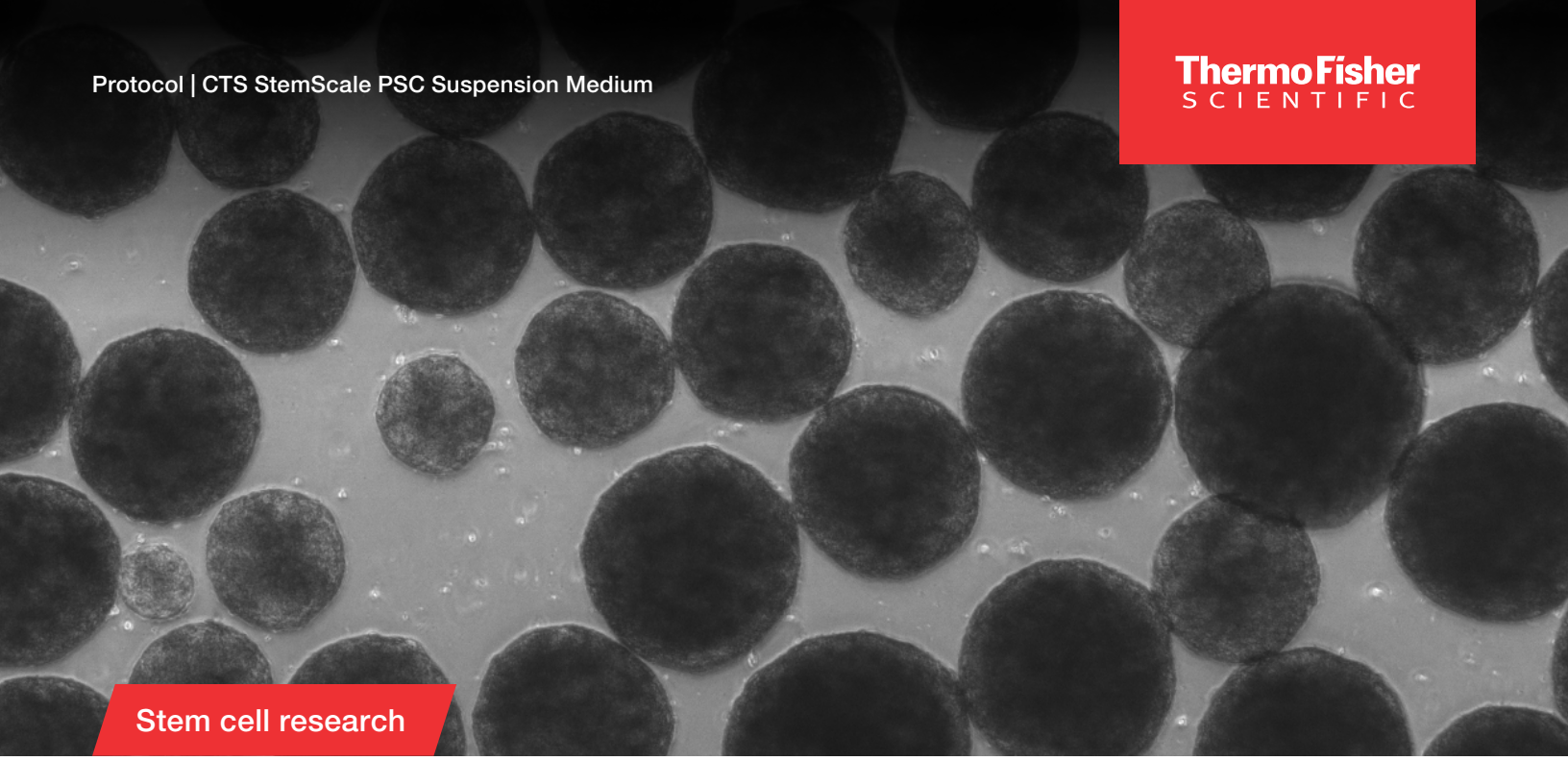

Stem cell research

A streamlined protocol for generating iNK cells from PSC spheroids

The cell therapy field has grown exponentially, offering promising therapeutics for several diseases. However, significant obstacles must still be overcome, including the ability to obtain large yields of the appropriate cell type. Natural killer (NK) cells are a cytotoxic, innate, lymphoid immune cell of interest in cancer cell therapy research. These innate immune cells can kill malignant cells without the need for HLA matching. NK cell therapy clinical trials require ~5 x 10^6 to 1 x 10^8 NK cells per kilogram of body weight, which underscores the need for improved scalability of cell manufacturing. To address this issue, scientists are exploring pluripotent stem cells (PSCs) as an easy-to-scale source of allogeneic, PSC-derived NK cells, or invariant NK (iNK) cells. Current PSC-to-iNK cell differentiation protocols include an embryoid body formation step and/or co-culture with feeder cells, which are inconsistent and lack scalability, hampering the manufacturing of large quantities of functional iNK cells. Here, we have developed a protocol to differentiate PSCs to iNK cells, which features the 3D PSC expansion capabilities of Gibco™ CTS™ StemScale™ PSC Suspension Medium.

In this protocol, iNK cells are generated by selective application of several protein/small molecule cocktails, and without the use of embryoid body formation, scaffolds, or exogenous feeder cells. This differentiation protocol is presented in three sections: 1) PSC-to-induced hematopoietic progenitor cell (iHPC) differentiation, 2) iHPC-to-iNK cell differentiation, and 3) iNK expansion. PSC culture and iHPC differentiation are performed under constant agitation via an orbital shake platform, followed by a static culture to promote maturation and expansion of iNK cells.

