Protocol for culturing iPSCs

General Notes

All cell handling **except thawing and centrifugation** is performed inside a **biological safety cabinet (BSC)**. Keep all reagents and plasticware sterile. We culture iPSCs **without antibiotics** whenever possible, so maintaining sterility is essential. Always use **sterile filter tips** when pipetting. Be careful not to have cell outside of incubator for too long to reduce the time on non-physiological temperature and decrease the chances of contamination and exposure to the air, which changes the pH of the medium. Most cell-culture media contain phenol red (a pH indicator) and its colour changes as follows:

Acidic (pH drops, e.g., heavy cell metabolism, contamination)
→ Turns yellow
Phenol red becomes its acidic form, which is yellow at low pH.

Physiological pH (~7.2–7.4)
→ Orange / reddish-orange
This is what healthy, equilibrated medium should look like in the incubator.

Basic / alkaline (pH rises, e.g., too much CO_2 loss, poor buffering, opened bottle)
→ Bright pink → purple
High pH shifts phenol red to its alkaline form, which is pink/magenta.

Media and Reagents

Reagent	Manufacturer	Catalogue No.	Storage
Vitronectin XF	StemCell Technologies	07180	Aliquoted, −20°C
Vitronectin (VTN-N) Recombinant Human Protein, truncated, 1 mL	Gibco	A400457	Aliquoted, −80°C
DPBS (+) with Mg^{2+}/Ca^{2+}	Sigma-Aldrich	D8662	Aliquoted, +4°C
DPBS (–) without ions	Sigma-Aldrich	D8537	Aliquoted, +4°C
TeSR-AOF medium	StemCell Technologies	100-0401	Aliquoted, −20°C; working aliquot in fridge

Reagent	Manufacturer	Catalogue No.	Storage
Rock Inhibitor (ROCKi) Y-27632 (Dihydrochloride)	StemCell Technologies	72304	Aliquoted (10 mM), -20ºC.
Gentle Cell Dissociation Reagent (GCDR)	StemCell Technologies	100-0485	Room temperature
EZ-Lift	Millipore	SCM139	+4°C
CryoStor CS10	StemCell Technologies	07930	+4°C
Normocin	InvivoGen	ant-nr-1	Aliquoted, −20°C

Note on TeSR-AOF

We aliquot TeSR-AOF into 50-ml tubes and store them at −20°C or +4°C (when in use). Although the manufacturer recommends warming the medium in a water bath, we instead let it warm to **room temperature for ~1 hour**, because elevated temperatures can degrade growth factors (e.g., FGF2). TeSR-AOF is animal-origin-free, cGMP, and supports both daily and reduced feeding schedules.

Plate Coating

We use various coatings (Matrigel, iMatrix, Vitronectin), but Vitronectin XF is currently preferred due to its defined, xeno-free composition and batch consistency.

Vitronectin XF (VN) – from StemCell

- For one well of a 6-well plate:
  1 ml DPBS (–) + **40 µl Vitronectin XF**
- Incubate **1 hour at room temperature**.
- After incubation, tilt plate to remove coating solution from the edge and **immediately add medium** to prevent drying.

Vitronectin VTN-N (VN) – from Gibco

- For one well of a 6-well plate:
  1 ml DPBS (–) + **10 µl Vitronectin**
- Incubate **1 hour at room temperature**.
- After incubation, tilt plate to remove coating solution from the edge and **immediately add medium** to prevent drying.

Matrigel (MG)

- Thaw aliquot on ice.
- Prepare dilution in DMEM/F12 to **0.34 mg/ml** (concentration varies by batch).
- Add **750 µl** per well of a 6-well plate.
- Incubate **30 min at 37°C**.
- Remove excess by tilting plate, then immediately add medium.

Cell Thawing

1. Coat a 6-well plate with VN (as above). Write down on plate: cell line name, passage, date of seeding, your initials, and type of coating. For passage nr. make sure it is the same passage as written on the vial.
2. Bring needed reagents to room temperature and prepare a 15-ml tube containing **5 ml DPBS (+)**.
3. Retrieve the cryovial from liquid nitrogen only when everything is ready. Transport on dry ice.
4. Thaw vial in a **37°C water bath** or incubator. Do **not** wait until completely thawed; transfer to BSC while a small ice chunk remains.
5. Transfer cell suspension into the DPBS (+) tube.
6. Centrifuge at **1500 rpm, 5 min**, room temperature.
7. Meanwhile, remove VN from the coated well and replace with **1.5 ml TeSR-AOF + 2 µl ROCKi**. ROCKi improves attachment; morphology next day may look more star-shaped. Replace medium the next day without ROCKi.
8. After centrifugation, aspirate supernatant and gently resuspend the pellet in **500 µl TeSR-AOF**. Slight clumping is normal—avoid very large clumps or a fully single-cell suspension.
9. Plate desired volume into each well.
   - How much you plate really depends on how many cells were originally frozen in the vial. In most cases, because we don't always know the exact number of cells in older vials, we usually assume that one vial contains roughly enough cells for one well of a 6-well plate. So the most common approach is simply to plate the entire contents of one vial into one well (**1 vial → 1 well**).
   - If you know the number of cells in the vial or if you have counted them, you can split them more precisely. In that case, people often use a 1:6 ratio (meaning that the amount of cells from one well or one vial can be divided into six new wells). This ratio is also commonly used when passaging cells, but again it depends on how many cells you have and how confluent you want them to be after plating.

