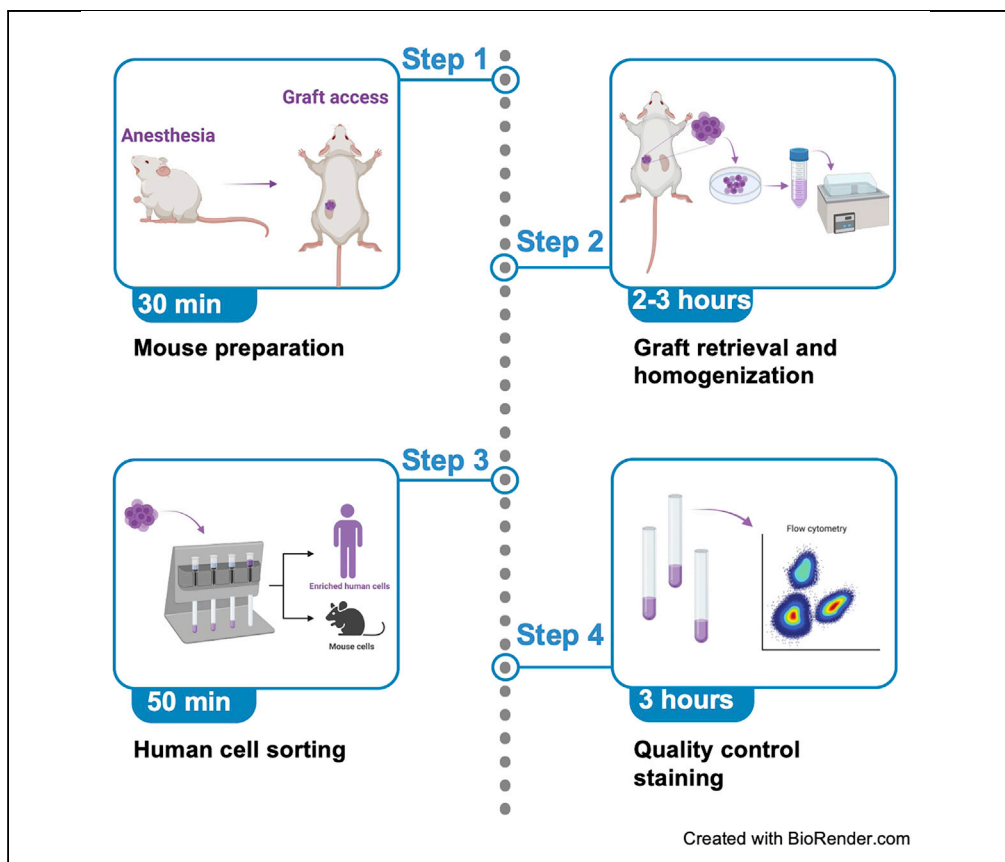


## Protocol

# Retrieval and purification of human $\beta$ cells from stem cell-derived islet engrafted mice



Stem cell-derived beta cells (SC- $\beta$ -cells) engrafted into mice serve as a pre-clinical model of diabetes. It is helpful to recover viable  $\beta$  cells following transplantation to perform tests on the graft. We developed a protocol to retrieve and purify a sufficient number of live  $\beta$  cells from mice following long-term human SC- $\beta$ -cell engraftment. The protocol enables examination of SC- $\beta$ -cells undergoing developmental and metabolic changes *in vivo* and may facilitate the understanding of metabolic demand on SC- $\beta$ -cells.

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### Highlights

Retrieval of a large number of highly viable stem cell-derived  $\beta$  cells (SC- $\beta$ -cells)

Purification of graft cells using target cell-specific magnetic sorting

Molecular and functional graft cell analysis

Kenty et al., STAR Protocols 2,  
100675  
September 17, 2021 © 2021  
The Authors.  
<https://doi.org/10.1016/j.xpro.2021.100675>



