grow brain from skin
How to:

Chemical/Material	Supplier	Use	Details
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	Growth medium for fibroblast culture	Advanced DMEM. Includes glucose, essential amino acids, and vitamins.
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	Serum supplement for cell growth	Fetal Bovine Serum. Heat-inactivated, tested for cell culture applications.
Collagenase	Sigma-Aldrich	Enzyme for tissue digestion	Collagenase from Clostridium histolyticum. Used to release fibroblasts from tissue.
SB431542	STEMCELL Technologies	TGF-beta pathway inhibitor	SB431542. Encourages neural lineage commitment.
LDN193189	STEMCELL Technologies	BMP pathway inhibitor	LDN193189. Used in neural induction protocols.
Brain-Derived Neurotrophic Factor (BDNF)	PeproTech	Supports neuronal differentiation	BDNF. Enhances survival and differentiation of neurons.
mTeSR1 Medium	STEMCELL Technologies	Reprogramming medium for iPSCs	mTeSR1. Defined medium for maintenance of iPSCs in feeder-free conditions.
Matrigel	Corning	Coating for feeder-free culture	Matrigel Basement Membrane Matrix. Mimics extracellular matrix.
Antibodies (e.g., Nanog)	Cell Signaling Technology	Validation of iPSC pluripotency	Nanog (D73G4) XP® Rabbit mAb. Detects pluripotency.

Detailed Steps

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Fibroblast Isolation and Culture

Perform a skin biopsy or use other tissue sources.

Here is a comprehensive step-by-step guide for obtaining a skin biopsy, detailing all aspects of preparation, procedure, and post-procedure care. This process is performed under sterile conditions and typically requires trained personnel.

Item	Description
Sterile biopsy punch	Instrument for obtaining a circular sample (sizes range from 2 mm to 8 mm).
Scalpel or blade	For excisional biopsies if required.
Sterile gloves	To maintain aseptic technique.
Chlorhexidine or iodine	Antiseptic solution for cleaning the skin.
Sterile drapes	To maintain a sterile field.
Local anesthetic	Lidocaine 1% or 2% (with or without epinephrine).
Syringe and needle	1 mL or 3 mL syringe with a 25- to 30-gauge needle for anesthetic injection.
Sterile gauze	For blotting and cleaning during the procedure.
Forceps	For holding skin during biopsy.
Sutures or adhesive strips	For closing the wound if necessary.
Specimen container	Sterile container with appropriate medium (e.g., PBS, DMEM, or formalin) depending on use.
Adhesive bandage	ite aftTo cover the biopsy ser the procedure.

Prepare the Patient

- **Consent**: Explain the procedure to the patient, including risks, benefits, and expected outcomes. Obtain informed consent.
- **Positioning**: Position the patient to provide clear access to the biopsy site while ensuring comfort.
- **Mark the Biopsy Site**: Use a sterile skin marker to outline the area to be biopsied.

Create a Sterile Field

- Clean the biopsy site with an antiseptic solution such as chlorhexidine or iodine, starting at the center and moving outward in a circular motion.
- Drape the area with sterile drapes, exposing only the marked site.

Procedure
Administer Local Anesthetic

- Fill a syringe with lidocaine (1% or 2%).
- Inject a small amount intradermally at the biopsy site to create a wheal.
- Advance the needle deeper while injecting to anesthetize the dermis and subcutaneous tissue. Use aspiration before injecting to avoid intravascular injection.
- Wait 1–2 minutes for the anesthetic to take effect, testing for numbness by gently pricking the area with a sterile needle.

Punch Biopsy (Preferred for small samples):

- Select an appropriately sized biopsy punch (commonly 4 mm).
- Hold the punch perpendicular to the skin and press down while rotating the instrument in a circular motion.
- Continue until the punch reaches the subcutaneous fat layer (a slight loss of resistance is felt).
- Use sterile forceps to gently lift the tissue plug and cut it free from the base using scissors or a scalpel.

Shave Biopsy (Superficial layers only):

- Use a scalpel to shave off the epidermis and part of the dermis.
- Hold the blade parallel to the skin and use smooth, gliding motions.

Excisional Biopsy (Larger or deeper samples):

- Use a scalpel to excise the entire lesion or tissue sample.
- Extend the incision to include a margin of normal skin around the lesion.
- Remove the tissue with sterile forceps and scissors.

Handle the Specimen

- Immediately place the sample in a sterile container with the appropriate medium:
    - **For tissue culture**: Use DMEM or PBS with antibiotics.
    - **For histology**: Use 10% formalin.

Post-Procedure Wound Care

Close the Wound

- **Small punch biopsy sites (<4 mm)**: Leave to heal by secondary intention or close with adhesive strips.
- **Larger or excisional biopsy sites**:
    1. Suture with 4-0 or 5-0 nylon or absorbable sutures.
    2. Approximate edges with minimal tension.

Apply a Dressing

- Place sterile gauze over the site and secure it with adhesive tape or a bandage.
- Advise the patient to keep the area clean and dry for 24 hours.
- Instruct on daily dressing changes and signs of infection (redness, swelling, discharge).
- For sutures, schedule a removal appointment (7–10 days for facial areas, 10–14 days for other areas).

Post-Procedure Follow-Up

- Ensure the sample is transported to the laboratory under appropriate conditions (temperature, medium).
- Document the procedure, including site, method, and complications.

Enzymatically digest the tissue with **collagenase** at 37°C for 30–60 minutes to release fibroblasts.

Item	Purpose
Collagenase enzyme	Digest extracellular matrix (e.g., Collagenase Type I or Type II).
Dispase (optional)	Complementary enzyme to assist in separating the epidermis from the dermis.
DMEM or PBS	Buffer for preparing enzyme solution and washing tissue.

Sterile Petri dish	For tissue handling and preparation.
Scalpel and forceps	To mince and handle tissue.
Sterile culture tubes	For digestion and processing (15 mL or 50 mL tubes recommended).
Humidified incubator	Maintains 37°C for enzyme activity.
Centrifuge	To pellet released fibroblasts.
Sterile filter (40 µm)	Filters debris from the digested sample.