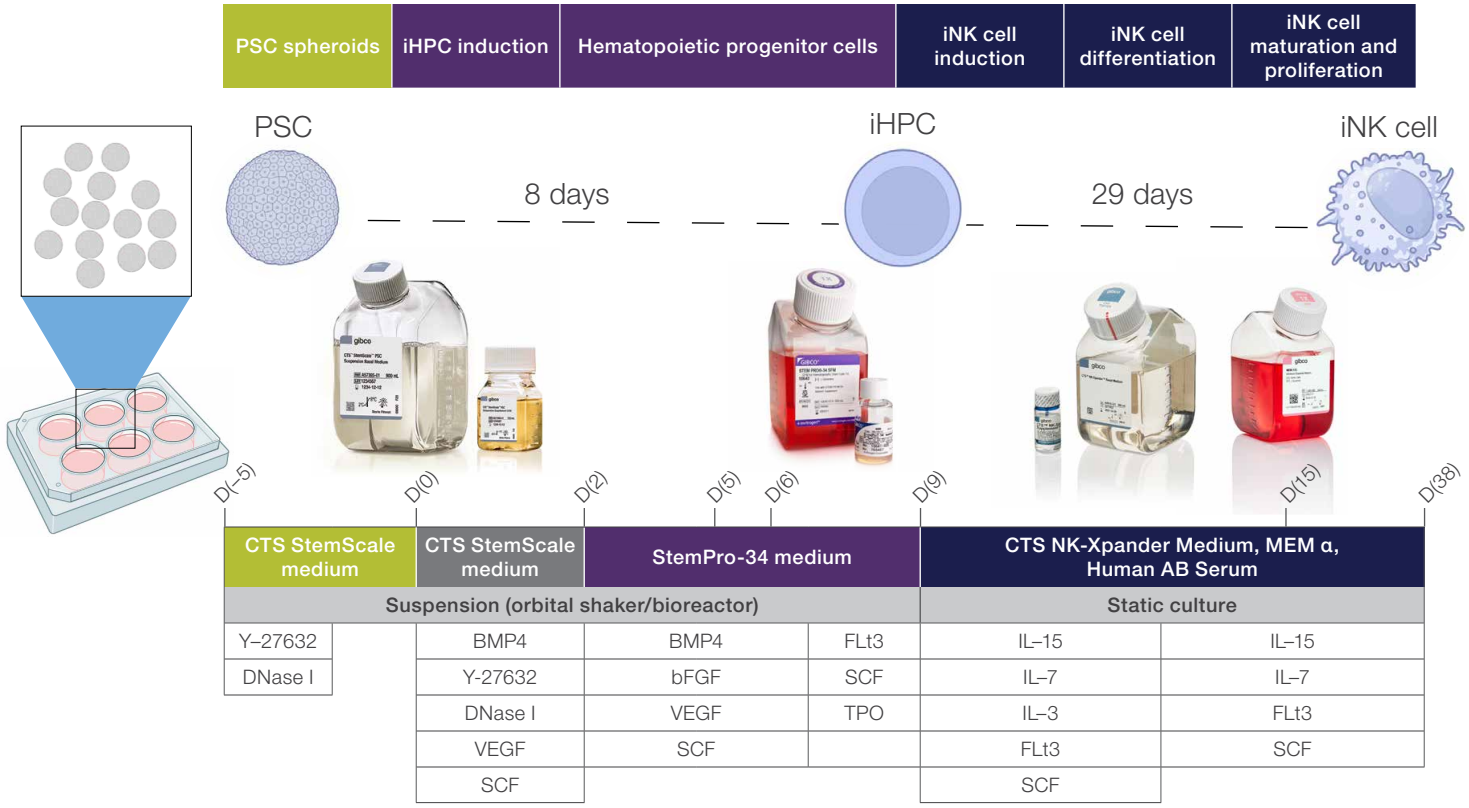

Figure 1. Overview of the PSC-to-iNK cell differentiation protocol. Images created by BioRender.com.

Preparing growth factors and side solutions

Reconstitute all growth factors according to manufacturer's recommendations. Store aliquots of appropriate working concentrations at –80°C.

Schedule for PSC spheroid culture:

Day –5	Day –4	Day –3	Day –2	Day –1	Day 0
Seed 3D PSC culture	Half media change	Half media change	Half media change	Half media change	Passage

Day –5: 3D PSC culture

1. For detailed instructions on PSC suspension culture, follow guidelines provided in the CTS StemScale PSC Suspension Medium user guide.

Notes:

- For best differentiation results, adapt PSCs for suspension culture with three consecutive passages in CTS StemScale medium prior to starting the differentiation protocol.
- This protocol's guidance is based on using 6-well plates. Other formats are referenced throughout where steps may differ.
- It will be necessary to determine an appropriate stirring speed when using equipment other than what is listed in this protocol. Please see the CTS StemScale PSC Suspension Medium user guide for help with speed conversions.
- Optimization of protein/small molecule concentrations, seeding densities, and culture durations are recommended as cell line variability occurs. Prior to initiating this protocol, PSCs should be cultured in suspension for at least three passages in CTS StemScale PSC Suspension Medium.

Schedule for iHPC differentiation:

Day 0	Day 1	Day 2	Day 5	Day 7	Day 9
Passage of 3D culture	Half media change	Full media change	Full media change	Half media change	Half media change

Day 0: Spheroid formation

Mesoderm induction:

Mesoderm induction medium 1		
Component	Stock conc.	Final conc.
CTS StemScale basal medium	1X	1X
CTS StemScale supplement	10X	1X
Antibiotic-Antimycotic	100X	1X
Y-27632	10 mM	10 µM
DNase I	1 U/µL	0.5 U/mL
SCF	100 µg/mL	50 ng/mL
BMP4	100 µg/mL	40 ng/mL
VEGF	100 µg/mL	50 ng/mL

1. Prepare mesoderm induction medium 1 fresh and allow it to warm to room temperature.
2. Transfer the entire volume of spheroids from each well into separate 15 or 45 mL conical tubes.