## Protocol

Retrieval and purification of human  $\beta$  cells from stem cell-derived islet engrafted miceJennifer Hyoje-Ryu Kenty,<sup>1,2,\*</sup> Gabriela Cristina Pelayo,<sup>1</sup> and Nayara Carvalho Leite<sup>1,3,\*</sup><sup>1</sup>Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [hryu@mcg.harvard.edu](mailto:hryu@mcg.harvard.edu) (J.H.-R.K.), [nayara\\_leite@harvard.edu](mailto:nayara_leite@harvard.edu) (N.C.L.)  
<https://doi.org/10.1016/j.xpro.2021.100675>

## SUMMARY

**Stem cell-derived beta cells (SC- $\beta$ -cells) engrafted into mice serve as a pre-clinical model of diabetes. It is helpful to recover viable  $\beta$  cells following transplantation to perform tests on the graft. We developed a protocol to retrieve and purify a sufficient number of live  $\beta$  cells from mice following long-term human SC- $\beta$ -cell engraftment. The protocol enables examination of SC- $\beta$ -cells undergoing developmental and metabolic changes *in vivo* and may facilitate the understanding of metabolic demand on SC- $\beta$ -cells.**

## BEFORE YOU BEGIN

The protocol below describes the specific steps to recover SC- $\beta$ -cells transplanted under the kidney capsule of immune compromised mice (Alvarez-Dominguez et al., 2020; Davis et al., 2020). In this protocol we show results from grafts retrieved 5 and 12 months post-transplantation.

**Note:** This protocol is an optimization of previous protocols for SC- $\beta$ -cell retrieval. While other protocols focus on human islet retrieval (Redick et al., 2020), this protocol focuses on retrieving SC- $\beta$ -cells as done in Davis et al. (2020); Alvarez-Dominguez et al. (2020).

The main difference between this protocol and previous protocols, is that previous protocols (Redick et al., 2020; Davis et al., 2020; Alvarez-Dominguez et al., 2020) used fluorescence-activated cell sorting (FACS) to sort SC- $\beta$ -cells. Here, we use magnetic-activated cell sorting (MACS), similar to (Aug-sornworawat et al., 2020). The advantages of using MACS compared to FACS include greater cell recovery and faster processing of both single and multiple samples due to the ability to run samples in parallel when using MACS (Sutermaster and Darling, 2019).

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Rat anti-C-Peptide	Developmental Studies Hybridoma Bank (DSHB)	Cat# GN-ID4, RRID:AB_2255626
Donkey anti-Rabbit Alexa 488	Thermo Fisher Scientific	Cat# A-21208, RRID:AB_2535794
Mouse anti-HLA-ABC PE-conjugated	BioLegend	Cat# 311406, RRID:AB_314875
Mouse lineage cocktail-PB antibody	BioLegend	Cat#133310, RRID:AB_11150779

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse Cell Depletion Cocktail	Miltenyi	Cat# 130-104-694
PE anti-human CD274 (PD-L1)	BioLegend	Cat# 329705, RRID:AB_940366
PE anti-human CD255 (Tweak-TNF $\alpha$ -R)	BioLegend	Cat# 308305, RRID:AB_314709
PE anti-human CD254 (Trance-TNF $\alpha$ -R)	BioLegend	Cat# 347503, RRID:AB_2256265
PE anti-human CD119 (IFN $\gamma$ -R $\alpha$ chain)	BioLegend	Cat# 308703, RRID:AB_2121591
PE anti-human IFN $\gamma$ -R $\beta$ chain	BioLegend	Cat# 308504, RRID:AB_314718

**Chemicals, peptides, and recombinant proteins**

RPMI	Thermo Fisher Scientific	Cat# 11875119
PBS (1 $\times$ ) (no calcium, no magnesium)	Thermo Fisher Scientific	Cat# 14190144
Horse Serum	Thermo Fisher Scientific	Cat# 1605130
Collagenase I	Life Technologies	Cat# 17018029
Perm/Wash buffer	BD Biosciences	Cat# 554723, RRID:AB_2869011
Cytofix/Ccytoperm Buffer	BD Biosciences	Cat# 554714, RRID:AB_2869008
Fetal Bovine Serum (FBS)	Sigma-Aldrich	Cat# 12103C
Bovine Serum Albumin (BSA)	MilliporeSigma	Cat# 12659

**Experimental models: Cell lines**

HUES-8 cell line	Harvard University	RRID:CVCL_B207
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**Experimental models: Organisms/strains**

