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Description

The Cas9 Inducible (Tet-On) iPS Cell Pool expresses the Cas9 (Streptococcus pyogenes CRISPR associated protein 9) gene under the control of a tight TRE tetracycline-inducible promoter, introduced into PBMC-derived human iPS cells via lentiviral transduction [Cas9 Lentivirus (inducible TET on), BPS Bioscience #78794]. Cas9 expression can be induced with doxycycline treatment, allowing temporal control of its expression. Cas9 Inducible (Tet-On) iPS cells can be transduced with single-guide RNA (sgRNA) targeting gene(s) of interest to generate genetically engineered cell pools or cell lines.

Background

Cas9 (Streptococcus pyogenes CRISPR associated protein 9) is an endonuclease enzyme that, when recruited to a specific DNA sequence by the appropriate sgRNA (single guide RNA), introduces a double stranded break into the DNA. This double stranded break can then be repaired through either Non-Homologous End Joining (NHEJ) or Homologous Recombination (HR). While NHEJ is an error prone process and causes insertions or deletions which may result in functional inactivation of the target gene, HR, in conjunction with a single stranded ssDNA repair construct, can be used to introduce mutations at specific base pair(s). Gene modifications introduced via Cas9 are now used in multiple fields of research, aimed at both understanding cellular mechanisms and developing therapeutic solutions.

The discovery by Yamanaka and colleagues in 2007 that 4 factors were sufficient to reprogram terminally differentiated fibroblasts into pluripotent stem cells launched the advent of human induced pluripotent stem (iPS) cell technology. These human iPS cells are capable of both self-renewal and differentiation down all three germline lineages and provide both a tool to model human development and disease in the relevant differentiated human cell types. Together with CRISPR/Cas9 technology, gene edited iPS cells provide a unique opportunity to study disease-specific mutations or knockout genes of interest, alongside isogenic control cell pools or cell lines.

Application(s)

- Study molecular pathways using temporally controlled induction of gene editing.
- Generation of sgRNA-edited iPS cell pools.
- Screening of pooled sgRNAs libraries to identify pluripotency, differentiation, and drug toxicity genetic modulators.

Considerations

Maintenance of the cells requires specific reagents such as specialty culture media, Matrigel™, Accutase™, RelesR™, and Thiazovivin that are not provided with the cells. Ensure that you have all reagents on hand prior to thawing the cells. Prepare media as indicated in section *"Media Required for Cell Culture"* below. Thiazovivin is a Rho Kinase inhibitor used to ensure that sensitive cell types such as iPS cells survive cell dissociation process and re-plate successfully. Thiazovivin is not stable in solution and should be added to the medium immediately before use.

Materials Provided

Components	Format
1 vials of frozen cells	Each vial contains 2 x 10^6 cells in 1 ml of iPS Cell Freezing Medium

Parental Cell Line

PBMC-derived, non-Disease Human iPS Cell Line (iXCells 30HU-002)

Mycoplasma Testing
The cell pool has been screened to confirm the absence of Mycoplasma species.

Materials Required but Not Supplied

These materials are not supplied with the cell line but are necessary for cell culture and cellular assays. BPS Bioscience's reagents are validated and optimized for use with this cell line and are highly recommended for best results. Media components are provided in the Media Formulations section below.

Media Required for Cell Culture

Name	Ordering Information
mTeSR™ Plus	Stem Cell Technologies #100-0276
Matrigel™	Corning #354230
DMEM/F12	Thermo Fisher #11330032
Thiazovivin	BPS Bioscience #78506
RelesR	Stem Cell Technologies #05872
Accutase™	Thermo Fisher #A1110501
Geneticin (G418)	Thermo Fisher #11811-013
Doxycycline	Sigma #D9891

Storage Conditions

Cells are shipped in dry ice and should immediately be thawed or stored in liquid nitrogen upon receipt. Do not use a -80°C freezer for long term storage.
Contact technical support at support@bpsbioscience.com if the cells are not frozen in dry ice upon arrival.

Stability
As this is a cell pool and not a cell line, BPS Bioscience cannot guarantee the stability of the genetic modifications over time. Clonal selection can be performed. We recommend freezing cell vials very early on and growing the cells for a limited number of passages. Cells should be cultured using Growth Media, which contain selection antibiotics.

Media Formulations
For best results, the use of validated and optimized media from BPS Bioscience is *highly recommended*. Other preparations or formulations of media may result in suboptimal performance.