Protocol Steps

Prepare the Collagenase Solution

- Dissolve collagenase enzyme in **DMEM** or **PBS** to the desired concentration. Typical ranges:
  - **Collagenase Type I**: 0.5–1 mg/mL.
  - **Collagenase Type II**: 1–2 mg/mL (stronger digestion).
- Add antibiotics (e.g., penicillin-streptomycin, 100 U/mL each) to prevent contamination.
- Filter-sterilize the solution using a 0.22 µm filter.
- Pre-warm the solution to 37°C in a water bath.

Tissue Preparation

Mince Tissue:

- Place the biopsy sample in a sterile Petri dish.
- Using a scalpel, mince the tissue into small pieces (~1 mm^3) to increase the surface area for digestion.

Optional Epidermis Removal:

- If targeting fibroblasts only, treat the tissue with **Dispase** (2 U/mL in PBS) at 4°C overnight or 37°C for 1–2 hours to separate the epidermis.
- Peel off the epidermis with sterile forceps.

Enzymatic Digestion

- **Add Collagenase Solution**:
    - Transfer the minced tissue to a sterile 15 mL or 50 mL tube.
    - Add enough collagenase solution to submerge the tissue completely (e.g., 5 mL for small biopsies).
- **Incubation**:
    - Place the tube in a 37°C humidified incubator or a water bath.
    - Shake gently or agitate intermittently to enhance digestion.
    - Digest for 30–60 minutes, monitoring the progress every 15 minutes.
        - **Signs of Progress**: Tissue pieces become smaller and more translucent.

Termination of Digestion

- **Neutralize Collagenase Activity**:
    - Add an equal volume of **complete culture medium** (e.g., DMEM with 10% FBS) to stop enzymatic activity.
    - FBS contains protease inhibitors that neutralize collagenase.
- **Filter and Collect Cells**:
    - Pass the digested solution through a 40 μm sterile cell strainer into a fresh sterile tube to remove undigested debris.

Pellet the Cells

- **Centrifuge**:
    - Spin the filtered solution at **300g for 5 minutes** at room temperature.
- **Discard Supernatant**:
    - Carefully remove the supernatant without disturbing the cell pellet.

Resuspend Fibroblasts

- Resuspend the cell pellet in **complete culture medium** (DMEM with 10–15% FBS and antibiotics).
- Plate the cells in a sterile culture dish or flask.

Incubate and Expand

- Place the culture dish in a 37°C, 5% CO_2 incubator.
- Change the medium every 2–3 days.
- Observe for fibroblast attachment and growth under a microscope:

- **Morphology**: Fibroblasts appear elongated and spindle-shaped.

Notes

1. Over-digestion can damage cells; monitor closely during the incubation period.
2. Minimized tissue handling ensures higher cell viability.
3. For high fibroblast yield, optimize enzyme concentration and digestion time based on tissue type and biopsy size.

Wash and plate the fibroblasts in a **DMEM** medium supplemented with:

- **FBS**: 10–15%.
- **Penicillin-Streptomycin**: 100 U/mL each.

Item	Purpose
DMEM	Basal medium for fibroblast culture.
Fetal Bovine Serum (FBS)	Provides nutrients and growth factors.
Penicillin-Streptomycin	Prevents bacterial contamination.
Sterile centrifuge tubes	For washing fibroblast pellet.
Sterile culture dishes/flasks	For plating cells.
Micropipettes and sterile tips	For precise handling of solutions.
Centrifuge	For pelleting cells during washing steps.

	Humidified CO_2 incubator	Maintains optimal conditions (37°C, 5% CO_2) for cell growth.

Protocol Steps

Prepare the Culture Medium

Prepare Complete DMEM:

- Use sterile DMEM as the base medium.
- Add **10–15% Fetal Bovine Serum (FBS)**:
  - If unsure, start with 10%, as higher concentrations (15%) may benefit early attachment.
- Add **Penicillin-Streptomycin** at a final concentration of **100 U/mL each**:
  - Typically, add 1% of a 100x stock solution to the medium.
- Filter-sterilize the complete medium using a 0.22 μm sterile filter if needed.

Warm the Medium:

- Pre-warm the complete medium to 37°C in a water bath to prevent temperature shock to cells.

Wash the Fibroblast Pellet

Resuspend Pellet:

- Add 5–10 mL of sterile PBS or DMEM to the fibroblast pellet in the centrifuge tube.
- Pipette gently up and down to resuspend the cells completely.

Centrifuge:

- Spin the tube at **300g for 5 minutes** at room temperature to pellet the cells again.

Remove Supernatant:

- Carefully aspirate the supernatant without disturbing the pellet.

Repeat (if necessary):

- Perform a second wash if the pellet contains visible debris.

Resuspend in Complete DMEM

Add Complete Medium:

- Add 5–10 mL of the pre-warmed DMEM medium to the fibroblast pellet.
- Resuspend cells thoroughly by pipetting up and down, ensuring no clumps.

Cell Counting (Optional):

- Take a small aliquot (10 µL) and mix with trypan blue to count viable cells using a hemocytometer.
- Adjust cell density for plating:
  - Ideal density: **5,000–10,000 cells/cm²**.

Plate the Fibroblasts

Prepare Culture Dish/Flask:

- Use sterile culture-treated plasticware (e.g., T-25 flask, 6-well plates, or 10 cm dishes).
- Add a sufficient volume of the cell suspension:
  - **T-25 flask**: 5 mL of medium.
  - **6-well plate**: 2 mL per well.

Distribute Evenly:

- Gently swirl the plate or flask to evenly distribute the cells across the surface.

Incubate the Cells

Place in Incubator:

- Transfer the culture dish/flask to a **37°C, 5% CO_2 humidified incubator**.
- Avoid disturbing the dish for the first 24 hours to allow cells to adhere.