Mouse ( <i>Mus musculus</i> ): SCID beige	Charles River Laboratories	Cat# 250
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**Software and algorithms**

FlowJo 10.7.2	Tree Star	RRID:SCR_008520
Attune NxT 4.2	Thermo Fisher Scientific	RRID:SCR_019590
GraphPad Prism 9.1.1	GraphPad	RRID:SCR_002798

**Other**

Strainer 37 $\mu$ m	STEMCELL Technologies	Cat# 27250
Strainer 200 $\mu$ m	pluriSelect	Cat# 435020003
QuadroMACS Separator	Miltenyi	Cat# 130-090-976
LS columns	Miltenyi	Cat# 130-042-401
Wide Bore Filtered Pipette Tips	Thermo Fisher Scientific	Cat# 2079GPK
Povidone iodine prep solution	Medline Industries	Cat# MDS093904
Eye ointment	Amazon	Systane Nighttime Lubricant Eye Ointment, 3.5 g
Far Infrared Warming Pad with Controller	Kent Scientific Corporation	Cat# RT-0520
Micro Dissecting Scissors	Roboz	Cat # RS-5914

## MATERIALS AND EQUIPMENT

**Collagenase I solution (5 mL of Collagenase I solution per mouse/graft)**

Reagent	Final concentration	Amount
Collagenase	2 mg/mL	20 mg
RPMI	n/a	10 mL
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

**Note:** Dissolve and filter the above before each experimental case. This solution cannot be stocked.

**Sorting buffer (30 mL of sorting buffer per mouse/graft)**

Reagent	Final concentration	Amount
BSA	0.5%	0.25 g
PBS	n/a	50 mL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>

**Note:** Dissolve and filter the above. Aliquot and store at  $-20^{\circ}\text{C}$  for up to 6 months or at  $4^{\circ}\text{C}$  for up to 2 weeks. Prepare 30 mL per mouse/graft.

Surface staining buffer (10 mL of sorting buffer per mouse/graft)		
Reagent	Final concentration	Amount
FBS	2%	1 mL
PBS	n/a	50 mL
Total	n/a	50 mL

**Note:** Dissolve and filter the above. Aliquot and store at  $-20^{\circ}\text{C}$  for up to 6 months or at  $4^{\circ}\text{C}$  for up to 2 weeks.

### STEP-BY-STEP METHOD DETAILS

#### Preparation on the day of experiment

⌚ Timing: 15 min

In this step, the solution preparation is described.

1. Prepare 2 mg/mL Collagenase I solution in RPMI media.
  - a. Make the Collagenase I solution by weighing 20 mg of collagenase and adding it to 10 mL RPMI.
  - b. Prepare 5 mL of Collagenase I solution per mouse/graft.  
*Keep Collagenase I solution on ice at all times to protect it from degradation.*
2. Prepare sorting buffer (0.5% BSA in PBS).
  - a. Prepare 30 mL of sorting buffer per mouse/graft.

#### Mouse preparation

⌚ Timing: 30 min

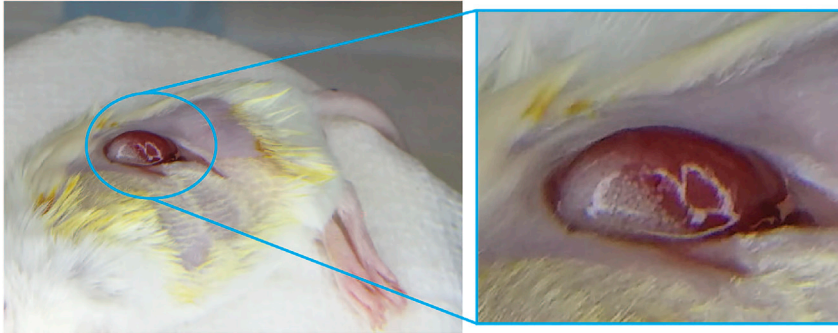
In this step, the mouse preparation is described.

