Inhibitor)

Adapted from: BCA Protein Assay V2 (translated from German)

Introduction

Cell-lysis buffers for the BCA Protein Assay and Western blots. Dissolve in ~70 mL (RIPA) or ~300 mL (NP-40) dH_2O before making up to volume. Store at 4 °C, stable 12 months; add protease inhibitor fresh.

Materials

› Reagents

- NaCl (Merck #106406); Na_2EDTA (Serva #11280)
- Tris (Trizma base, Sigma #T1503); HCl
- Sodium deoxycholate (Sigma #D6750); SDS pellets (Roth #CN30.3)
- Triton X-100 (Fluka #93420); NP-40
- cOmplete Mini protease inhibitor tablets (Roche #11836170001)

Procedure

1. Assemble the following reagents in the given proportions.

4× RIPA Lysis Buffer (100 mL) ^

	A	B	C
1	**Component**	**Amount**	**Stated concentration**
2	NaCl	0.876 g	600 mM
3	EDTA	0.02 g	4 mM
4	Tris-HCl	2.5 mL	40 mM
5	Na-deoxycholate	4 g	4%
6	Triton X-100	4 mL	4% (v/v)
7	SDS	0.4 g	0.4%
8	A. dest.	to 100 mL	

Alternative: NP-40 Lysis Buffer, pH 8 (500 mL) ^

	A	B
1	**Component**	**Amount**
2	NaCl	4.38 g
3	Na_2EDTA	0.74 g
4	Tris	3.0 g
5	NP-40	2.5 mL
6	pH	adjust to 8 with HCl
7	A. dest.	to 500 mL

NOTE As written in the source. The NaCl, EDTA and Tris amounts work out to about 150 mM, 0.5 mM and 25 mM (1×) rather than the stated 4× values — confirm before relying on the concentrations.

2. Protease inhibitor 10×: dissolve 1 tablet in 1 mL dH_2O; store 100 µL aliquots at −20 °C. Working lysis buffer, fresh: 900 µL lysis buffer + 100 µL 10× protease inhibitor.
3. Mix thoroughly, label, and store as noted.

Electrophoresis Buffer Formulations (Bio-Rad Bulletin 6199)

Adapted from: Bio-Rad Bulletin 6199, Buffer Formulations (excerpt of the Electrophoresis Guide, Bulletin 6640)

Introduction

Reference formulations for sample preparation, gel casting, sample buffers and running buffers. Bio-Rad catalog numbers are given for the premade equivalents. Where the same buffer appears on Western-Blot Buffers, use that page for routine work.

Materials

Reagents

- Tris base, glycine, Tricine, SDS, HCl
- Acrylamide, N,N′-bis-methylene-acrylamide
- Glycerol, bromophenol blue, Coomassie Blue G-250, β-mercaptoethanol, DTT
- NaCl, KCl, Na_2HPO_4, KH_2PO_4, EDTA, NP-40 or Triton X-100, sodium deoxycholate
- Urea, thiourea, CHAPS, Bio-Lyte® ampholytes, TCA, acetone, n-butanol

Procedure

1. Sample preparation buffers.

Sample Preparation Buffers

	A	B	C
1	**Buffer**	**Preparation**	**Storage**
2	1 M Tris-HCl, pH 7.6 (100 mL)	12.11 g Tris base, 80 mL diH_2O; pH 7.6 with HCl; to 100 mL	
3	0.5 M Tris-HCl, pH 6.8 (100 mL; #161-0799)	6.06 g Tris base, ~60 mL diH_2O; pH 6.8 with HCl; to 100 mL	4 °C
4	10% SDS (10 mL; #161-0416)	1.00 g SDS, diH_2O to 10 mL	
5	1.0% bromophenol blue (10 mL; #161-0404)	100 mg bromophenol blue, diH_2O to 10 mL	
6	RIPA solubilization buffer (100 mL) — 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS	0.88 g NaCl, 0.15 g EDTA, 1 g NP-40 or Triton X-100, 1 g sodium deoxycholate, 0.10 g SDS, 80 mL diH_2O, 2.5 mL 1 M Tris-HCl pH 7.6; diH_2O to 100 mL	
7	PBS (1 L) — 0.9% NaCl in 10 mM phosphate, pH 7.4	8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 800 mL diH_2O; pH 7.4 with HCl or NaOH; to 1 L	
8	SDS-PAGE sample buffer 2× (8 mL; #161-0737) — 62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue	1.0 mL 0.5 M Tris-HCl pH 6.8, 2.0 mL glycerol, 0.08 mL 1.0% bromophenol blue, 1.6 mL 10% SDS, 2.92 mL diH_2O; add β-ME (0.4 mL) or 3% DTT immediately before use	1–2 mL aliquots, −70 °C