First Medium Change:

- After 24–48 hours, aspirate the old medium carefully, avoiding disturbing attached cells.
- Replace with fresh, pre-warmed DMEM medium (same formulation).

Subsequent Changes:

- Change the medium every 2–3 days, or as needed based on pH changes (medium turns yellow when acidic).

Monitor Cell Growth

Inspect Daily:

- Use a phase-contrast microscope to observe cell attachment and morphology.
- Fibroblasts should appear elongated and spindle-shaped once adhered.

Confluence:

- Allow the cells to grow until 70–80% confluence before passaging. Change the medium every 2–3 days and expand fibroblasts until confluent.

Reprogramming to iPSCs

Transfect fibroblasts with Yamanaka factors (**Oct4, Sox2, Klf4, c-Myc**) using a **viral system** (lentivirus preferred) or a **non-viral system** (episomal plasmids).

Item	Purpose
Fibroblasts	Source cells to be reprogrammed.
Yamanaka Factor Vectors	Plasmids encoding **Oct4, Sox2, Klf4, and c-Myc**.
Lentiviral Packaging Kit	For generating lentiviral particles (if using a viral system).
Transfection Reagent	Facilitates plasmid delivery (e.g., Lipofectamine, Fugene).
Complete DMEM Medium	Maintains fibroblast viability during transfection.
Biosafety Cabinet	For handling lentiviral particles in a sterile environment.
Humidified CO_2 Incubator	Maintains optimal conditions (37°C, 5% CO_2) during the experiment.
Antibiotic Selection Agents	For plasmid-based systems, e.g., puromycin.

Reagents for Lentiviral System

Reagent	Purpose
Lentiviral Vectors	Encode Yamanaka factors.
Packaging Plasmids	Provide essential viral proteins (e.g., pCMV-VSV-G, psPAX2).
HEK293T Cells	Host cells for producing lentiviral particles.
Transfection Reagent	For introducing vectors into HEK293T cells.

Prepare Fibroblasts for Transfection

Plate Fibroblasts:

- Seed fibroblasts in a 6-well plate or T-25 flask 24 hours before transfection.
- Ensure cells are **70–80% confluent** on the day of transfection.

Change Medium:

- Replace the medium with fresh complete DMEM (without antibiotics) 1–2 hours before transfection.

Choose Delivery System

A. Viral System (Lentivirus Preferred)

Produce Lentiviral Particles:

- Transfect HEK293T cells with the following plasmids:
  - Lentiviral vector encoding Yamanaka factors.
  - Packaging plasmids (e.g., psPAX2, pCMV-VSV-G).
- Use a transfection reagent (e.g., Lipofectamine) according to the manufacturer's instructions.
- Incubate HEK293T cells for 48–72 hours, collecting the supernatant containing lentiviral particles every 24 hours.
- Filter the supernatant through a 0.45 µm filter to remove cell debris.

Infect Fibroblasts:

- Add the filtered lentiviral supernatant to fibroblasts.
- Supplement with **polybrene** (4–8 µg/mL) to enhance infection efficiency.

- Incubate for 24 hours in a humidified CO_2 incubator.

Replace Medium:

- After 24 hours, replace the medium with fresh complete DMEM to remove excess viral particles.

Monitor for Expression:

- Transduction efficiency can be monitored using GFP or another marker (if included in the lentiviral vector).

Non-Viral System (Episomal Plasmids)
Prepare Episomal Plasmid Mixture:

- Use plasmids encoding Yamanaka factors with a non-integrating backbone.
- Typical episomal vectors include the pEP4 series (Addgene).

Transfect Fibroblasts:

- Dilute plasmid DNA (2–5 µg per well for a 6-well plate) in **serum-free DMEM**.
- Add transfection reagent (e.g., Lipofectamine 3000) to the diluted DNA at the recommended ratio.
- Incubate the DNA-reagent mixture at room temperature for 15–20 minutes to form complexes.

Add Transfection Complex:

- Replace the medium on fibroblasts with fresh complete DMEM (without antibiotics).
- Add the transfection complex dropwise to the fibroblasts.
- Incubate at 37°C in a humidified CO_2 incubator for 4–6 hours.

Replace Medium:

- After incubation, replace the medium with fresh complete DMEM.

Optional Selection:

- If plasmids include a selection marker (e.g., puromycin resistance), add the appropriate antibiotic to the medium after 48 hours.

Monitor Reprogramming
Daily Observations:

- Monitor cells daily for morphological changes indicative of iPSC reprogramming:

- Appearance of compact, tightly packed colonies with a high nucleus-to-cytoplasm ratio.

Passage Colonies:

- After 14–21 days, isolate colonies manually using a pipette or mechanical scraping.
- Plate colonies on **Matrigel-coated plates** in **mTeSR1 medium**.

Validate Reprogramming
Pluripotency Markers:

- Perform immunostaining for markers such as **Nanog**, **Tra-1-60**, and **SSEA-4**.

Gene Expression Analysis:

- Confirm expression of reprogramming genes (Oct4, Sox2) using qPCR or RNA-seq

Culture transfected cells on a feeder-free substrate (**Matrigel**) in **mTeSR1 medium**.

Item	Purpose
Matrigel Basement Membrane Matrix	Coating substrate for feeder-free culture of pluripotent stem cells.
mTeSR1 Medium	Defined medium optimized for maintaining pluripotency.
Sterile 6-well plates	Cell culture vessel.
Sterile PBS	For rinsing plates and cells.

Complete DMEM or basal DMEM	Diluent for Matrigel and cell medium.
Humidified CO_2 incubator	Maintains culture conditions (37°C, 5% CO_2, high humidity).
Pipettes and sterile tips	For precise handling of liquids.
Cell culture hood	For aseptic handling of materials.