Note: Thaw Media do *not* contain selective antibiotics. However, Growth Media *do* contain selective antibiotics, which are used for maintaining selective pressure on the cell population expressing the gene of interest over passages. Cells should be cultured at 37°C with 5% CO_2.

Media Required for Cell Culture

iPSC Thaw Medium:
mTeSR™ Plus supplemented with 1% Penicillin/Streptomycin.

Complete iPSC Thaw Medium:
mTeSR™ Plus supplemented with 1% Penicillin/Streptomycin and 1 µM Thiazovivin.

Thiazovivin is unstable once diluted in the medium. Prepare just enough to proceed with thawing of the cells.

iPSC Growth Medium
mTeSR™ Plus supplemented with 1% Penicillin/Streptomycin and 200 µg/ml Geneticin (G418).

iPSC Passage Medium
mTeSR™ Plus supplemented with 1% Penicillin/Streptomycin and 200 µg/ml Geneticin (G418) and 1 µM Thiazovivin.

2X Freezing Medium
80% mTeSR™ Plus supplemented with 1% Penicillin/Streptomycin, 1 µM Thiazovivin and 20% DMSO (vol/vol).

Induction Media

iPSC Thaw Medium:
mTeSR™ Plus supplemented with 1% Penicillin/Streptomycin (does NOT contain Thiazovivin) and doxycycline at various concentrations (0.5-1.5 µg/mL).

Cell Culture Protocols
Note: iPS cells are derived from human material and thus the use of adequate safety precautions is recommended.

Matrigel®-coated plates

Matrigel™ solidifies rapidly when warm. Keep everything on ice and work in sterile conditions. Matrigel™ coated plates can be prepared up to two weeks ahead of time. We recommend following the manufacturer's instructions for Matrigel™ handling. Matrigel™ should not be subjected to repeated freeze-thaw cycles.

When first using a vial of Matrigel™, it is recommended to aliquot ~100 µl and/or ~200 µl into microcentrifuge tubes for future use.

1. Prepare cold, sterile cell culture medium such as DMEM/F12 containing 1% Penicillin/Streptomycin (no serum).

2. Thaw Matrigel™ at 4°C.

3. While the Matrigel™ is thawing, transfer the desired volume of ice-cold DMEM/F12 into a 50 ml conical tube.

Table 1: Example of volumes to be used with various size plates or flasks.

Cell culture plate	Matrigel™ volume	Volume medium	Coating volume
2x 6-well plate	~100 µl*	25 ml	2 ml/Well
4x 6-well plate	~200 µl*	50 ml	2 ml/Well
4x 96-well plate	~100 µl*	40 ml	100 µl/Well
4x T25 Flask	~100 µl*	12 ml	3 ml/Flask
3x T75	~200 µl*	30 ml	10 ml/Flask
2x T175	~ 300 µl*	40 ml	20 ml/Flask

**Amount is lot-specific, please refer to manufacturer's CoA.*

4. Once Matrigel™ is thawed, add 500 µl of cold DMEM/F12 to the microcentrifuge tube containing the Matrigel™.

5. Pipette up and down using a 1 ml pipette tip.

6. Transfer the diluted Matrigel™ aliquot to the 50 mL conical tube containing the ice-cold medium.

7. Plate the Matrigel™ solution in the cell culture plates according to coating volumes shown in Table 1.

8. Transfer to a 5% CO_2 Incubator at 37°C for a minimum of 1 hour and up to 2 weeks.

   Note: The DMEM/F12 medium must be gently removed from the Matrigel™-coated wells immediately before adding the cells.

Cell Thawing

1. Ensure that you have prepared the Matrigel™-coated culture plates or flasks at least 1 hour in advance.

2. Bring iPSC Thaw Medium to Room Temperature (RT). **iPSC Thaw Medium should NOT be pre-warmed in a water bath.**

3. Prepare 15 ml of Complete iPSC Thaw Medium by adding Thiazovivin to a final concentration of 1 µM.

⚠ *Thiazovivin is unstable once diluted in the medium. Prepare just enough to proceed with thawing the cells.*

4. Retrieve a cell vial from liquid nitrogen storage. Keep on dry ice until ready to thaw. When ready to thaw, swirl the vial of frozen cells for approximately 60 seconds in a 37°C water bath. Once cells are thawed (it may be slightly faster or slower than 60 seconds), quickly transfer the entire contents of the vial to an empty 50 ml conical tube. **Leaving the cells in the water bath at 37°C for too long will result in rapid loss of viability.**

5. Using a 10 ml serological pipette, slowly add 10 ml of Complete iPSC Thaw Medium to the conical tube containing the cells. iPSC Thaw Medium should be added dropwise while gently rocking the conical tube to permit gentle mixing and avoid osmotic shock.