3. Anesthetize mouse with 1–2% isoflurane and assess anesthetic depth by a firm toe pinch using front toes (pedal reflex).
4. Place mouse on infrared heat pad for temperature regulation and apply sterile eye ointment before shaving to minimize the risk of introducing hair in the eye and drying of the eyes.
5. Shave and disinfect the surgical site with betadine swabs alternated with alcohol at least three times.
6. Make a less than 1 cm incision on the back above the kidney to allow access to the organ. If necessary, free kidney from adhesion and fat by carefully cutting fat away from the kidney without damaging the kidney using micro dissecting scissors. Identify the graft (filmy white graft is visible on ventral side of left kidney – [Figure 1](#)).

#### Graft collection

⌚ Timing: 2–3 h

In this step, the graft collection and homogenization are described. After engraftment, the typical graft size of 5–12 month old  $\sim 5$  million cell transplantation is around 0.5 cm/0.2 inches and covers a small portion of the kidney as shown in [Figure 2](#).



**Figure 1. Representative images of SC- $\beta$ -cells transplanted under the kidney capsule (image taken right after the injection)**

7. Euthanize mouse by performing cardiac puncture if blood collection is needed or by giving lethal amount of isoflurane inhalant, 4% isoflurane, followed by cervical dislocation.
8. Trim fat as closely as possible from kidney.
9. Remove the kidney with the graft from the body.
10. Place kidney with graft in a petri dish that contains 5 mL of PBS to keep the graft hydrated.
11. Using a razor blade, dissect kidney into hemispheres and gently peel graft-containing capsule using forceps.
12. Slice the graft into several small pieces by pulling apart using two plastic tips (Figure 3).

**△ CRITICAL: Make sure you chop the graft into small pieces (approximately 1 mm) before proceeding to the next step.**

13. Place the graft pieces into a 50 mL tube with 5 mL of Collagenase I solution. Keep it on ice until ready to proceed to the next step.

**△ CRITICAL: This is an important step for survival of the cells. Make sure you proceed to the next step as soon as possible. We recommend not more than 20 min.**

14. Incubate the graft in a water bath at 37°C for 45 min. Vigorously shake or vortex every 15 min.

**Note:** If after 45 min you still see a lot of graft not dissociated, incubate it for additional 10 min. We do not recommend incubating the graft longer than total of 55 min, it will cause decrease of cell viability.

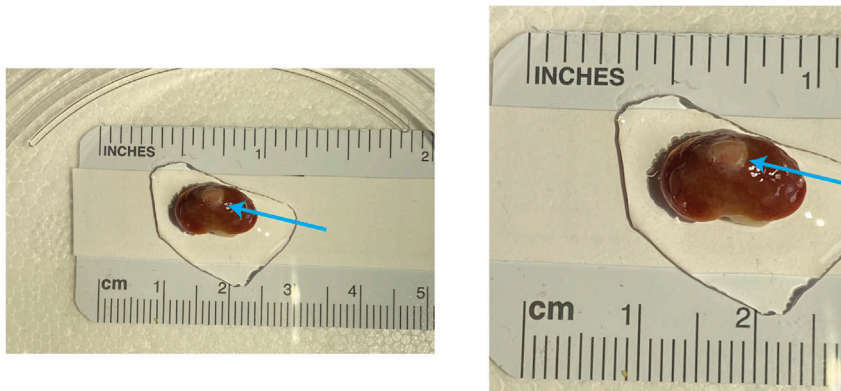
15. After incubation, breakup the tissue with a wide mouth pipette and filter the homogenized graft through a 200  $\mu$ m cell strainer into a new 15 mL tube.
16. Centrifuge the graft homogenate at 500  $\times$  g for 5 min, remove the supernatant, and resuspend the cell pellet with 10 mL sorting buffer (0.5% BSA in PBS).

### Human cell sorting

⌚ Timing: 50 min

In this step, the cell suspension preparation and human fraction enrichment are described. All the steps in this section, including centrifugations, are performed at room temperature, 20°C–22°C, unless indicated otherwise.

17. Filter the cell suspension from step 16 using a 37  $\mu$ m filter and determine cell number using a manual or automatic cell counter.