    - Over time we are trying to standardize our freezing protocol to always freeze ~300,000 cells per vial. If this standardization works, then we'll know that one vial consistently gives enough cells for exactly one well, making plating much more predictable and reproducible. Until then, the "1 vial → 1 well" rule is a safe default.

10. Incubate at **37°C, 5% CO_2**.
    - We previously used hypoxic conditions (5% O_2), but standard incubators (**20% O_2**) are also fine and support faster growth without noticeable spontaneous differentiation.

Medium Changes

We change medium every **second day**.
From Monday–Thursday we use **2 ml** per well; on Friday we use **3 ml** to support the weekend.

1. Let all reagents warm to room temperature (~1 h).
2. Aspirate old medium and gently wash cells with **1 ml DPBS (+)**.
3. Add fresh medium (2 or 3 ml depending on the day).
4. Return plate to incubator.

Note on Antibiotics

We normally avoid antibiotics because they can:

- mask low-grade contamination,
- alter gene expression, proliferation, differentiation, or cell survival.

For long-term protocols like **skin organoid differentiation**, antibiotics are needed due to higher risk of contamination.
We use **Normocin** (active against mycoplasma, bacteria, fungi).

- Stock: 50 mg/ml
- Working: dilute **1:500** → e.g., add **4 µl** Normocin to **2 ml medium**.

Normocin **prevents** contamination; it does **not** cure existing contamination.
For precious cultures, stronger agents (Plasmocin, Normocure, Fungin, etc.) are used cautiously.
Expect slightly increased cell death with antibiotic use.

Cell Passaging

Passage cells at **70–80% confluence** or when differentiation is visible.
We use either **GCDR** (standard passaging) or **EZ-Lift** (removal of differentiating cells).

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1. GCDR Passaging

1. Prepare a VN-coated target plate. Write down on plate: cell line name, passage, date of passage, your initials, and type of coating. For passage nr. make sure it is one passage higher than currently written on plate.
2. Warm reagents and prepare one 15-ml centrifuge tube.
3. Aspirate medium and wash the cells **twice** with **1 ml DPBS (–)**. (DPBS (–) helps detach cells.)
4. Add **1 ml GCDR** per well.
5. Incubate:
   - **6–12 min** on VN
   - **6–8 min** on Matrigel
6. Prepare new culture wells with **1 ml TeSR-AOF + 1 µl ROCKi**.
7. After ~6 min, check under microscope. Colonies should look "whitish" and loosened.
   - If still tightly attached → incubate a bit longer.
   - If already floating → follow the backup procedure (*) below.
8. Remove GCDR and add **1 ml TeSR-AOF + 1 µl ROCKi**.
9. Detach cells by gentle pipetting and transfer to a 15-ml tube. Rinse the well with another 1 ml medium if needed.
10. Break up large clumps gently.
11. Seed desired number of cells.
    - For counting, mix 10 µl cells + 10 µl Trypan Blue and count with **Countess II (Full-gating iPSC protocol)**.
    - Recommended: **200,000 cells** per well for weekly passaging; **300,000 cells** for freezing.
12. Gently shake plate to distribute cells evenly.
13. Incubate.
14. Replace ROCKi-containing medium the following day.

(*) Backup procedure if cells over-detach

1. Transfer cells + GCDR directly into 15-ml centrifuge tube and top-up with **5 ml DPBS (+)**.
2. Centrifuge **1500 rpm, 5 min**.

3. Remove supernatant and resuspend in **1 ml TeSR-AOF + 1 µl ROCKi**.
4. Plate as usual.

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2. EZ-Lift Passaging (Selective removal of differentiated cells)

1. Prepare VN-coated target plate. Write down on plate: cell line name, passage, date of passage, your initials, and type of coating. For passage nr. make sure it is one passage higher than currently written on plate.
2. Warm reagents and prepare a 15-ml tube with **5 ml DPBS (+)**.
3. Aspirate medium and wash cells **twice** with **1 ml DPBS (–)**.
4. Add **1 ml EZ-Lift** per well and incubate **4 min at 37°C**.
5. Tap the bottom of the well **20–25 times in ~5 seconds**.
6. Incubate another **4 min**, then tap again.
7. Transfer the entire content (do **not** wash the well) into the DPBS (+) tube.
8. Centrifuge **1500 rpm, 5 min**.
9. Meanwhile, prepare new culture wells with **1 ml TeSR-AOF + 1 µl ROCKi**.
10. After centrifugation, remove supernatant and resuspend pellet in **1 ml TeSR-AOF + 1 µl ROCKi**.
11. Plate desired number of cells. Count if needed.
12. Gently shake to distribute.
13. Incubate.
14. Replace ROCKi-containing medium the next day.

Cell Freezing

1. Ensure **Mr. Frosty** is ready. If needed, thaw it in water bath and let it equilibrate to room temperature (~1 h).
2. Warm reagents and prepare a 15-ml tube with **5 ml DPBS (+)**.
3. Aspirate medium and wash cells **twice with DPBS (–)**.
4. Detach cells using GCDR or EZ-Lift protocols above.
5. Transfer cells into DPBS (+) tube and centrifuge **1500 rpm, 5 min**.

6. Label cryovials (cell line, passage, date, initials, coating; if you know, also nr. of cells). For passage nr. make sure it is one passage higher than currently written on plate.
7. Remove supernatant and resuspend pellet in **500 µl CryoStor per well**.
8. Transfer suspension to cryovials.
9. Place cryovials into **room-temperature Mr. Frosty** and freeze at **−80°C for at least 1 day.**
10. Transfer frozen vials to **liquid nitrogen** for long-term storage.