Prepare Matrigel-Coated Plates

- **Thaw Matrigel**:
  - Remove Matrigel from the freezer and thaw on ice overnight in a 4°C refrigerator. Keep cold to prevent gelling.
- **Dilute Matrigel**:
  - Dilute Matrigel with **cold DMEM** or **PBS** to the manufacturer-recommended concentration (typically 1:30 to 1:100).
  - Mix gently to avoid bubbles and degradation of the protein matrix.
- **Coat the Plates**:
  - Add the diluted Matrigel solution to the wells of a 6-well plate:
    - Use approximately **1 mL per well**.
  - Gently swirl the plate to ensure even coating of the well surface.
- **Incubate the Plates**:
  - Place the coated plates in a 4°C refrigerator overnight or at room temperature for **1 hour**.
- **Remove Excess Matrigel**:
  - Before use, aspirate the excess Matrigel solution from the wells.
  - Rinse each well once with **sterile PBS** or basal DMEM to remove unbound material.
  - Do not let the wells dry out.

Prepare mTeSR1 Medium

- **Thaw Components**:
  - Thaw the basal mTeSR1 medium and growth factor supplements (typically provided separately in the kit).
- **Mix Medium**:
  - Combine the basal medium with supplements as per the manufacturer's instructions.
  - Store unused medium at 4°C and use within the recommended time frame.
- **Warm the Medium**:

- Pre-warm the prepared medium to **37°C** in a water bath before use.

Plate Transfected Cells

- **Harvest Cells**:
  - Detach transfected fibroblasts gently:
    - Use **Accutase** or **TrypLE Express** to lift cells from the culture dish without damaging them.
    - Add the detachment reagent to the dish and incubate for **5 minutes at 37°C**.
    - Gently pipette up and down to dislodge cells.
- **Centrifuge and Wash**:
  - Centrifuge the cell suspension at **300g for 5 minutes**.
  - Aspirate the supernatant and resuspend the cell pellet in **mTeSR1 medium**.
- **Count and Seed Cells**:
  - Use a hemocytometer or automated cell counter to count cells.
  - Adjust the cell density to **20,000–50,000 cells/cm²**.
  - Plate the cell suspension evenly across the Matrigel-coated wells:
    - Add **2 mL of cell suspension per well** in a 6-well plate.
- **Incubate the Cells**:
  - Place the plate in a **humidified CO_2 incubator (37°C, 5% CO_2)**.
  - Avoid disturbing the plate for at least **24 hours** to allow cells to adhere.

Maintain the Culture

- **Change the Medium**:
  - Replace the spent medium with fresh, pre-warmed mTeSR1 medium **daily**.
  - Use a pipette to carefully aspirate the old medium from the edge of the well to avoid disturbing the cells.
- **Monitor Cell Morphology**:
  - Observe the cells under a phase-contrast microscope:
    - Look for **tight, compact colonies with high nucleus-to-cytoplasm ratio** indicating successful reprogramming.
    - Discard cultures showing contamination or abnormal growth.

Passaging Cells

- **Prepare Matrigel-Coated Plates**:
  - Pre-coat fresh plates with Matrigel as described above.
- **Detach iPSC Colonies**:
  - After 10–14 days, detach colonies manually using a pipette tip or gently enzymatically using Accutase.
  - Avoid over-digestion to preserve colony integrity.

- **Replate and Observe**:
    - Plate the detached colonies on new Matrigel-coated plates in fresh mTeSR1 medium.
    - Observe for colony formation over 14–21 days, changing the medium daily.
    - After colonies form, mechanically isolate and expand them on Matrigel-coated dishes

Validation of iPSCs

Immunostain colonies for **Nanog, Tra-1-60, and SSEA-4** to confirm pluripotency.

Item	Purpose
Primary antibodies	Specific to pluripotency markers:
- Anti-Nanog antibody	Confirms nuclear expression of Nanog.
- Anti-Tra-1-60 antibody	Detects surface antigen associated with pluripotency.
- Anti-SSEA-4 antibody	Identifies surface marker of pluripotent stem cells.
Secondary antibodies	Fluorescently labeled antibodies targeting primary antibodies (e.g., Alexa Fluor conjugates).
Fixative (e.g., 4% PFA)	Fixes cell structure for staining.
Permeabilization buffer	Detergent-containing buffer (e.g., 0.1% Triton X-100 in PBS).
Blocking buffer	Prevents non-specific binding (e.g., 5% serum in PBS).
PBS (Phosphate Buffered Saline)	Used for washing cells.
DAPI (optional)	Nuclear counterstain.
Microscope slides or coverslips	For imaging.

Fluorescence microscope	For visualizing labeled colonies.

Fix the Colonies

Prepare Fixative:

- Use freshly prepared **4% paraformaldehyde (PFA)** in PBS.
- Pre-warm fixative to room temperature.

Aspirate Medium:

- Carefully remove the mTeSR1 medium from the culture plate without disturbing colonies.

Add Fixative:

- Add enough PFA to cover the colonies (e.g., 1 mL per well in a 6-well plate).
- Incubate at room temperature for **15 minutes**.

Wash with PBS:

- Aspirate the fixative and wash cells gently with PBS 2–3 times to remove residual PFA.

Permeabilize the Cells

Prepare Permeabilization Buffer:

- Use **0.1% Triton X-100 in PBS** or a similar solution.

Add Permeabilization Buffer:

- Cover the colonies with the buffer and incubate for **10 minutes at room temperature**.

Wash with PBS:

- Gently wash cells 2–3 times with PBS to remove the detergent.

Block Non-Specific Binding

Prepare Blocking Buffer:

- Use **5% serum** (e.g., goat serum or donkey serum) in PBS, or a commercial blocking solution.

Add Blocking Buffer:

- Incubate colonies with blocking buffer for **1 hour at room temperature**.
- This step prevents non-specific binding of antibodies.

Add Primary Antibodies
Dilute Primary Antibodies:

- Prepare antibody solutions in blocking buffer at the manufacturer-recommended dilution (e.g., 1:100–1:500).