	A	B	C
1	**Buffer**	**Preparation**	**Storage**
9	Protein precipitation solution (100 mL) — 20% TCA, 0.2% DTT in ice-cold acetone	20.00 g TCA + 0.20 g DTT in 80 mL acetone; dissolve; acetone to 100 mL	−20 °C
10	Wash solution (100 mL) — 0.2% DTT in ice-cold acetone	0.20 g DTT in 80 mL acetone; dissolve; acetone to 100 mL	−20 °C
11	Lysis buffer (50 mL) — 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% carrier ampholytes pH 3–10	21.00 g urea + 7.60 g thiourea, diH_2O to 45 mL; add 2.00 g CHAPS, 1.0 mL Bio-Lyte® ampholytes, 0.50 g DTT; diH_2O to 50 mL	
12	SDS sample solubilization buffer (50 mL) — 1% SDS, 100 mM Tris-HCl pH 9.5	0.50 g SDS + 0.60 g Tris base in 40 mL diH_2O; titrate to pH 9.5 with dilute HCl; to 50 mL	

2. Gel casting reagents.

Gel Casting Reagents ^

	A	B	C
1	**Reagent**	**Preparation**	**Storage**
2	Acrylamide/bis, 30% T, 2.67% C (300 mL)	87.60 g acrylamide + 2.40 g bis-acrylamide, diH_2O to 300 mL; filter	4 °C, dark, 30 days (premade: #161-0125 powder, #161-0158 solution)
3	1.5 M Tris-HCl, pH 8.8 (150 mL; #161-0798)	27.23 g Tris base, 80 mL diH_2O; pH 8.8 with 6 N HCl; to 150 mL	4 °C
4	0.5 M Tris-HCl, pH 6.8 (100 mL; #161-0799)	6.00 g Tris base, 60 mL diH_2O; pH 6.8 with 6 N HCl; to 100 mL	4 °C
5	10% (w/v) SDS (100 mL; #161-0416)	10.00 g SDS in 90 mL diH_2O, dissolve with gentle stirring; to 100 mL	
6	10% (w/v) APS	0.10 g ammonium persulfate in 1 mL diH_2O	fresh daily
7	Water-saturated n-butanol	50 mL n-butanol + 5 mL diH_2O; shake; use the top phase only	RT

3. Sample buffers (2×, 30 mL).

Sample Buffers (30 mL) ^

	A	B	C
1	**Buffer**	**Composition**	**Preparation**
2	2× SDS-PAGE (Laemmli; #161-0737)	62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% β-ME (added fresh)	3.75 mL 0.5 M Tris-HCl pH 6.8, 15.0 mL 50% glycerol, 0.3 mL 1.0% bromophenol blue, 6.0 mL 10% SDS, diH_2O to 30 mL; before use add β-ME (50 µL to 950 µL sample buffer)
3	2× Native PAGE (#161-0738)	62.5 mM Tris-HCl pH 6.8, 40% glycerol, 0.01% bromophenol blue	3.75 mL 0.5 M Tris-HCl pH 6.8, 24.0 mL 50% glycerol, 0.3 mL 1.0% bromophenol blue, diH_2O to 30 mL
4	2× Tricine (#161-0739)	200 mM Tris-HCl pH 6.8, 2% SDS, 40% glycerol, 0.04% Coomassie Blue G-250, 2% β-ME (added fresh)	12.0 mL 0.5 M Tris-HCl pH 6.8, 24.0 mL 50% glycerol, 6.0 mL 10% SDS, 12.00 mg Coomassie Blue G-250, diH_2O to 30 mL; before use add β-ME (20 µL to 980 µL sample buffer)