   Note: Healthy spheroids should be ~400 µm in diameter and appear rounded with well-defined borders. The culture time to reach the recommended spheroid size may differ by cell line; it may take ~4–6 days.
3. Centrifuge the spheroids at 200 x *g* at room temperature for 2 minutes and aspirate medium, being careful not to disturb the spheroid pellet. Alternatively, allow the spheroids to settle by gravity for approximately 5 minutes and aspirate the medium.
4. Resuspend the cell pellet in 25% Gibco™ CTS™ TrypLE™ Select Enzyme (dilute with Gibco™ CTS™ DPBS).

   Note: Recommended dissociation conditions differ by vessel type (Table 1).

Table 1. Dissociation conditions based on vessel type.

Dissociation considerations	Volume		
	6-well plate	125 mL flask	100 mL bioreactor
25% CTS TrypLE Select Enzyme	1 mL per well	5 mL	15 mL (50% CTS TrypLE Select Enzyme)
Time	5 minutes	10 minutes	10–15 minutes

5. Place the conical tubes at 37°C in a water bath for 5 minutes.

   Note: Agitate the tube every few minutes to mix the spheroids in CTS TrypLE Select Enzyme to facilitate dissociation.
6. Triturate the cell suspension gently 2–3 times with a P1000 pipette to ensure spheroids are dissociated into single cells, and add 3 mL of Gibco™ DMEM/F12 + Gibco™ GlutaMAX™ Supplement per 1 mL of diluted CTS TrypLE Select Enzyme to the cell suspension.
7. Centrifuge the cells at 200 x *g* for 4 minutes at room temperature and aspirate supernatant, being certain not to disturb the cell pellet.
8. Resuspend the cell pellet in mesoderm induction medium 1 and perform assessment of total cell count and viability.

   Note: Resuspension volume varies by vessel type (Table 2).

Table 2. Resuspension volume of mesoderm induction medium 1, based on vessel type.

	Working volume for different vessels		
Vessel	Non–tissue culture treated 6-well plate	Non–tissue culture treated 125 mL flask	Non–tissue culture treated 100 mL bioreactor
Volume	2 mL	5 mL	10 mL

9. Seed single cell suspension into a non–tissue culture treated vessel (6-well plate–100 mL bioreactor) at a final concentration of 3 x 10^5 cells/mL. To support proper spheroid formation in different vessels, add mesoderm induction medium 1 to a final volume as listed in Table 3.

   Note: Seeding density of starting PSCs may vary depending on cell line. Make sure to optimize this step for your cell line of interest. We recommend 3 x 10^5 cells/mL as a starting point for day 0.

Table 3. Volume of mesoderm induction medium 1, based on vessel type.

	Working volume for different vessels		
Vessel	Non–tissue culture treated 6-well plate	Non–tissue culture treated 125 mL flask	Non–tissue culture treated 100 mL bioreactor
Volume	2 mL	20 mL	100 mL

10. Incubate plates at 37°C, 5% CO_2 on an orbital shaker platform set to 70 RPM.

    Note: The recommended stir speed for a 100 mL vertical wheel bioreactor is 30–40 RPM but may require additional optimization.

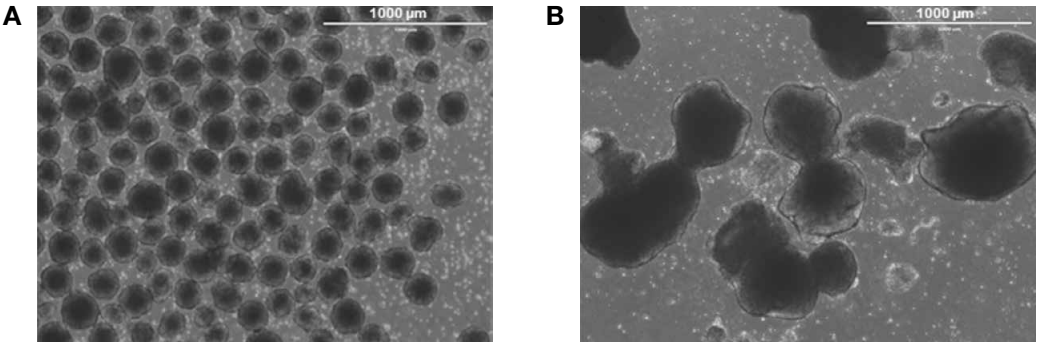

Figure 2. Morphological observations during iHPC differentiation. (A) Day 2: PSC spheroids aggregate and grow to ~150–250 µm diameter. At this stage, the individual cells that did not aggregate into spheroids are lost during subsequent medium changes. **(B)** Day 8: Increased iHPC population detaches from spheroids to further mature and expand in suspension.

Day 1: Medium change

Mesoderm induction:

Mesoderm induction medium 2		
Component	**Stock conc.**	**Final conc.**
CTS StemScale basal medium	1X	1X
CTS StemScale supplement	10X	1X
Antibiotic-Antimycotic	100X	1X
SCF	100 µg/mL	50 ng/mL
BMP4	100 µg/mL	40 ng/mL
VEGF	100 µg/mL	50 ng/mL

1. Prepare mesoderm induction medium 2 fresh and allow it to warm to room temperature.
2. Remove the plate containing spheroids from the incubator.
3. Gently swirl the plate so all spheroids cluster to the middle of the well.
4. Tilt the plate at an ~45° angle for 4–5 minutes to ensure spheroids settle via gravity sedimentation.
5. With the plate tilted, carefully remove 1 mL of culture medium from each well by using a P1000 pipette.