6. Immediately spin down the cells at 300 *x g* for 5 minutes, remove the medium and gently resuspend the cells in 5 ml of Complete iPSC Thaw Medium.

7. Aspirate coating solution from 2 wells of a 6-well Matrigel™-coated plate.

8. Immediately transfer the resuspended cells to the 2 wells of the Matrigel™-coated plate and incubate at 37°C in a 5% CO_2 incubator. Each well contains approximately 1 million cells.

9. Rock the plate to ensure uniform distribution of cells.

10. After 24 hours in culture, check for cell attachment and viability. Change the culture medium to fresh iPSC Thaw Medium and continue growing cells in a 5% CO_2 incubator at 37°C until the cells are ready to passage.

11. Cells should be passaged before they reach 80% confluency or before colonies become too large, whichever comes first. For the first passage and subsequent passages, use iPS Cell Growth Medium.

12. Perform media changes as recommended in the cell maintenance schedule below. For Cas9 Inducible (Tet-On) iPS Cell Pool cultivated in mTeSR Plus, we recommend one media change on either Saturday or Sunday. This is a recommended schedule only, cells should be fed and passaged based on daily visual observation.

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Passage	Change medium	No Change	Passage	Change medium	One medium change	

Routine Cell Passage

1. Monitor iPS Cell cultures for both colony size and plate confluence. Passage once the colonies are large with a dense, tightly packed central region or when the well is ~80% confluent, whichever occurs first.

2. Ensure that you have prepared Matrigel™-coated culture plates or flasks at least 1 hour in advance.

3. Prepare 15 ml of Passage Medium by adding Thiazovivin to a final concentration of 1 µM to 15 ml of Growth Medium.

4. Aspirate spent cell culture medium, and gently wash the cells with phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+}.

5. Add 1 ml of RelesR™ per well of a 6-well plate and rock the plate to evenly distribute RelesR™. Immediately aspirate all but ~60 µl of RelesR™ leaving a very thin film.

6. Incubate at 37°C for 3-5 minutes or until the edges of the iPS Cell colonies have begun to detach (the colonies will appear to be “curling up” from the edges).

7. Once the cells have detached, add iPSC Passage Medium, transfer to a tube and dilute with Passage Medium to seed into new Matrigel-coated culture vessels at a sub-cultivation ratio of 1:10 to 1:20. Be sure to aspirate the coating solution from Matrigel™-coated plates before plating the cells.

Cell Freezing

1. Add Thiazovivin to make a final concentration of 1 µM to 15 ml of iPSC Thaw Medium to make Complete iPSC Thaw Medium.

2. Prepare 2X Freezing Medium: 80% Complete iPSC Thaw Medium + 20% DMSO.

3. Aspirate the cell culture medium and wash the cells with PBS without Ca^{2+}/Mg^{2+}.

4. Add 1 ml of RelesR™ per well of a 6-well plate and rock the plate to evenly distribute RelesR™. Immediately aspirate all but ~60 µl of RelesR™ in order to leave a very thin film of liquid covering the cells.

5. Incubate at 37°C for 3-5 minutes or until the edges of the iPS Cell colonies have begun to detach (the colonies will appear to be "curling up" from the edges).

6. Once the cells have detached, add Complete iPSC Thaw Medium and count the cells. For routine use, two vials can be frozen from a ~80% confluent well of a 6-well plate. Alternatively, cells can be frozen at 2 million cells/vial.

7. Spin down the cells at 300 *x g* for 5 minutes, remove the supernatant and resuspend the cells in Complete iPSC Thaw Medium using 0.5 ml of medium per vial to be frozen.

8. Using a 10 ml serological pipette, slowly add an equal volume of 2X Freezing Medium (0.5 ml per vial to be frozen) to the conical tube containing the iPS Cells. The 2x Freezing Medium should be added dropwise while softly rocking the conical tube to permit gentle mixing and avoid osmotic shock.

9. Dispense 1 ml of cell suspension into each cryogenic vial. Place the vials in an insulated container for slow cooling and store at -80°C overnight.

10. Transfer the vials to liquid nitrogen the next day for long term storage.

Note: It is recommended to expand the cells and freeze at least 10 vials at an early passage for future use.

