TITLE:
Ex Vivo Expansion and Genetic Manipulation of Mouse Hematopoietic Stem Cells in Polyvinyl Alcohol-Based Cultures

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SUMMARY:
Presented here is a protocol to initiate, maintain, and analyze mouse hematopoietic stem cell cultures using *ex vivo* polyvinyl alcohol-based expansion, as well as methods to genetically manipulate them by lentiviral transduction and electroporation.

ABSTRACT:
Self-renewing, multipotent, hematopoietic stem cells (HSCs) are an important cell type due to their abilities to support hematopoiesis throughout life and reconstitute the entire blood system following transplantation. HSCs are used clinically in stem cell transplantation therapies, which represent curative treatment for a range of blood diseases. There is substantial interest in both understanding the mechanisms that regulate HSC activity and hematopoiesis, and developing new HSC-based therapies. However, the stable culture and expansion of HSCs *ex vivo* has been a major barrier in studying these stem cells in a tractable *ex vivo* system. We recently developed a polyvinyl alcohol-based culture system that can support the long-term and large-scale expansion of transplantable mouse HSCs and methods to genetically edit them. This protocol describes methods to culture and genetically manipulate mouse HSCs *via* electroporation and lentiviral transduction. This protocol is expected to be useful to a wide range of experimental hematologists interested in HSC biology and hematopoiesis.

INTRODUCTION:
The hematopoietic system supports a range of essential processes in mammals, from oxygen supply to fighting pathogens, through specialized blood and immune cell types. Continuous blood production (hematopoiesis) is required to support blood system homeostasis, which is sustained by hematopoietic stem and progenitor cells (HSPCs)[1]. The most primitive hematopoietic cell is the hematopoietic stem cell (HSC), which has unique capacities for self-renewal and multilineage differentiation[2,3]. This is a rare cell population, mainly found in the adult bone marrow[4], where

45 they occur at a frequency of just approximately one every 30,000 cells. HSCs are thought to
46 support life-long hematopoiesis and help to re-establish hematopoiesis following hematological
47 stress. These capacities also allow HSCs to stably reconstitute the entire hematopoietic system
48 following transplantation into an irradiated recipient[5]. This represents the functional definition
49 of an HSC and also forms the scientific basis for HSC transplantation therapies—curative
50 treatment for a range of blood and immune diseases[6]. For these reasons, HSCs are a major focus
51 of experimental hematology.
52
53 Despite a large focus of research, it has remained challenging to stably expand HSCs *ex vivo*[7]. We
54 recently developed the first long-term *ex vivo* expansion culture system for mouse HSCs[8]. The
55 approach can expand transplantable HSCs by 234–899-fold over a 4 week culture. In comparison
56 to alternative approaches, the major change in the protocol was the removal of serum albumin
57 and its replacement with a synthetic polymer. Polyvinyl alcohol (PVA) was identified as an optimal
58 polymer for the mouse HSC cultures[8], which has now also been used to culture other
59 hematopoietic cell types[9]. However, another polymer called Soluplus (a polyvinyl caprolactam-
60 acetate-polyethylene glycol graft copolymer) has also recently been identified, which appears to
61 improve clonal HSC expansion[10]. Prior to the use of polymers, serum albumin in the form of fetal
62 bovine serum, bovine serum albumin fraction V, or recombinant serum albumin were used, but
63 these had limited support for HSC expansion and only supported short-term (~1 week) *ex vivo*
64 culture[7]. However, it should be noted that HSC culture protocols that retain HSCs in a quiescent
65 state can support a longer *ex vivo* culture time[11,12].
66
67 In comparison with other culture methods, a major advantage of PVA-based cultures is the
68 number of cells that can be generated and the length of time the protocol can be used to track
69 HSCs *ex vivo*. This overcomes several barriers in the field of experimental hematology, such as
70 the low numbers of HSCs isolatable per mouse (only a few thousand) and the difficulty to track
71 HSCs over time *in vivo*. However, it is important to remember that these cultures stimulate HSC
72 proliferation, while the *in vivo* HSC pool is predominantly quiescent at a steady state[13].
73 Additionally, although the cultures are selective for HSCs, additional cell types do accumulate
74 with the cultures over time, and transplantable HSCs only represent approximately one in 34
75 cells after 1 month. Myeloid hematopoietic progenitor cells appear to be the major
76 contaminating cell type in these HSC cultures[8]. Nevertheless, we can use these cultures to enrich
77 for HSCs from heterogeneous cell populations (e.g., c-Kit$^+$ bone marrow HSPCs[14]). It also supports
78 transduction or electroporation of HSCs for genetic manipulation[14–16]. To help identify HSCs from
79 the heterogeneous cultured HSPC population, CD201 (EPCR) has recently been identified as a
80 useful *ex vivo* HSC marker[10,17,18], with transplantable HSCs restricted to the CD201$^+$CD150$^+$c-
81 Kit$^+$Sca1$^+$Lineage$^-$ fraction.
82
83 This protocol describes methods to initiate, maintain, and assess PVA-based mouse HSC
84 expansion cultures, as well as protocols for genetic manipulation within these cultures using
85 electroporation or lentiviral vector transduction. These methods are expected to be useful for a
86 range of experimental hematologists.
87
88 **PROTOCOL:**