   Note: For medium replacement in a vertical wheel bioreactor (100 mL working volume), remove vessel from the platform for 5–10 minutes to ensure spheroids settle via gravity sedimentation. Manually aspirate 50 mL of medium. Add an equal volume (50 mL) of fresh mesoderm induction medium 2 to replace the aspirated volume.
6. Replace the 1 mL spent medium with equal volume prewarmed mesoderm induction medium 2 per well.
7. Incubate the plate at 37°C, 5% CO_2 on an orbital shaker platform set to 70 RPM or a vertical wheel bioreactor set to 40 RPM.

   Note: iHPC induction can be initiated between days 1–3. Due to differences in PSC line nucleation efficiency and proliferation, spheroid growth may vary. We recommend initiating iHPC induction when most PSC spheroids are between 150–250 µm in diameter.

Day 2: iHPC induction

iHPC induction medium		
Component	Stock conc.	Final conc.
StemPro-34 basal medium	1X	1X
StemPro-34 supplement	40X	1X
1-thioglycerol	11.5 M	400 µmol/L
L-(+)-Ascorbic Acid	10 mg/mL	20 µg/mL
CTS GlutaMAX-I Supplement	200 mM	2 mM
ITS	100X	1X
SCF	100 µg/mL	50 ng/mL
BMP4	100 µg/mL	40 ng/mL
VEGF	100 µg/mL	50 ng/mL
bFGF	100 µg/mL	10 ng/mL

1. Prepare iHPC induction medium fresh (without growth factors or cytokines) and filter before use. The medium is stable for 30 days when stored in the dark at 4–8°C. Growth factors and cytokines are added immediately prior to medium changes.

   Note: Working volumes vary by vessel type (Table 4).

Table 4. Volume of iHPC induction medium 1, based on vessel type.

	Working volume for different vessels		
Vessel	Non–tissue culture treated 6-well plate	Non–tissue culture treated 125 mL flask	Non–tissue culture treated 100 mL bioreactor
Volume	3 mL	20 mL	100 mL

2. Gently swirl the plate so all the spheroids cluster to the middle of the well.
3. Tilt the plate at an ~45° angle for 4–5 minutes to ensure spheroids settle via gravity sedimentation.
4. With the plate still tilted, carefully remove ~80% of the medium from each well by using a P1000 pipette.

   Note: For medium replacement in a vertical wheel bioreactor, remove the vessel from the platform for 5–10 minutes to allow spheroids to settle via gravity sedimentation. Manually aspirate 50 mL of media. Add an equal volume (i.e. 50 mL) of iHPC induction medium to replace the aspirated volume.
5. Add enough iHPC induction medium to obtain the appropriate working volume, as listed in step 1.
6. Incubate the plate at 37°C, 5% CO_2 on an orbital shaker platform set to 60 RPM (or 30–40 RPM for vertical wheel bioreactor cultures).

Day 5: iHPC full medium change

iHPC maturation medium 1		
Component	**Stock conc.**	**Final conc.**
StemPro-34 basal medium	1X	1X
StemPro-34 supplement	40X	1X
1-thioglycerol	11.5 M	400 µmol/L
L-(+)-Ascorbic Acid	10 mg/mL	20 µg/mL
CTS GlutaMAX-I Supplement	200 mM	2 mM
ITS	100X	1X
SCF	100 µg/ml	50 ng/mL
FLT3	100 µg/mL	20 ng/mL
TPO	100 µg/mL	30 ng/mL

1. Prepare iHPC maturation medium 1 fresh and allow it to warm to room temperature.
2. Gently swirl the plate so all the spheroids cluster to the middle of the well.
3. Tilt the plate at an ~45° angle for 4–5 minutes to ensure spheroids settle via gravity sedimentation.
4. Manually remove as much medium per well as possible using a 1,000 µL or 10 mL pipette. Be careful not to remove any spheroids.

   Notes:

   - For medium replacement in a vertical wheel bioreactor, remove vessel from the platform for 5–10 minutes to ensure spheroids settle via gravity sedimentation. Manually aspirate 50 mL of medium. Add an equal volume (50 mL) of iHPC maturation medium 1 to replace the aspirated volume.
   - If spheroids are accidentally aspirated, add the spheroid suspension back to the well and repeat steps 1–3 to minimize sample loss.
5. Add 3 mL iHPC maturation medium 1 to each well.
6. Incubate the plate at 37°C, 5% CO_2 on an orbital shaker platform set to 60 RPM (or 30–40 RPM for bioreactor cultures).

Day 8: iHPC half medium change

iHPC maturation medium 2		
Component	**Stock conc.**	**Final conc.**
StemPro-34 basal medium	1X	1X
StemPro-34 supplement	40X	1X
1-thioglycerol	11.5 M	400 µmol/L
L-(+)-Ascorbic Acid	10 mg/mL	20 µg/mL
CTS GlutaMAX-I Supplement	200 mM	2 mM
ITS	100X	1X
SCF	100 µg/mL	100 ng/mL
FLT3	100 µg/mL	40 ng/mL
TPO	100 µg/mL	60 ng/mL

1. Prepare iHPC maturation medium 2 fresh and allow it to warm to room temperature.
2. Gently swirl the plate so all the spheroids cluster to the middle of the well.
3. Tilt the plate at an ~45° angle for 4–5 minutes to ensure spheroids settle via gravity sedimentation.
4. Medium change is needed while minimizing any loss of maturing iHPCs. With the plate tilted, carefully remove 1.5 mL of culture medium from each well and transfer to a 15 mL conical tube. Be careful not to disturb the settled spheroids.

   Note: For medium replacement in a vertical wheel bioreactor, remove vessel from the platform for 5 minutes to ensure spheroids settle via gravity sedimentation. Remove 50 mL medium without disturbing the settled spheroids and put in a 50 mL tube.
5. Centrifuge the conical tube at 300 x *g* for 5 minutes at room temperature.
6. Aspirate the supernatant without disturbing the cell pellet.
7. Resuspend the cell pellet in 1.5 mL of iHPC maturation medium 2.
8. Add cell suspension back into the same well.
9. Incubate the plate at 37°C, 5% CO_2 on an orbital shaker platform set to 60 RPM (or 30–40 RPM for bioreactor cultures).