**Figure 2. Kidney grafts collected post-transplantation**  
SC- $\beta$ -cell graft is indicated by the arrow.

18. Centrifuge cell suspension at  $300 \times g$  for 10 min. Aspirate supernatant completely.
19. Resuspend cell pellet in 80  $\mu$ L of sorting buffer per  $1 \times 10^7$  total cells.
20. Add 20  $\mu$ L of Mouse Cell Depletion Cocktail (Miltenyi, 130-104-694) per  $1 \times 10^7$  total cells.
21. Mix well and incubate for 15 min at  $2^{\circ}\text{C}$ – $8^{\circ}\text{C}$ .
22. Centrifuge cell suspension at  $300 \times g$  for 5 min. Discard the supernatant.
23. Resuspend the cell pellet with 5 mL of sorting buffer and centrifuge at  $300 \times g$  for 5 min. Discard the supernatant.
24. Adjust volume to 500  $\mu$ L using sorting buffer for up to  $1 \times 10^7$  total cells. Keep the cells at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  until ready to proceed to step 25.

**△ CRITICAL:** Up to  $1 \times 10^7$  cells can be processed on one LS Column. If more cells were used, split the sample into multiple LS Columns.

Proceed to magnetic separation.

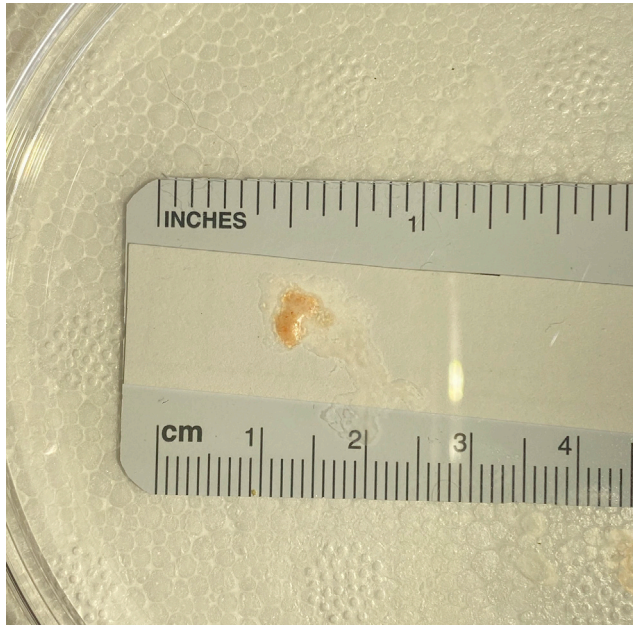
25. Place column in the magnetic field of a QuadroMACS Separator (130-090-976).
26. Prepare column by rinsing with 3 mL of sorting buffer. Wait until it stops dripping.
27. Put a 15 mL tube under the column; this tube will be used to collect the unlabeled fraction of human cells.
28. Apply cell suspension (500  $\mu$ L) onto the column. Collect flow-through containing unlabeled cells, representing the enriched human cells.
29. Wash column twice with 1 mL of sorting buffer. Collect the unlabeled cells that pass through at the same tube.
30. Remove column from the separator and place it in a new 15 mL collection tube, this tube will be used to collect magnetic labeled fraction of mouse cells. Pipette 3 mL of buffer onto the column. Immediately flush out the magnetically labeled mouse cells by firmly pushing the plunger into the column.

**Note:** If one originally transplanted 5 million cells into the kidney capsule, on average one can recover 1–2 million human cells per mouse graft following transplantation for 5–12 months.

### Quality control staining

⌚ Timing: 3 h

In this section, standard staining for quality control is described.



**Figure 3.** SC- $\beta$ -cell graft after being cut into small pieces ( $\sim$ 1 mm)

**Note:** You can add antibodies of your choice for quality control assessment here if you would like.

**△ CRITICAL:** Perform staining on ice until the fixation step to protect cells from dying.

31. Collect a small fraction ( $\sim$ 300  $\mu$ L) of both the human and mouse final resuspension for quality control assessment and transfer each to new 1.5 mL tubes.
32. Centrifuge cell suspension at 300  $\times$  g for 5 min at 4°C. Discard the supernatant.
33. Add 100  $\mu$ L of surface staining buffer (PBS + 2% FBS) to each tube and incubate for 30 min. This is the blocking step.