All animal procedures, including breeding and euthanasia, must be performed within institutional and national guidelines. The experiments detailed below were approved by the UK Home Office. See the **Table of Materials** for a list of all materials, reagents, and equipment used in this protocol.

1. Preparing stock solutions

1.1. PVA stock solution

1.1.1. Take 50 mL of tissue culture quality water in a small glass bottle (suitable for autoclaving). Warm the water to near boiling in a microwave.

1.1.2. Weigh out 5 g of PVA powder and add to the water.

NOTE: It is recommended to take 1–5 g of PVA. Dissolving larger amounts may result in incomplete reconstitution.

1.1.3. Close the lid tightly and shake to mix. Then, loosen the lid.

NOTE: Be careful when re-opening, as the pressure may change due to the changing water temperature.

1.1.4. Autoclave and allow to cool. Close the lid tightly and shake to mix. Make aliquots in sterile tubes and store for up to 3 months at 4 °C.

1.2. Dissolve lyophilized cytokines in F-12 medium containing 1 mg/mL PVA. Reconstitute the stem cell factor to 10 µg/mL and thrombopoietin to 100 µg/mL to generate 1:1,000 stocks. Make aliquots in sterile tubes and store long-term at -80 °C. Alternatively, store the aliquots at 4 °C for up to 1 week.

NOTE: Avoid the reconstitution of cytokines in bovine serum albumin due to the negative impact on mouse HSC expansion.

2. HSC bone marrow extraction and c-Kit$^+$ enrichment

2.1. Dissect the femurs, tibias, pelvises, and spine from freshly euthanized 8–12-week-old C57BL/6 mice (*via* CO_2 asphyxiation and/or cervical dislocation) and place the bones in PBS on ice.

NOTE: Ensure the surfaces and tools are sterilized with 70% ethanol.

2.2. Clean the bones using lint-free delicate task wipes, removing muscle and spinal cord, and add to a mortar with ~3 mL of PBS.

133
134 NOTE: Ensure the pestle and mortar are sterilized with 70% ethanol and then washed out once
135 with PBS.
136
137 2.3. Crush the bones with the pestle without grinding to minimize shearing forces. Break up
138 large bone marrow fragments released into the PBS using a 19 G needle attached to a 5 mL
139 syringe. Transfer the cell suspension through a 70 µm filter into a 50 mL conical tube.
140
141 2.4. Once the suspension has been transferred, repeat with fresh PBS until the bones are
142 bleached and no marrow is visible. Aim for an end volume of ~30 mL per mouse and ~50 mL for
143 two mice.
144
145 2.5. Mix the bone marrow cells, collect 10 µL of cells, and count using Türks' solution at a
146 dilution of 1:10–1:20 with a hemocytometer.
147
148 NOTE: One mouse is expected to yield 2–5 × 10^8 whole bone marrow cells.
149
150 2.6. Spin at 450 × *g* for 5 min at 4 °C, discard the supernatant, and resuspend the pellet in cold
151 PBS (350 µL for one mouse or 500 µL for two mice).
152
153 2.7. Add 0.2 µL of allophycocyanin (APC) anti-c-Kit antibody per 10 million cells and incubate
154 for 30 min in the dark at 4 °C.
155
156 2.8. Add 5 mL of cold PBS to the incubated cells to wash off excess antibody and filter through
157 a 50 µm filter into a fresh 15 mL conical tube.
158
159 2.9. Wash the original tube with 7 mL of cold PBS and transfer through the filter. If filter
160 clogging occurs, scratch the filter surface with a P1000 tip.
161
162 2.10. Spin at 450 × *g* for 5 min at 4 °C, discard the supernatant, and resuspend the pellet in cold
163 PBS (350 µL for one mouse or 500 µL for two mice).
164
165 2.11. Add 0.2 µL of anti-APC microbeads per 10 million cells and incubate for 15 min in the dark
166 at 4 °C. Add 12 mL of sterile PBS to wash off excess microbeads.
167
168 2.12. Spin down the cells at 450 × *g* for 5 min at 4 °C and resuspend in 2 mL of cold PBS, or 500
169 µL for two mice. While the cells are spinning in this step, proceed to step 2.13.
170
171 2.13. Prepare for column enrichment by placing a magnetic filtration column into the magnet
172 of a magnetic column separator, with a 50 µm filter on top and a 15 mL conical tube below.
173
174 2.14. Run 3 mL of sterile PBS through the 50 µm filter and filtration column. Once the PBS has
175 run through, pass the cell suspension through the column, followed by three washes of 3 mL of
176 cold PBS each time. For each wash, wait for the column to stop dripping before adding the next