   Notes: Assessing iHPC phenotype:

   - To assess phenotype, we recommend culturing duplicate samples: one sample for iHPC phenotyping, and one sample to continue to iNK cell differentiation.
   - When optimizing this protocol, we recommend phenotyping for hematopoietic surface markers between days 6 and 8. Once a baseline has been established, this step can be optimized.
   - To the sample used for iHPC phenotyping, gently break apart cell clumps by triturating with a P1000 pipette ~10 times. There will be some clumps remaining that will get discarded in the next step.
   - Pass the entire 3 mL from one well through a 40 µm cell strainer into a conical tube.
   - Wash the well with 2 mL DMEM/F-12 and filter through the same 40 µm cell strainer.
   - Wash the cell strainer with 15 mL DMEM/F-12 to ensure all single cells have passed through.
   - Proceed with a flow cytometry protocol using the harvested single cell suspension, assessing for the following markers: CD34, CD45, CD90, CD43.

Schedule for iNK cell differentiation:

Day 9	Day 12	Day 15–38
Full medium change	Half medium change	Half medium change every 2–3 days Check iNK cell phenotype once a week beginning on day 25

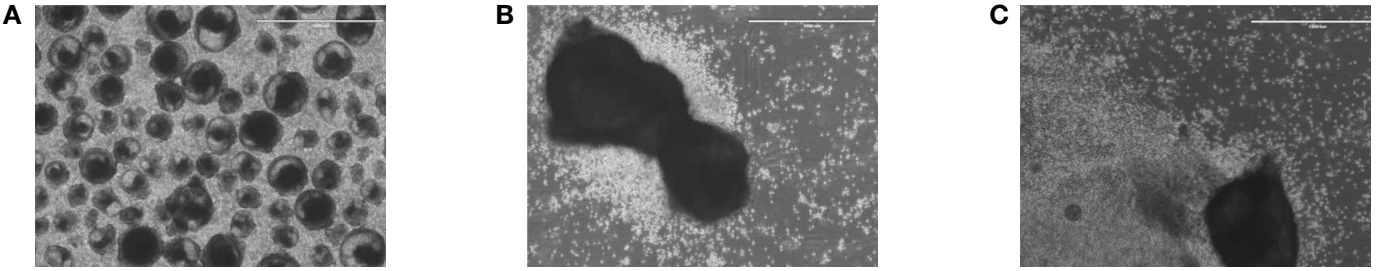

Figure 3. Morphological observations during iNK differentiation. (A) Day 12: Individual iHPC and spheroids begin attaching to vessel floor. **(B)** Day 21: Spheroids form a "feeder-like" layer to which the suspension iNK cells adhere and further mature. **(C)** Day 30: Further expansion of the spheroid monolayer and expansion of iNK cells. The iNK cells at this point will be loosely attached and may reside in suspension.

Day 9: Initiate iNK cell differentiation

iNK cell induction:

iNK cell induction medium		
Component	Stock conc.	Final conc.
CTS NK-Xpander Basal Medium	1X	56%
MEMα	1X	28%
Human AB Serum	1X	15%
CTS NK-Xpander Supplement	50X	1X
β-mercaptoethanol	11.5 M	1 µM
CTS GlutaMAX-I Supplement	200 mM	4 mM
IL-3	100 µg/mL	5 ng/mL
SCF	100 µg/mL	20 ng/mL
FLT3	100 µg/mL	10 ng/mL
IL-7	100 µg/mL	20 ng/mL
IL-15	100 µg/mL	10 ng/mL

1. Prepare iNK cell induction medium fresh (without growth factors or cytokines) and filter before use. Medium is stable for 30 days when stored in the dark at 4–8°C. Growth factors and cytokines are added immediately prior to medium changes.
2. Calculate the volume of iNK cell induction medium required (Table 5).

Table 5. Seeding volume based on vessel type.

PSC to iHPC vessel	Recommended iHPC to iNK cell vessel	Seeding volume
6-well plate	Non–tissue culture treated 6-well plate	3 mL/well
125 mL flask	Non–tissue culture treated T-75 flask	15 mL
100 mL vertical wheel bioreactor	Non–tissue culture treated T-175 flask	50 mL

3. Collect the entire volume of iNK cell induction medium (including residual spheroids) from each well. Place into separate conical tubes for each well.
4. To the emptied culture vessel, immediately add 2 mL of iNK cell induction medium to each well.
5. Centrifuge the conical tubes containing cells and supernatant at 300 x *g* for 5 minutes at room temperature.
6. Aspirate supernatant.
7. Resuspend the cell pellet in 1 mL of iNK cell induction medium.
8. Transfer resuspended cells back into the original well.

   Notes:
   125 mL flask: While the entire volume of the vessel is being centrifuged, add 10 mL of iNK induction medium to a non–tissue culture treated T-75 flask. Aspirate the supernatant and resuspend the cell pellet in 5 mL of iNK cell induction medium. Transfer the resuspended cells back into the new T-75 flask.

   100 mL bioreactor: While the entire volume of the vessel is being centrifuged, add 40 mL of iNK induction medium to a non–tissue culture treated T-175 flask. Aspirate the supernatant and resuspend the cell pellet in 10 mL of iNK cell induction medium. Transfer the resuspended cells back into the new T-175 flask.
9. Incubate plates at 37°C, 5% CO_2 for static culture.