Add to Colonies:

- Cover colonies with the primary antibody solution (e.g., anti-Nanog, anti-Tra-1-60, anti-SSEA-4).
- Incubate at **4°C overnight** for optimal binding.

Wash with PBS:

- Remove the antibody solution and wash cells 3 times with PBS for **5 minutes each**.

Add Secondary Antibodies
Dilute Secondary Antibodies:

- Use fluorescently labeled secondary antibodies (e.g., Alexa Fluor 488, Alexa Fluor 594).
- Dilute in blocking buffer (e.g., 1:500).

Incubate with Secondary Antibodies:

- Add secondary antibody solution to colonies.
- Incubate for **1 hour at room temperature in the dark** to protect fluorescence.

Wash with PBS:

- Wash cells 3 times with PBS for **5 minutes each** to remove unbound secondary antibodies.

Optional Counterstaining with DAPI
Dilute DAPI:

- Use DAPI at 1 µg/mL in PBS.

Add to Colonies:

- Incubate for **5 minutes at room temperature**.

Wash with PBS:

- Wash once with PBS to remove excess DAPI.

Imaging

Prepare for Microscopy:

- If cells are on coverslips, mount them onto microscope slides with an antifade mounting medium.
- If in a culture plate, leave the colonies in PBS for imaging.

Visualize Under a Fluorescence Microscope:

- Use appropriate excitation/emission filters for the secondary antibody fluorophores.
- **Expected results**:
  - **Nanog**: Nuclear staining.
  - **Tra-1-60**: Cell surface staining.
  - **SSEA-4**: Cell surface staining.

Analysis

Confirm Pluripotency:

- Colonies expressing all three markers (**Nanog, Tra-1-60, and SSEA-4**) indicate successful reprogramming to iPSCs.

Document Findings:

- Capture high-resolution images for records.

Perform qPCR or RNA-seq for expression of reprogramming genes.

Item	Purpose
RNA Extraction Kit	For isolating high-quality total RNA from iPSCs (e.g., TRIzol reagent or column-based kits).
DNase I	Removes genomic DNA contamination from RNA samples.
Reverse Transcription Kit	Converts RNA to complementary DNA (cDNA).
qPCR Master Mix	For performing quantitative PCR (e.g., SYBR Green or TaqMan).
Primers for Reprogramming Genes	Specific primers for **Oct4**, **Sox2**, **Klf4**, **c-Myc**, and control genes (e.g., GAPDH).
PCR Tubes or Plates	For qPCR reactions.
RNA-Seq Library Preparation Kit	If performing RNA-seq.
Fluorescence-capable qPCR Machine	For real-time PCR.
Sequencing Facility Access	For RNA-seq if performed externally.

RNA Extraction

Harvest Cells:

- Detach iPSCs from the culture plate using Accutase or gentle scraping.
- Centrifuge at **300g for 5 minutes** and remove the supernatant.

Lyse Cells:

- Add lysis buffer (from the RNA extraction kit or TRIzol reagent) to the cell pellet.
- Pipette up and down to homogenize the sample.

Isolate RNA:

- For column-based kits: Follow the manufacturer's protocol.
- For TRIzol:
  - Add chloroform, vortex, and centrifuge to separate phases.
  - Recover the aqueous phase containing RNA.
  - Precipitate RNA with isopropanol, wash with 70% ethanol, and resuspend in RNase-free water.

Quantify and Assess RNA Quality:

- Use a spectrophotometer (e.g., Nanodrop) to measure RNA concentration (A260/280 ratio should be ~2.0).
- Optionally, assess RNA integrity using an electropherogram (e.g., Agilent Bioanalyzer).

Remove Genomic DNA Contamination

DNase Treatment:

- Treat the extracted RNA with DNase I according to the manufacturer's instructions.
- Heat inactivate or clean up the reaction using the RNA extraction kit.

Reverse Transcription (cDNA Synthesis)

Prepare Reaction Mix:

- Combine the following components in PCR tubes:
  - RNA template: ~1 µg.
  - Reverse transcription buffer (provided in the kit).
  - dNTP mix.
  - Reverse transcriptase enzyme.
  - Random primers or oligo-dT primers.

Perform Reverse Transcription:

- Incubate the reaction as follows:

        - 25°C for 5 minutes (primer annealing).
        - 42°C for 30–60 minutes (cDNA synthesis).
        - 70°C for 10 minutes (enzyme inactivation).

Store cDNA:

- Store synthesized cDNA at **-20°C** for short-term use or **-80°C** for long-term storage.

Quantitative PCR (qPCR)

Prepare Reaction Mix:

- Combine the following in each qPCR well or tube:
    - SYBR Green or TaqMan Master Mix: 10 µL.
    - Forward and reverse primers (final concentration ~200 nM each): 1 µL.
    - cDNA template: 1–2 µL.
    - Nuclease-free water to 20 µL total volume.

Primers for Target Genes:

- Examples:
    - **Oct4**: Forward 5'-GAGAAGGATGTGGTCCGAGT-3', Reverse 5'-GCTGATTGGCGATGTGAGTG-3'.
    - **Sox2**: Forward 5'-GCTTAGCCTCGTCGATGTTG-3', Reverse 5'-ATCTTTGCAGTACACGTCCC-3'.
    - **Klf4**: Forward 5'-CTGCGTCAAGCGGGGCTTAC-3', Reverse 5'-GAGCGGCTGTCCGCCAGAGT-3'.
    - **c-Myc**: Forward 5'-AGTTCTGAGCAAGGACGACG-3', Reverse 5'-TTGTTCTGAGGCCCTTGCTG-3'.
    - **Housekeeping gene (e.g., GAPDH)**: Forward 5'-TGCACCACCAACTGCTTAGC-3', Reverse 5'-GGCATGGACTGTGGTCATGAG-3'.