Cas9 Induction with Doxycycline

1. Ensure that you have prepared a Matrigel™-coated, 12-well plate at least 1 hour in advance (can be prepared up to two weeks in advance).

2. Prepare 20 ml of iPSC Passage Medium.

3. Aspirate the cell culture medium and wash the cells with PBS without Ca^{2+}/Mg^{2+}.

4. Add 1 ml of RelesR™ per well of a 6-well plate and rock the plate to evenly distribute RelesR™. Immediately aspirate all but ~60 µl of RelesR™ in order to leave a very thin film of liquid covering the cells.

5. Incubate at 37°C for 3-5 minutes or until the edges of the iPS Cell colonies have begun to detach (the colonies will appear to be "curling up" from the edges).

6. Once the cells have detached, add iPSC Passage Medium and count the cells.

7. Dilute cells to ~120,000 cells/ml in iPSC Thaw Medium.

8. Transfer 1 ml of cell suspension into each well of a 12-well plate and incubate at 37°C in a 5% CO_2 incubator.

9. Rock the plate to ensure uniform distribution of cells and incubate overnight.

10. Prepare Induction Media (iPSC Thaw Media with varying concentrations of doxycycline, in order to determine the best expression level for your application).

11. Incubate Cas9 Inducible (Tet-On) iPS Cells with Induction Media for 72 hours, changing to fresh Induction Media daily.

12. Collect cells for Western Blot analysis to confirm induction of Cas9 expression. At this point cells may be used in the customer's desired assay or replated in media without doxycycline to reverse the induction of Cas9 expression.

    Note: We have observed that doxycycline concentrations between 0.5 µg/ml and 1.5 µg/ml induce expression of Cas9 protein that is reversible after incubation in iPSC Thaw Media without doxycycline for an additional 96 hours (Figure 2).

Validation Data

A. Pluripotency marker expression in Cas9 Inducible (Tet-On) iPS Cell Pool

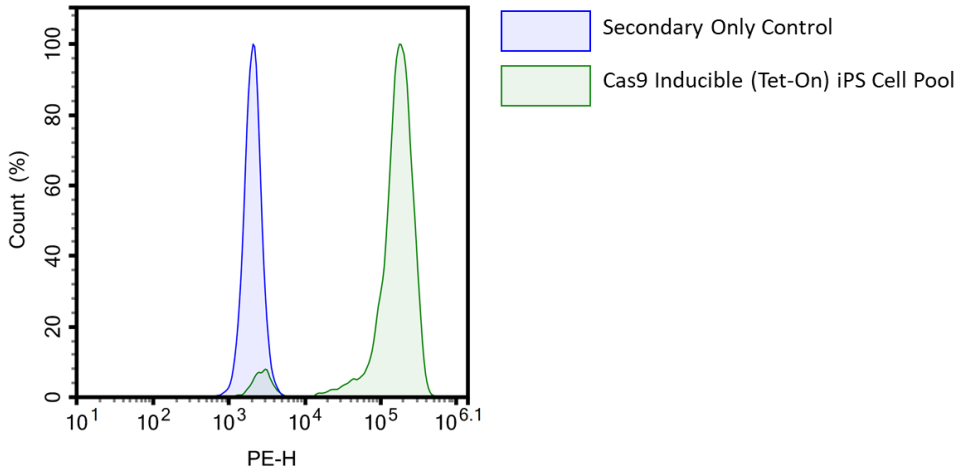

Figure 1. Oct4 Expression in Cas9 Inducible (Tet-On) iPS Cell Pool by flow cytometry.
Cells were fixed with Fixation Buffer (BioLegend #42080) and intracellular staining was performed with an anti-Oct4 antibody (BioLegend #653701) followed by a PE-labeled anti-mouse secondary antibody (BioLegend #405307). Cas9 Inducible (Tet-On) iPS Cell Pool expression of Oct4 (green) was compared to cells stained with secondary antibody only as control (blue).