Day 12: iNK cell differentiation half medium change

iNK cell induction medium		
Component	**Stock conc.**	**Final conc.**
CTS NK-Xpander Basal Medium	1X	56%
MEM α	1X	28%
Human AB Serum	1X	15%
CTS NK-Xpander Supplement	50X	1X
β-mercaptoethanol	11.5 M	1 µM
CTS GlutaMAX-I Supplement	200 mM	4 mM
IL-3	100 µg/mL	10 ng/mL
SCF	100 µg/mL	40 ng/mL
FLT3	100 µg/mL	20 ng/mL
IL-7	100 µg/mL	40 ng/mL
IL-15	100 µg/mL	20 ng/mL

1. Prepare iNK cell induction medium fresh (without growth factors or cytokines) and filter before use. Medium is stable for 30 days when stored in the dark at 4–8°C. Growth factors and cytokines are added immediately prior to medium changes.
2. Collect 1.5 mL of culture medium from each well and transfer to a 15 mL conical tube.
3. Centrifuge the tubes at 300 x *g* for 5 minutes at room temperature.
4. Aspirate supernatant.
5. Resuspend cells in 1.5 mL of iNK cell induction medium per well to replace the aspirated volume.
6. Transfer resuspended cells back into the original well/vessel.

   Notes:
   T-75 flask: Collect 8 mL of culture medium and transfer to a 15 mL conical tube. Centrifuge the tubes at 300 x *g* for 5 minutes, then aspirate supernatant. Resuspend in 8 mL of iNK cell induction medium to replace the aspirated volume. Transfer resuspended cells back into the original vessel.

   T-175 flask: Collect 25 mL of culture medium and transfer to a 25 mL conical tube. Centrifuge the tubes at 300 x *g* for 5 minutes then aspirate supernatant. Resuspend in 25 mL of iNK cell induction medium to replace the aspirated volume. Transfer resuspended cells back into the original vessel.
7. Incubate plates/flasks at 37°C, 5% CO_2 for static culture.

Day 15: iNK cell differentiation half medium change

iNK cell maturation medium		
Component	Stock conc.	Final conc.
CTS NK-Xpander Basal Medium	1X	56%
MEM α	1x	28%
Human AB Serum	1x	15%
CTS NK-Xpander Supplement	50X	1X
β-mercaptoethanol	11.5 M	1 µM
CTS GlutaMAX-I Supplement	200 mM	4 mM
SCF	100 µg/mL	40 ng/mL
FLT3	100 µg/mL	20 ng/mL
IL-7	100 µg/mL	40 ng/mL
IL-15	100 µg/mL	20 ng/mL

1. Prepare iNK cell maturation medium fresh (without growth factors or cytokines) and filter before use. Medium is stable for 30 days when stored in the dark at 4–8°C. Growth factors and cytokines are added immediately prior to medium changes.
2. Calculate the volume of iNK cell maturation medium required for a half medium change.
3. Collect 1.5 mL of culture medium from each well and transfer to a 15 mL conical tube.
4. Centrifuge the tubes at 300 x *g* for 5 minutes at room temperature.
5. Aspirate supernatant.
6. Resuspend cells in 1.5 mL of iNK cell maturation medium per well to replace the aspirated volume.
7. Transfer resuspended cells back into the original well/vessel.

   Notes:
   T-75 flask: Collect 8 mL of culture medium and transfer to a 15 mL conical tube. Spin tubes at 300 x *g* for 5 minutes then aspirate supernatant. Resuspend in 8 mL of iNK cell maturation medium to replace the aspirated volume. Transfer resuspended cells back into the original vessel.

   T-175 flask: Collect 25 mL of culture medium and transfer to 25 mL conical tube. Spin tubes at 300 x *g* for 5 minutes then aspirate supernatant. Resuspend in 25 mL of iNK cell differentiation medium to replace the aspirated volume. Transfer resuspended cells back into the original vessel.
8. Incubate plates/flasks at 37°C, 5% CO_2 for static culture.

Day 18–36: iNK cell differentiation half medium change

1. Calculate the volume of iNK cell maturation medium required for a half medium change.
2. Collect 1.5 mL of culture medium from each well and transfer to a 15 mL conical tube.
3. Centrifuge the tubes at 300 x *g* for 5 minutes at room temperature.
4. Aspirate supernatant.
5. Resuspend cells in 1.5 mL of iNK cell maturation medium per well to replace the aspirated volume.
6. Transfer resuspended cells back into the original well.

   Notes:
   T-75 flask: Collect 8 mL of culture medium and transfer to a 15 mL conical tube. Spin tubes at 300 x *g* for 5 minutes then aspirate supernatant. Resuspend in 8 mL of iNK cell maturation medium to replace the aspirated volume. Transfer resuspended cells back into the original vessel.

   T-175 flask: Collect 25 mL of culture medium and transfer to 25 mL conical tube. Spin tubes at 300 x *g* for 5 minutes then aspirate supernatant. Resuspend in 25 mL of iNK cell differentiation medium to replace the aspirated volume. Transfer resuspended cells back into the original vessel.
7. Incubate plates/flasks at 37°C, 5% CO_2 for static culture.
8. Perform medium changes every 2–3 days.

   Notes:
   We recommend performing flow cytometry weekly starting the week of day 25, checking for CD45 and CD56 expression to monitor differentiation. Collect aliquots of the cell suspension from the well plate/vessel. Once the population of suspension cells reaches 60%, they can be harvested for downstream applications or cryopreserved.

Day 38: iNK cell harvest and cryopreservation

1. iNK cells weakly attach to the bottom of the culture vessel. Resuspend iNK cells in the culture vessel by gently triturating with a P1000 pipette.
2. Collect entire 3 mL of medium from each well and filter using a 100 µm cell strainer into a conical tube.

   Notes:
   T-75 flask: Collect entire 15 mL of medium and filter using a 100 µm cell strainer into a conical tube. Rinse the bottom of the flask with 10 mL of DMEM/F12, GlutaMAX Supplement. Pass through the same cell strainer.