Run qPCR:

- Place the plate in a fluorescence-capable qPCR machine.
- Set the program:
    - Initial denaturation: 95°C for 2 minutes.
    - Amplification (40 cycles):
        - Denature: 95°C for 15 seconds.
        - Annealing/extension: 60°C for 30 seconds.

Analyze Results:

- Confirm gene expression via Ct (cycle threshold) values.
- Normalize target gene expression against the housekeeping gene (e.g., GAPDH) using the ΔΔCt method.

RNA-Seq (Optional)
Prepare RNA-Seq Library:

- Use a library preparation kit suitable for your sequencing platform (e.g., Illumina).
- Steps:
  - RNA fragmentation.
  - cDNA synthesis.
  - Adapter ligation.
  - PCR amplification.

Sequence and Analyze:

- Send libraries to a sequencing facility or use an in-house sequencer.
- Analyze data using bioinformatics pipelines to confirm expression of reprogramming genes.

Validation
qPCR:

- Successful reprogramming is indicated by robust expression of **Oct4**, **Sox2**, **Klf4**, and **c-Myc**, normalized against the housekeeping gene.

RNA-Seq:

- Confirm high expression of pluripotency genes and low expression of fibroblast markers.

Neural Induction
Plate iPSCs on Matrigel-coated plates.

Item	Purpose
Matrigel Basement Membrane Matrix	Coating substrate for iPSC culture.
DMEM/F12 Medium	Basal medium for neural induction.
N2 Supplement	Provides essential nutrients for neural differentiation.
Heparin	Stabilizes growth factors to support neural induction.

SB431542	TGF-beta inhibitor to drive neural lineage differentiation.
LDN193189	BMP pathway inhibitor to promote neural induction.
Sterile 6-well plates	For culturing iPSCs.
Humidified CO_2 incubator	Maintains culture conditions (37°C, 5% CO_2).
Pipettes and sterile tips	For handling cells and media.
Phase-contrast microscope	For observing neural rosette formation.

Prepare Matrigel-Coated Plates
Thaw and Dilute Matrigel:

- Thaw Matrigel on ice overnight in a 4°C refrigerator.
- Dilute in cold DMEM/F12 medium according to the manufacturer's instructions (typically 1:30 to 1:100).

Coat Plates:

- Add 1 mL of diluted Matrigel to each well of a 6-well plate.
- Swirl the plate to ensure even coating.

Incubate:

- Incubate plates at room temperature for 1 hour or at 4°C overnight.
- Remove excess Matrigel immediately before plating iPSCs.

Prepare Neural Induction Medium
Prepare Base Medium:

- Combine DMEM/F12 medium with the following supplements:
  - N2 Supplement: Add as per manufacturer's instructions.
  - Heparin: Add at a final concentration of 1 µg/mL.
  - SB431542: Add at a final concentration of 10 µM.
  - LDN193189: Add at a final concentration of 100 nM.
- Filter-sterilize the medium if necessary.

Pre-Warm Medium:

- Warm the prepared medium to 37°C before use.

Plate iPSCs on Matrigel
Harvest iPSCs:

- Detach iPSC colonies using a gentle dissociation method:
  - Use Accutase to avoid damage to cell viability.
  - Incubate for 5 minutes at 37°C, then pipette gently to dislodge colonies.
- Centrifuge the cell suspension at 300g for 5 minutes.

Resuspend Cells:

- Resuspend the cell pellet in pre-warmed neural induction medium.
- Adjust cell density to 100,000–200,000 cells/cm².

Seed Cells:

- Plate the cell suspension evenly on Matrigel-coated wells (e.g., 2 mL per well in a 6-well plate).

Incubate:

- Place the plate in a humidified CO_2 incubator at 37°C.
- Avoid disturbing the plate for the first 24 hours to allow cells to attach.

Neural Induction and Maintenance
Replace Medium Daily:

- Remove spent medium gently to avoid disturbing cells.
- Add fresh, pre-warmed neural induction medium.

Monitor Differentiation:

- Use a phase-contrast microscope daily to observe changes in cell morphology:
  - After 2–3 days, cells should elongate and form early neural structures.
  - By 7–10 days, distinct neural rosettes should form.

Observe and Identify Neural Rosettes
Morphological Characteristics:

- Neural rosettes are characterized by:
  - Radial organization of cells.
  - Dense, circular clusters resembling early neural tube structures.

Optional Staining for Confirmation:

- Immunostain for neural markers to confirm differentiation:
  - PAX6 (early neural marker).
  - Nestin (marker for neural progenitors).

Expected Outcomes

- Neural rosettes should form within 7–10 days, confirming successful neural induction.
- If rosettes are not forming:
  - Check medium formulation and small molecule concentrations.
  - Confirm the quality of Matrigel and iPSCs.

NPC Expansion

Item	Purpose
Pipette (manual or P200)	For mechanical isolation of neural rosettes.
DMEM/F12 Medium	Basal medium for NPC culture.
N2 Supplement	Provides essential nutrients for NPC survival and proliferation.
EGF (20 ng/mL)	Epidermal Growth Factor to support NPC proliferation.
bFGF (20 ng/mL)	Basic Fibroblast Growth Factor for NPC self-renewal.
Matrigel	Coating substrate for NPC adhesion.
6-well plates or T-25 flasks	For NPC culture and expansion.
Sterile PBS	For washing isolated rosettes.
Humidified CO_2 incubator	Maintains culture conditions (37°C, 5% CO_2).

Phase-contrast microscope	For observing and identifying neural rosettes and NPC growth.

Isolate Neural Rosettes

Identify Rosettes:

- Use a phase-contrast microscope to locate **neural rosettes** in the culture plate:
  - Dense, circular clusters with radial organization of cells.
- Mark the rosettes if needed to aid manual isolation.

Manually Isolate Rosettes:

- Using a sterile pipette (P200 or a glass pipette):
  - Gently aspirate medium around a rosette.
  - Loosen the rosette by applying gentle mechanical force with the pipette tip.
  - Aspirate the rosette into the pipette.