**Note:** At this point you can transfer your samples to a round bottom 96-well plate. It is useful to do the staining in a plate if you have preparations from more than 5 grafts/mice.

34. Prepare 100  $\mu$ L of 2 $\times$  concentrated surface staining antibody mix for each sample.
  - a. For one sample: Add the mouse lineage cocktail-PB antibody (biolegend 133310, dilution 1:50) + HLA-ABC-PE antibody (biolegend 311405, dilution 1:50) to 100  $\mu$ L of surface staining buffer.

**Note:** To calculate the volume needed, multiply the number of samples (n) plus 1 by 100.  $(n + 1) * 100 = \text{volume } (\mu\text{L})$  needed for staining. We use this calculation to account for pipette errors.

35. Add 100  $\mu$ L of 2 $\times$  concentrated surface staining antibody mix into each sample (from step 33) and incubate on ice for 45 min.
36. Centrifuge cell suspension at 300  $\times$  g for 5 min at 4°C, discard the supernatant, and add 200  $\mu$ L of surface staining buffer. This is the wash.
37. Repeat step 36 once.
38. Centrifuge cell suspension at 300  $\times$  g for 5 min at 4°C, discard the supernatant, and add 100  $\mu$ L of cytofix/cytoperm buffer solution (BD 554714) in each sample for 15 min at 20°C–22°C.

39. Centrifuge cell suspension at  $300 \times g$  for 5 min at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ , discard the supernatant, and add 200  $\mu\text{L}$  of perm/wash buffer (1 $\times$ ) (BD 554723) at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ .
40. Repeat step 39 once.

▮▮ **Pause point:** Store the samples  $4^{\circ}\text{C}$  for up 3 days or proceed to step 41

41. Incubate for 15 min at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ .
42. Prepare 200  $\mu\text{L}$  of 2 $\times$  concentrated intracellular staining antibody mix for each sample.
  - a. For one sample: Add the Rat anti-C-Peptide antibody (DSHB, GN-ID4, dilution 1:50) to 200  $\mu\text{L}$  of surface staining buffer.

**Note:** To calculate the volume needed, multiply the number of samples (n) plus 1 by 200.  $(n + 1) * 200 = \text{volume needed for staining}$ . We use this calculation to account for pipette errors.

43. Add 200  $\mu\text{L}$  of 2 $\times$  concentrated intracellular staining antibody mix into each sample (from step 38) and incubate at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  for 1 h.
44. Centrifuge cell suspension at  $300 \times g$  for 5 min at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ , discard the supernatant, and add 200  $\mu\text{L}$  of perm/wash buffer (1 $\times$ ) (BD 554723) at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ .
45. Repeat step 44 once.
46. Prepare 200  $\mu\text{L}$  of 2 $\times$  concentrated secondary antibody mix for each sample.
47. For one sample: Add the Donkey anti-Rat IgG Secondary Antibody, Alexa Fluor 488 (ThermoFisher, A-21208, dilution 1:150) to 200  $\mu\text{L}$  of surface staining buffer.

**Note:** To calculate the volume needed, multiply the number of samples (n) plus 1 by 200.  $(n + 1) * 200 = \text{volume needed for staining}$ . We use this calculation to account for pipette errors.