   T-175 flask: Collect entire 50 mL of medium and filter using a 100 µm cell strainer into a conical tube. Place the cell strainer to a new conical tube. Rinse the bottom of the flask with 25 mL of DMEM/F12, GlutaMAX Supplement. Pass through the same cell strainer. Combine contents of both conical tubes to perform cell counts.
3. Rinse each well with 2 mL of DMEM/F12 + GlutaMAX Supplement to collect any remaining iNK cells. Pass through the same cell strainer.
4. Wash the cell strainer with 15 mL of DMEM/F12 + GlutaMAX Supplement.
5. Centrifuge the tubes containing cells and supernatant at 300 x *g* for 5 minutes at room temperature.
6. Aspirate supernatant. Resuspend cell pellet in 1–5 mL of DMEM/F12 + GlutaMAX Supplement to perform cell counts.

   Note: iNK cells can be sampled from this step for surface marker analysis. We recommend flow cytometry analysis of surface markers CD56, CD45, CD16, CD3 (Figure 4).
7. Record the viability and concentration of cells. Calculate the total number of cells harvested by multiplying cell concentration by the total volume.
8. Calculate the volume of Gibco™ CTS™ PSC Cryomedium required to generate 2.5 x 10^6 cells/mL.
9. Centrifuge the tubes containing cells and supernatant at 300 x *g* for 5 minutes at room temperature.
10. Aspirate supernatant.
11. Resuspend cell pellet in calculated volume of CTS PSC Cryomedium to achieve a cell density of 2.5 x 10^6 cells/mL.
12. Transfer resuspended cells into cryovials and store in a Thermo Scientific™ Mr. Frosty™ Freezing Container at –80°C.
13. Move to liquid nitrogen for long-term storage within a week.

    Note: iNK cells can be expanded prior to cryopreservation or post-cryopreservation. Please see CTS NK-Xpander Medium **user guide** for help with expansion.

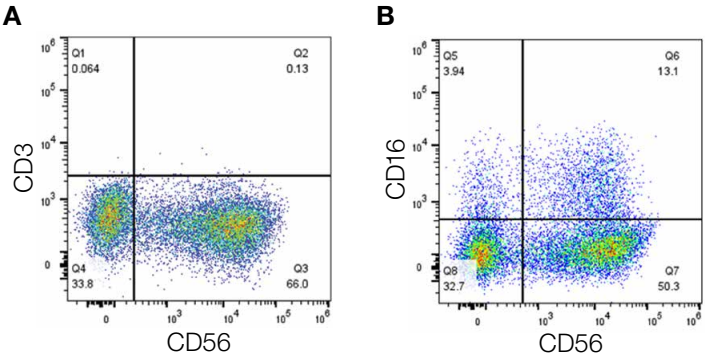

Figure 4. iNK cell phenotyping prior to expansion. (A) Flow cytometry analysis of day-31 iNK cells shows 66% $CD56^+$ populations with only 0.16% $CD56^+CD3^+$ double positive cells indicating a very low level of T-cell markers. **(B)** Detection of 13.1% $CD56^+CD16^+$ double positive cells highlights the differentiated iNK cells expressing markers of activation.