Transfer to a Collection Tube:

- Deposit the isolated rosettes into a sterile 15 mL centrifuge tube containing **1–2 mL of PBS** to wash debris.
- Repeat until all desired rosettes are collected.

Centrifuge and Wash:

- Centrifuge the rosettes at **300g for 5 minutes** to pellet the cells.
- Aspirate the supernatant and resuspend the pellet in **pre-warmed NPC expansion medium**.

Prepare Matrigel-Coated Plates

Coat Plates:

- Prepare plates coated with **Matrigel** as described in the neural induction protocol.
- Incubate plates with Matrigel solution for 1 hour at room temperature or overnight at 4°C.

Remove Excess Matrigel:

- Aspirate Matrigel solution and rinse gently with PBS.

Plate Neural Rosettes in NPC Expansion Medium

Prepare NPC Expansion Medium:

- Combine the following components:
  - **DMEM/F12** (basal medium).
  - **N2 Supplement**: As per the manufacturer's instructions.
  - **EGF**: Final concentration of 20 ng/mL.
  - **bFGF**: Final concentration of 20 ng/mL.
- Filter-sterilize if necessary.
- Pre-warm the medium to **37°C**.

Plate Rosettes:

- Resuspend the neural rosettes in the prepared NPC expansion medium.
- Seed the rosettes evenly in the coated wells:
  - For a 6-well plate: Add 2 mL of medium per well.
- Place the plate in a **humidified CO_2 incubator at 37°C**.

Maintain and Expand NPCs

Monitor Daily:

- Observe cultures under a phase-contrast microscope daily:
  - Neural rosettes should attach and begin spreading out within 1–2 days.
  - Proliferating NPCs appear as elongated, spindle-shaped cells.

Change Medium:

- Replace the spent medium with fresh pre-warmed NPC expansion medium every 2–3 days.

Passage NPCs:

- When cultures reach **70–80% confluence** (typically every 5–7 days):
  - Detach NPCs using **Accutase** or gentle scraping.
  - Wash cells with PBS and centrifuge at 300g for 5 minutes.
  - Resuspend the pellet in fresh NPC expansion medium and replate on new Matrigel-coated plates.

Tips for Success

- Avoid over-digestion or vigorous pipetting during isolation to maintain cell viability.
- Use fresh EGF and bFGF stocks to ensure growth factor activity.
- Monitor cultures for contamination or signs of differentiation (e.g., loss of NPC morphology).

Expected Outcomes

- Neural rosettes should expand into a homogenous population of NPCs over multiple passages.
- NPCs should maintain expression of neural markers such as **Nestin** and **PAX6**, which can be validated via immunostaining.

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Neuronal Differentiation

Item	Purpose
Neurobasal Medium	Basal medium optimized for neuronal culture.
B27 Supplement	Provides nutrients and growth factors for neuronal differentiation.

BDNF (20 ng/mL)	Brain-Derived Neurotrophic Factor to promote neuronal survival and differentiation.
GDNF (20 ng/mL)	Glial Cell Line-Derived Neurotrophic Factor to support neuronal differentiation.
Retinoic Acid (100 nM)	Drives differentiation into neuronal phenotypes.
Laminin	Enhances neuronal adhesion.
Poly-D-Lysine (PDL)	Coating substrate for neuronal adhesion.
NPCs (neural progenitor cells)	Cells to be differentiated into neurons.
6-well plates or coverslips	Culture vessels for neuronal differentiation.
Humidified CO_2 incubator	Maintains culture conditions (37°C, 5% CO_2).
Pipettes and sterile tips	For handling cells and media.

Prepare Laminin/Poly-D-Lysine-Coated Plates

Prepare Poly-D-Lysine Solution:

- Dissolve PDL in sterile water to a final concentration of **10 µg/mL**.
- Filter-sterilize the solution.

Coat Plates with PDL:

- Add enough PDL solution to cover the surface of the wells (e.g., 1 mL per well in a 6-well plate).
- Incubate at room temperature for **1 hour** or overnight at 4°C.

Wash with Sterile Water:

- Aspirate the PDL solution and rinse wells 2–3 times with sterile water to remove excess PDL.

Prepare Laminin Solution:

- Dilute laminin in sterile PBS or culture medium to a final concentration of **10 µg/mL**.

Coat Plates with Laminin:

- Add laminin solution to the PDL-coated wells (e.g., 1 mL per well in a 6-well plate).
- Incubate at 37°C for **1 hour**.

Aspirate Laminin Solution:

- Remove the laminin solution immediately before plating cells. Do not let the wells dry.

Prepare Neuronal Differentiation Medium

Prepare the Medium:

- Use **Neurobasal Medium** as the basal medium.
- Add the following supplements:
  - **B27 Supplement**: As per manufacturer's instructions (usually 2% v/v).
  - **BDNF**: Final concentration of **20 ng/mL**.
  - **GDNF**: Final concentration of **20 ng/mL**.
  - **Retinoic Acid**: Final concentration of **100 nM**.
- Filter-sterilize if needed.

Pre-Warm Medium:

- Warm the prepared medium to **37°C** in a water bath before use.

Plate NPCs

Harvest NPCs:

- Detach NPCs gently using **Accutase** to avoid damaging cells.
- Incubate with Accutase for **5 minutes at 37°C**, then gently pipette up and down to dislodge cells.
- Centrifuge the cell suspension at **300g for 5 minutes** and discard the supernatant.

Resuspend NPCs:

- Resuspend the NPC pellet in neuronal differentiation medium.
- Count cells using a hemocytometer or automated cell counter.
- Adjust the cell density to **50,000–100,000 cells/cm²**.

Seed NPCs:

- Plate the cell suspension onto the laminin/PDL-coated wells (e.g., 2 mL per well in a 6-well plate).
- Place the plate in a **humidified CO_2 incubator at 37°C**.

Neuronal Differentiation and Maintenance

Daily Observation:

- Use a phase-contrast microscope to monitor cells for attachment and morphological changes.
- NPCs should adhere within 24 hours and begin extending neurites (projections).