177 wash.
178
179 2.15. Remove the column from the magnet and place it on top of a fresh 15 mL tube. Add 5 mL
180 of cold PBS, fit the column plunger onto the column, and elute the cells by pushing the plunger.
181
182 2.16. Mix the c-Kit-enriched cells, collect 10 µL of cells, and count using Türks' solution at a
183 dilution of 1:2 with a hemocytometer. The typical yield from one mouse is 2–5 × 10^6 c-Kit^+ cells.
184
185 NOTE: At this point, the cells can be directly seeded into HSC media or prepared for HSC
186 fluorescence-activated cell sorting (FACS) purification.
187
188 **3. Initiating cell cultures with c-Kit-enriched HSPCs**
189
190 3.1. Prepare fresh medium (**Table 1**) for the number of cells/wells required. Seed 0.5–1 million
191 cells per mL for c-Kit-enriched HSPCs.
192
193 3.2. Spin down the c-Kit-enriched cells and resuspend in HSC medium at the desired cell
194 density.
195
196 3.3. Transfer the cells to fibronectin-coated or negative surface charged plates, at 200 µL per
197 96-well plate or 1 mL per 24-well plate.
198
199 3.4. Place the cells in a tissue culture incubator set to 37 °C and 5% CO_2.
200
201 **4. Initiating cell cultures with FACS purified HSCs**
202
203 4.1. Prepare an appropriate volume of the biotinylated lineage antibody stain: 3 µL of master
204 mix (**Table 2**) per 10 million cells, diluted 1:100 in PBS.
205
206 4.2. Spin down the c-Kit-enriched cells and resuspend in the lineage antibody stain for 30 min
207 at 4 °C.
208
209 4.3. Wash with 10 mL of sterile PBS and spin down at 450 × *g* for 5 min at 4 °C.
210
211 4.4. Prepare an appropriate volume of the fresh HSC antibody stain (**Table 3**): 300 µL per 10
212 million cells. Alongside this sample staining, prepare appropriate staining control samples for
213 compensation and gating.
214
215 NOTE: Work in a tissue culture hood with the light off when using dye-conjugated antibodies.
216
217 4.5. Resuspend the cells in the HSC antibody stain and incubate at 4 °C for 90 min. Mix the
218 cells every 20–30 min by tapping to prevent cell pelleting.
219
220 4.6. Wash with 10 mL of sterile PBS and spin down at 450 × *g* for 5 min at 4 °C.