B. Reversible induction of Cas9 protein expression in Cas9 Inducible (Tet-On) iPS Cell Pool

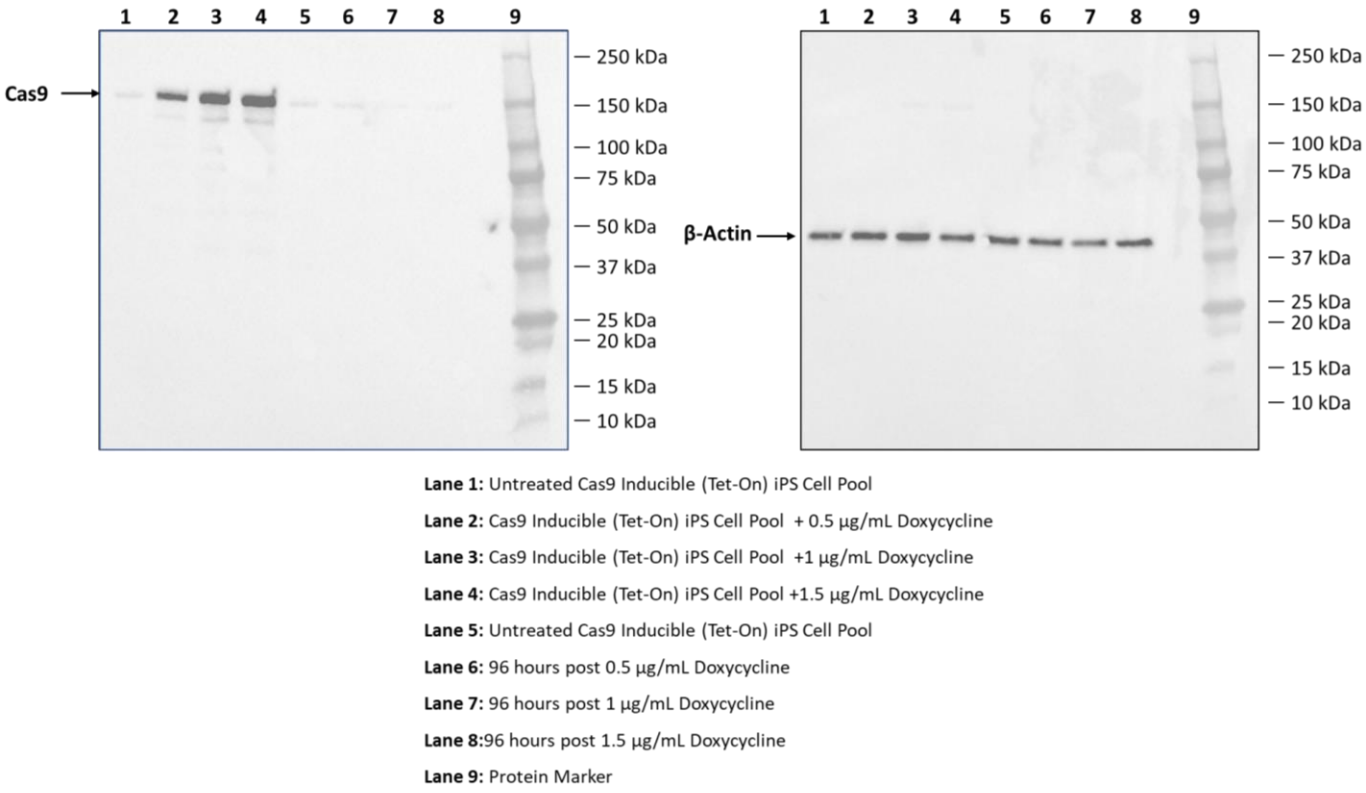

Figure 2. Cas9 protein expression analysis by Western Blot in Cas9 Expressing iPS Cell Pool. Cas9 Inducible (Tet-On) iPS Cells were collected and analyzed by Western Blot after 72 hours of treatment with various concentrations of doxycycline, or 96 hours after withdrawal of doxycycline-containing media. Cell lysates were analyzed by Western Blot using either Mouse Anti-Cas9 (Biolegend #844301) or Rabbit Anti-Actin (Cell Signaling Technology #4970) primary antibody, and Anti-Mouse (Biolegend #405306) or Anti-Rabbit (SBCT #2357) HRP conjugated secondary antibody respectively.

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License Disclosure

The iPSC technology is protected by several patents, including US patent Nos. 8048999, 8058065, 8129187, 8278104, 8530238, 8900871, 9404124, 9499797, 10519425, and patent pending, for which iPS Academia Japan, Inc. has been granted license rights with a sub-licensable right.

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Products	*Catalog #*	*Size*
StemBright™ Luciferase iPSC-Derived Neural Progenitor Cell Pool	78593	1 vial
StemBright™ GFP iPSC-Derived Neural Progenitor Cell Pool	78574	1 vial
TCF/LEF StemBright™ Luciferase Reporter iPS Cell Pool	78515	1 vial
Cas9 Expressing iPS Cell Pool	78578	1 vial