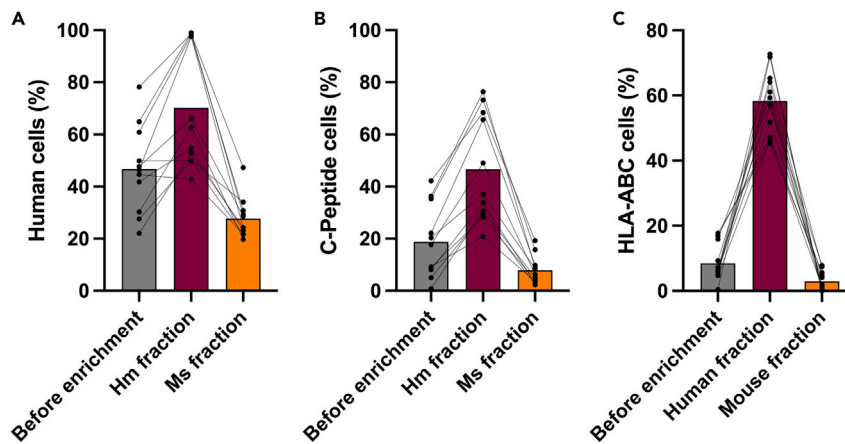
48. Add 200  $\mu\text{L}$  of 2 $\times$  concentrated secondary antibody mix into each sample (from step 44) and incubate at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  for 30 min.
49. Centrifuge cell suspension at  $300 \times g$  for 5 min at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ , discard the supernatant, and add 200  $\mu\text{L}$  of perm/wash buffer (1 $\times$ ) (BD 554723) at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ .
50. Repeat 49.
51. Proceed to flow cytometry analysis.

## EXPECTED OUTCOMES

Starting with 5 million SC- $\beta$ -cells transplanted for 5–12 months, we retrieved 1–2 million human cells per graft. After the mouse cell depletion step, we assessed the percentage of (A) total human cells (gated on the mouse lineage negative population), (B) cells that express a specific human antigen (HLA-ABC+ cells), and (C) cells that express a  $\beta$  cell specific protein (C-peptide+ cells) (Figure 4). As a quality control check, we expect that HLA-ABC+, C-Peptide+ cell populations are enriched in the human fraction after sorting.

Here, we describe an example of downstream analysis that can be performed with recovered SC- $\beta$ -cells. The recovery of viable SC- $\beta$ -cells from grafts for downstream analyses would facilitate the expansion of *in vivo* pre-clinical studies for  $\beta$  cell replacement therapy. As one consideration, a challenge for  $\beta$  cell replacement therapy is shielding the cells from immune attack. With this protocol, SC- $\beta$ -cells can be recovered and used for assays on  $\beta$  cell protection. We retrieved SC- $\beta$ -cells at different time points post-transplantation and performed protein expression analysis at the single cell level using flow cytometry. We assessed, over-time, the expression of the cytokine receptors, tumor necrosis factor alpha receptor (TNF $\alpha$ -R) and interferon gamma receptor (IFN $\gamma$ -R), and PD-L1 (Figure 5).





**Figure 4. Enrichment of human cells**

Flow cytometry quantification of (A) Human cells, (B) C-Peptide+ cells, and (C) HLA-ABC+ cells after sorting using a mouse depletion cocktail (n = 11 mouse/grfts). Data are means.

In summary, we present a method to recover sufficient numbers of viable cells from long-term human SC- $\beta$ -cell grafts in mice to allow molecular and functional analyses post-graft retrieval.

## LIMITATIONS

Total cell yield may depend on the number of engrafted cells which could vary due to hypoxia-induced cell loss post-transplantation (2–3 weeks post-transplantation prior to the vascularization event). We typically measure plasmatic human insulin levels every 4 weeks post-transplantation to validate the success of engraftment.

Retrieval of SC- $\beta$ -cells post-transplantation also comes with challenges due to scar tissue forming around the graft over time. The above discussed cell yield and purity are based on isolation of grafts 5 and 12 months post-transplantation, however, retrieving the grafts at different time points could change the recovery yield.

## TROUBLESHOOTING

### Problem 1

Low viable cell yield after isolation (step 12 in [graft collection](#)).