4. Running buffers (10×, 1 L). Do not adjust the pH (~8.3).

Running Buffers (10×, 1 L) ^

	A	B	C
1	**Buffer**	**Composition**	**Preparation**
2	10× SDS-PAGE (#161-0732)	250 mM Tris, 1.92 M glycine, 1% SDS, pH 8.3	30.30 g Tris base, 144.10 g glycine, 10.00 g SDS, diH_2O to 1 L
3	10× Native PAGE (#161-0734)	250 mM Tris, 1.92 M glycine, pH 8.3	30.30 g Tris base, 144.10 g glycine, diH_2O to 1 L
4	10× Tris-Tricine (#161-0744)	1 M Tris, 1 M Tricine, 1% SDS, pH 8.3	121.10 g Tris base, 179.20 g Tricine, 10.00 g SDS, diH_2O to 1 L

5. Mix thoroughly, label, and store as noted.

Metabolomics Solvents & LC-MS Mobile Phases

Introduction

Extraction and reconstitution solvents for the Metabolomics section. Pre-cool the extraction solvent at −80 °C for at least 2 h before use.

Materials

- **Reagents**
  - Methanol, dichloromethane, ethyl acetate
  - Water, acetonitrile
  - Formic acid; ammonium formate; ammonium hydroxide

Procedure

1. Assemble the following reagents in the given proportions.

Metabolomics Solvents

	A	B	C
1	**Solvent**	**Composition**	**Notes**
2	Extraction solvent	methanol : dichloromethane : ethyl acetate 10:2:3	−80 °C ≥ 2 h before use; sample : solvent 2:10 (culture) or 200 µL : 900 µL (saliva)
3	Reconstitution solvent	water : methanol : acetonitrile 2:1:1	2 mL (culture) or 200 µL (saliva)
4	Mobile phase, positive mode	DI water containing 0.1% formic acid	
5	Mobile phase, negative mode	5 mM ammonium formate containing 1% methanol	pH adjusted to 8 with ammonium hydroxide

2. Mix thoroughly, label, and store as noted.

Cell-Culture & Cryo Media

Introduction

Media and supplement solutions for the MSC, MNC/MLR and hPL protocols. Keep MLR media on ice.

Materials

› Reagents

- DMEM low glucose; DMEM high glucose with L-glutamine; RPMI
- Platelet lysate (PL); FCS; human AB serum
- Pen/Strep (/Glutamine); sodium pyruvate; heparin
- DMSO; Benzonase; BD Pharm Lyse
- $CaCl_2$; 0.9% NaCl

Procedure

1. Assemble the following reagents in the given proportions.

Media & Supplement Solutions ^

	A	B	C
1	**Medium**	**Composition**	**Used in**
2	MSC medium	DMEM low glucose + 10% PL (centrifuged 10,000×g, 45 min) + 1% Pen/Strep/Glutamine + 0.1% heparin	MSC Isolation
3	MSC freezing medium	20% DMSO in PL	MSC Isolation
4	MSC differentiation culture medium	DMEM high glucose with L-glutamine + 10% FCS or PL (with PL: + 0.1% heparin) + 1% PenStrep + 1% NaPy	MSC Differentiation
5	MLR cultivation medium	RPMI + 10% hAB serum (heat-inactivated) + 1% PenStrep; keep on ice	MLR
6	MLR thawing medium	MLR cultivation medium + Benzonase 1:10,000 (1 µL per 10 mL); keep on ice	MLR
7	1× red-cell lysis buffer	100 mL BD Pharm Lyse (#555899) + 900 mL distilled water	MNC Pool
8	MNC freezing medium	FCS + 10% DMSO	MNC Pool
9	2 M $CaCl_2$ (200 mL)	58.8 g $CaCl_2$ in 150 mL 0.9% NaCl; stir until dissolved; to 200 mL with 0.9% NaCl; sterile-filter (0.2 µm bottle-top)	Fibrin-depleted hPL

2. Mix thoroughly, label, and store as noted.