Change Medium:

- Replace spent medium with fresh, pre-warmed neuronal differentiation medium every **2–3 days**.
- Be careful not to disturb the cells during medium changes.

Culture Duration:

- Maintain cultures for **3–6 weeks**, allowing cells to fully differentiate into neurons.

Validate Neuronal Differentiation

Morphological Changes:

- Differentiated neurons should exhibit:
  - Large cell bodies.
  - Extended and branched neurites.
  - Network-like structures forming over time.

Immunostaining (Optional):

- Stain cells for neuronal markers:
  - **MAP2**: A dendritic marker.
  - **Tuj1 (beta-III tubulin)**: A neuronal cytoskeletal protein.
  - **NeuN**: A marker of mature neurons.

Functional Assays (Optional):

- Confirm neuronal activity using patch-clamp electrophysiology or calcium imaging.

Tips for Success

- Ensure the quality and sterility of all materials, especially coating substrates and medium supplements.
- Avoid over-confluence of NPCs before differentiation to ensure efficient neuronal development.
- Use fresh neurotrophic factors (BDNF, GDNF) for consistent results.

Expected Outcomes

- By **3–6 weeks**, cells should form mature neurons, confirmed by morphology and marker expression.
- Differentiated neurons can be used for further experiments, such as disease modeling or electrophysiology studies.

Validation of Neurons

Item	Purpose
Primary Antibodies	Target neuronal markers: MAP2, Tuj1 (beta-III tubulin), and NeuN.
Secondary Antibodies	Fluorescently labeled antibodies targeting primary antibodies (e.g., Alexa Fluor dyes).
4% Paraformaldehyde (PFA)	Fixes cell structures.
Permeabilization Buffer	Detergent-containing buffer (e.g., 0.1% Triton X-100 in PBS).
Blocking Buffer	Prevents non-specific binding (e.g., 5% serum in PBS or BSA).
DAPI	Nuclear stain for counterstaining (optional).
PBS (Phosphate Buffered Saline)	Used for washing cells.
Fluorescence Microscope	For imaging stained cells.

Fix Cells:

- Remove medium and wash cells twice with PBS.
- Add **4% PFA** to wells to cover the cells.
- Incubate at room temperature for **15 minutes**.
- Wash cells 3 times with PBS to remove residual PFA.

Permeabilize Cells:

- Add permeabilization buffer (**0.1% Triton X-100 in PBS**) to each well.
- Incubate for **10 minutes at room temperature**.
- Wash cells twice with PBS.

Block Non-Specific Binding:

- Add blocking buffer (**5% serum in PBS or 1% BSA**) to each well.
- Incubate for **1 hour at room temperature**.

Add Primary Antibodies:

- Prepare primary antibodies in blocking buffer:
  - **Anti-MAP2**: 1:200 dilution.
  - **Anti-Tuj1 (beta-III tubulin)**: 1:200 dilution.
  - **Anti-NeuN**: 1:200 dilution.
- Add the antibody solution to the cells and incubate at **4°C overnight**.

Wash with PBS:

- Remove primary antibody solution and wash cells 3 times with PBS for **5 minutes each**.

Add Secondary Antibodies:

- Prepare secondary antibodies (e.g., Alexa Fluor 488 or 594) in blocking buffer at **1:500 dilution**.
- Add the secondary antibody solution to the cells.
- Incubate in the dark at room temperature for **1 hour**.

Optional: Counterstain with DAPI:

- Add DAPI (1 µg/mL in PBS) to each well.
- Incubate for **5 minutes at room temperature**.
- Wash once with PBS.

Image Cells:

- Use a fluorescence microscope to capture images of stained cells.
- **Expected Results**:
  - **MAP2**: Dendritic staining.
  - **Tuj1**: Cytoskeletal staining of neurons.
  - **NeuN**: Nuclear staining of mature neurons.

Electrophysiological Assays (Patch-Clamp)

Supplies and Materials

Item	Purpose
Patch-clamp amplifier	Records electrical activity from individual neurons.
Microelectrodes	For recording electrical signals (glass capillaries with ~2–5 MΩ resistance).
Recording chamber	Holds neurons and recording solution.
Extracellular solution	Simulates physiological conditions (e.g., HEPES-buffered saline with NaCl, KCl, $CaCl_2$, $MgCl_2$).
Intracellular solution	Fills microelectrodes and mimics cytoplasmic ion composition.
Micropipette puller	Fabricates glass microelectrodes.
Vibration isolation table	Reduces external noise and vibration for precise recordings.
Data acquisition system	Captures and analyzes electrical recordings.

Prepare Neurons:

- Maintain differentiated neurons in culture until they exhibit mature neuronal morphology (3–6 weeks).
- Transfer culture to a recording chamber filled with pre-warmed extracellular solution.

Prepare Microelectrodes:

- Use a micropipette puller to create glass microelectrodes with a tip resistance of 2–5 MΩ.
- Fill the electrodes with intracellular solution (e.g., K-gluconate or KCl-based).

Set Up Recording:

- Position the recording electrode using a micromanipulator.

- Gently approach the cell membrane until a high-resistance seal forms (>1 GΩ).

Record Membrane Potential:

- Break into the cell membrane to establish whole-cell configuration.
- Measure resting membrane potential and record action potentials in response to current injections.

Stimulate Neurons:

- Inject currents in steps (e.g., 10–50 pA) to elicit action potentials.
- **Expected Results**:
  - Mature neurons should fire single or repetitive action potentials.

Analyze Data:

- Use data acquisition software (e.g., pClamp, LabChart) to analyze:
  - Resting membrane potential.
  - Firing patterns.
  - Ion channel activity.

Expected Results

Immunocytochemistry:

- Positive staining for MAP2, Tuj1, and NeuN confirms neuronal identity.

Patch-Clamp:

- Action potential firing and synaptic activity validate functional maturity of neurons.