### Potential solution

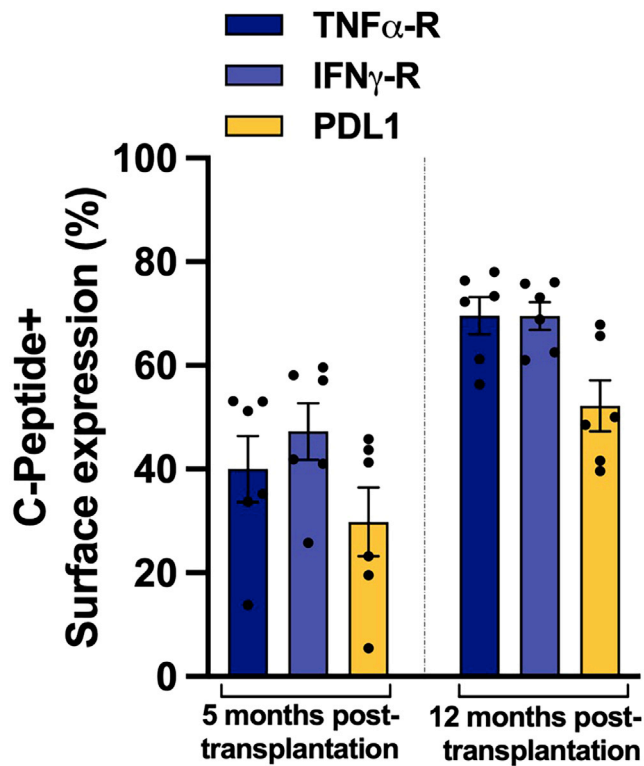
Optimizing time usage on each step during the graft collection is key to prevent cell loss. Practice of surgical steps is a good starting step for such optimization. Therefore, quick and smooth transition between tissue dissection and graft collection steps is a crucial factor. Another important optimization during isolation is the trituration of tissue. Insufficient trituration results in few single cells released from the tissue, whereas excessive trituration results in high mechanical cell stress and cell death.

### Problem 2

The graft did not dissociate after Collagenase I incubation and is not going through the 200  $\mu$ m filter (step 14 in [graft collection](#)).

### Potential solution

Make sure you vigorously shake or vortex during the Collagenase I incubation. If after 45 min you still see a lot of graft not dissociated, incubating it for additional 10 min. We do not recommend incubating the graft longer than total of 55 min, it will cause decrease of cell viability.



**Figure 5.** Cytokine receptors (TNF $\alpha$ -R and IFN $\gamma$ -R), and PD-L1 expression increase over time in transplanted SC- $\beta$ -cells. Data are means  $\pm$  SEMs.

### Problem 3

The columns are clogged, and the cell suspension does not pass through (step 27 in [human cell sorting](#)).

### Potential solution

The cell suspension is too concentrated. To fix, dilute the cell suspension 2–3 times and pass the sample through a newly prepared column.

### Problem 4

No positive staining of extracellular markers by flow cytometry (step 42 in [quality control staining](#)).

### Potential solution

If no positive staining of markers is detected, this may be due to:

Antibody of interest was not added to the antibody mix (step 42). Ensure the antibody is added when repeating the experiment. We further recommend using single-stain and unstained controls to appropriately select the correct voltages on the flow cytometry equipment.

### Problem 5

The human fraction contains a large amount of mouse cells (quality control outcome).

### Potential solution

The cell preparation has a lot of mouse tissue. Make sure you trim out all fat and kidney tissue before proceeding to step 12.

The cell suspension is too concentrated, and it saturates the column. Collect the human fraction and pass it through a newly prepared column.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Nayara Leite ([nayara\\_leite@harvard.edu](mailto:nayara_leite@harvard.edu)) or Jennifer Kenty ([hryu@mcb.harvard.edu](mailto:hryu@mcb.harvard.edu)).

### Materials availability

This stuanalyze dataset new unique reagents.

### Data and code availability

This stuanalyze dataset/analyze datasets.

## ACKNOWLEDGMENTS

We thank Ramona Pop and Doug Melton for discussions and feedback on the manuscript and the Melton lab Foundry for generating the SC- $\beta$ -cells used in this study. N.C.L. is supported by American Diabetes Association grant #1-19-PMF-024. This work was supported by grants from the Harvard Stem Cell Institute, the Helmsley Charitable Trust (2015PG-T1D044), JDRF (5-SRA-2014-284-Q-R), and the JPB Foundation (award no. 1094).

## AUTHOR CONTRIBUTIONS

N.C.L. and J.H.-R.K. conceived the study. The experiments and data interpretation were performed by N.C.L., G.C.P., and J.H.-R.K. J.H.-R.K. and N.C.L. performed the graft transplantation. N.C.L. retrieved the grafts and performed the functional and transcriptomics assays. G.C.P. and N.C.L. performed the flow cytometry assays. All authors wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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