iNK cell expansion

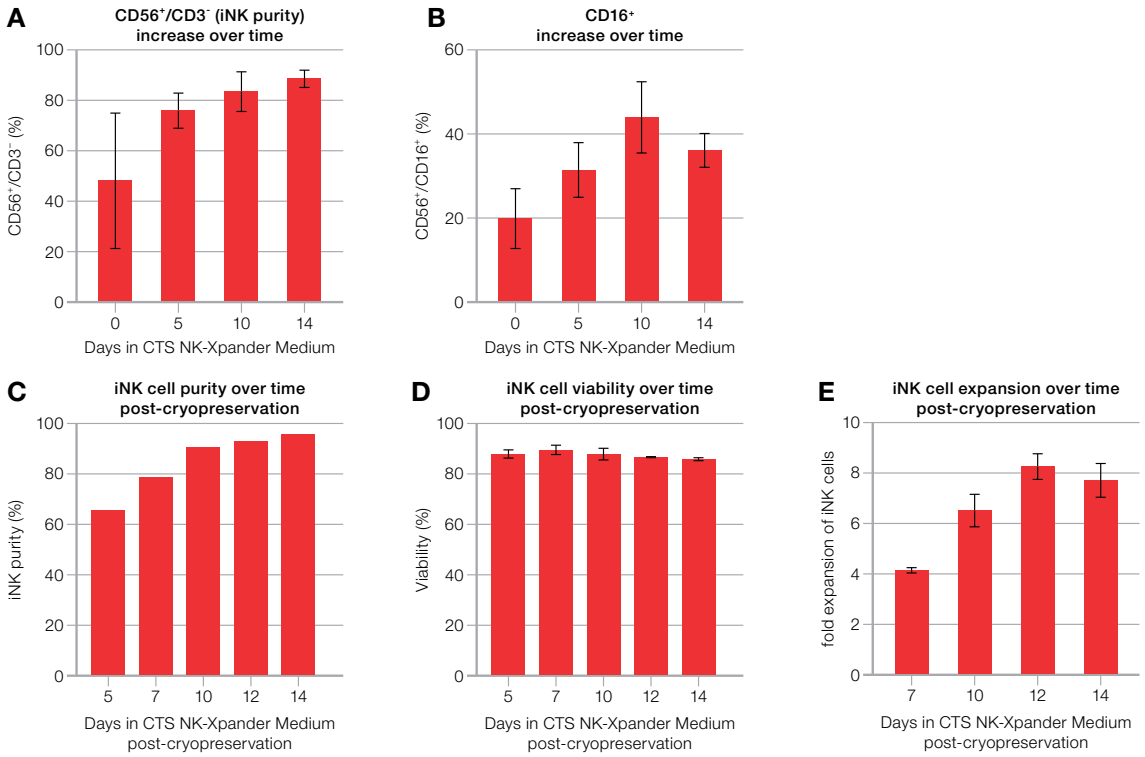

Figure 5. Characterization of iNK cells enriched in CTS NK-Xpander Medium. (A) Surface marker expression of $CD56^+/CD3^-$ indicates a highly enriched population of iNK cells. **(B)** Expression of the cytolytic $CD16^+$ marker increased over 14 days. **(C)** Further enrichment of the $CD56^+/CD3^-$ iNK cell population post-cryopreservation. **(D)** iNK cell viability remained stable post-cryopreservation. **(E)** iNK cells continue to proliferate post-cryopreservation, reaching an ~8-fold expansion over 12–14 days.

Thawing iNK cells from liquid nitrogen

iNK cell enrichment medium		
Component	**Stock conc.**	**Final conc.**
CTS NK-Xpander Basal Medium	1X	1x
Human AB Serum	1X	5%
CTS NK-Xpander Supplement	50X	1X
IL-2	1 x 10^6 U/mL	500 U/mL
IL-15	100 µg/mL	10 ng/mL

1. Prepare iNK enrichment medium fresh and filter before the addition of recombinant proteins, which should be added fresh during each media change.
2. Thaw the cryovial of iNK cells in a 37°C water bath. When only a sliver of ice remains, transfer contents into a 15 mL conical tube containing 9 mL of CTS NK-Xpander Medium. Rinse vial with 1 mL of medium to collect any remaining cells in the cryovial. Add to conical tube.
3. Centrifuge the 15 mL tube at 300 x *g* for 5 minutes to pellet the cells.
4. Aspirate supernatant.
5. Resuspend cell pellet in 1–2 mL of iNK cell enrichment medium, supplemented with 500 U/mL IL-2 and 10 ng/mL IL-15.
6. Calculate cell viability and cell concentration.
7. Seed iNK cells in the iNK cell enrichment medium at 3–5 x 10^5 cells/mL into each well of a 24-well plate.
8. After 4 days in culture, perform a full medium change. Re-seed iNK cells at 3 x 10^5 cells/mL in iNK cell enrichment medium containing fresh 500 U/mL IL-2 and 10 ng/mL IL-15.

   Note: Scale up to a larger plate format (6- to 12-well plate) if needed.
9. Spike fresh 500 U/mL IL-2 and 10 ng/mL IL-15 every 1–2 days. Perform a full medium change every 4–5 days.

   Note: If confluency reaches 2 x 10^6 cells/mL, passage cells and resuspend in fresh iNK enrichment medium containing fresh 500 U/mL IL-2 and 10 ng/mL IL-15 at 3 x 10^5 to 5 x 10^5 cells/mL.

Conclusions

Cultivation of human PSCs grown in CTS StemScale PSC Suspension Medium enables efficient scale-up potential that can be further harnessed to generate large quantities of differentiated cells such as iNK cells. With this protocol, we have demonstrated how the use of cytokine cocktails can drive the differentiation of PSC spheroids into iNK cells that express markers of activation. The iNK cells can be further expanded to increase yields and purity. Additionally, the iNK cells maintain expansion capabilities post-cryopreservation.

Ordering information

Description	Cat. No.
Reagents	
2-Mercaptoethanol	21985023
Antibiotic-Antimycotic	15240112
CTS DPBS, without calcium chloride, without magnesium chloride	A1285601
CTS GlutaMAX-I Supplement	A1286001
CTS NK-Xpander Medium	A5019001
CTS PSC Cryomedium	A4238801
CTS StemScale PSC Suspension Medium	A5869601
CTS TrypLE Select Enzyme	A1285901
CD16 APC	17-0168-42
CD3 FITC	11-0037-42
CD45 PE-Cyanine7	25-0459-42
CD56 PE	12-0567-42
DMEM/F-12, GlutaMAX Supplement	10565018
DNase I, RNase-free	89836
DPBS, no calcium, no magnesium	14190144
Fisherbrand Sterile Cell Strainers	22-363-549
Insulin-Transferrin-Selenium (ITS -G) (100X)	41400045
L-(+)-Ascorbic Acid	A15613.36
MEM α, nucleosides	12571-063
Monothioglycerol, cell culture tested	M6145

Description	Cat. No.
Reagents (continued)	
PeproGMP Human BMP-4 Recombinant Protein	GMP120-05ET
PeproGMP Human FGF-basic (FGF-2/bFGF) Recombinant Protein	GMP100-18B
PeproGMP Human Flt-3 Ligand (FLT3L) Recombinant Protein	GMP300-19
PeproGMP Human IL-15 Recombinant Protein	GMP200-15
PeproGMP Human IL-2 Recombinant Protein	GMP200-02
PeproGMP Human IL-3 Recombinant Protein	GMP200-03
PeproGMP Human IL-7 Recombinant Protein	GMP200-07
PeproGMP Human SCF Recombinant Protein	GMP300-07
PeproGMP Human TPO (Thrombopoietin) Recombinant Protein	GMP300-18
PeproGMP Human VEGF-165 Recombinant Protein	GMP100-20
StemPro-34 SFM	10639011
Trypan Blue	15250061
Y-27632 2HCl	129830-38-2
Equipment	
Attune NxT Flow Cytometer	A24861
Countess II Automated Cell Counter	AMQAF1000
CO_2 Resistant Shaker	88881101
Mr. Frosty Freezing Container	5100-0001
Nunc Non-Treated Multidishes (6-well plates)	150239
Sterile Single-Use Erlenmeyer Flasks with Plain Bottom (125 mL)	4117-0125

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