221
222 4.7. Aspirate the supernatant, flick the pellet to disrupt, and resuspend in sterile PBS with 0.5
223 µg/mL propidium iodide (PI).
224
225 4.8. Prepare fresh medium (**Table 1**) for the number of wells required, and plate into
226 fibronectin or negative surface charged plates (200 µL per 96-well plate or 1 mL per 24-well
227 plate).
228
229 4.9. Prepare the FACS machine for sorting and sort $CD150^{+}CD34^{-}c\text{-}Kit^{+}Sca1^{+}Lineage^{-}$ HSCs
230 directly into media-containing wells (see **Figure 1** for the standard FACS gating strategy used
231 here). Sort up to 200 cells per 96-well plate well or up to 1,000 cells per 24-well plate well.
232
233 NOTE: FACS machines should be operated by a trained scientist. It is recommended that users
234 contact their local FACS facility to discuss this sorting strategy if they are not experienced in FACS
235 isolation of mouse HSCs.
236
237 **5. Performing media changes**
238
239 5.1. For cell cultures initiated from c-Kit-enriched cells, begin media changes after 2 days. For
240 cell cultures initiated from FACS-isolated HSCs, begin media changes after 5 days.
241
242 5.2. Prepare sufficient fresh prewarmed (~37 °C) HSC medium (**Table 1**) for all wells.
243
244 5.3. Gently remove the plate from the tissue culture incubator.
245
246 NOTE: As HSPCs on negative surface charged plates are more easily disturbed than those on
247 fibronectin, extra care should be taken when changing the medium on negative surface charged
248 plates to avoid disturbing the cell cultures.
249
250 5.4. Using a pipette or vacuum pump, slowly remove ~90%–95% of the medium from the well
251 meniscus.
252
253 NOTE: Avoid drawing up the medium from the base of the well, otherwise many cells will be
254 removed.
255
256 5.5. Add 200 µL (for 96-well plates; 1 mL for 24-well plates) of fresh medium to the well.
257
258 5.6. Return the plate to the tissue culture incubator.
259
260 5.7. Repeat steps 5.1–5.6 every 2–3 days until the experimental end point.
261
262 5.8. For cell cultures initiated with c-Kit-enriched HSPCs (section 3), split the cultures at a ratio
263 of 1:2–1:3 after ~3 weeks. For cell cultures initiated with FACS purified HSCs (section 4), split the
264 cultures at a ratio of 1:2–1:3 after ~3 weeks and when the cultures are >90% confluent.

265
266 NOTE: The exact timeline will depend on the experimental interests. These cultures have been
267 characterized for 4–8 weeks[8], but additional culture lengths may be possible.
268
269 **6. Electroporating cultured HSPCs**
270
271 NOTE: This protocol is for electroporation of Cas9/sgRNA ribonucleoprotein, but could be
272 adapted for electroporation of mRNA or other recombinant proteins. Initiate cultures with
273 sufficient numbers of cells in order to perform this at the desired experimental time point.
274
275 6.1. Perform a medium change 1 day before electroporation, as described in section 5.
276
277 NOTE: A minimum of an overnight culture before electroporation is recommended. However,
278 cells are typically cultured for 1–3 weeks before transduction.
279
280 6.2. On the day of electroporation, set up the nucleofector. Turn on the machine. On the
281 touchscreen, select the **X** module and then the **cuvette size** used.
282
283 6.3. Prepare sufficient P3 solution (according to the manufacturer's instructions) for the scale
284 of the electroporation (100 µL per cuvette or 20 µL per microcuvette) and allow to equilibrate to
285 room temperature.
286
287 6.4. Prepare sufficient fresh medium (**Table 1**) for the number of wells being plated and add
288 500 µL of medium for a 24-well plate well or 100 µL of media to a 96-well plate well.
289
290 6.5. Thaw out sgRNA (prediluted to 2 µg/mL in RNase-free water) on ice and mix 16 µg of
291 sgRNA with 30 µg of Cas9 enzyme (at 10 µg/mL) in a sterile PCR tube. Include an extra PCR tube
292 containing only Cas9 protein as a control. Mix by flicking the tube, then briefly spin down.
293 Incubate at 25 °C for 10 min in a thermocycler to complex the ribonucleoprotein, and then keep
294 the tubes on ice.
295
296 NOTE: This can be scaled down fivefold if performing electroporation in microcuvettes.
297
298 6.6. Mix and transfer the HSPCs for electroporation into a tube; collect 10 µL of the cells and
299 count using Türks' solution at a dilution of 1:2 with a hemocytometer.
300
301 NOTE: It is recommended to electroporate 1–5 million cells per 100 µL cuvette (scaled down
302 fivefold for the microcuvette).
303
304 6.7. Spin down the cells in a 1.5 mL tube at 450 × *g* for 5 min at 4 °C. Aspirate as much of the
305 supernatant as possible and resuspend with 100 µL of nucleofection buffer.
306
307 6.8. Transfer the cell suspension immediately into the PCR tube containing the complexed RNP
308 and pipette up and down slowly to gently mix and transfer to a 100 µL electroporation cuvette.

309 Eject the mixture into the cuvette slowly and in one fluid motion to avoid the formation of air
310 bubbles in the cuvette.
311
312 6.9. On the electroporator, select the position of the wells being electroporated. Using the
313 touchscreen, select the cell type program **CD34⁺**, **human**, or type in the pulse code **EO100**. Press
314 the **OK** button.
315
316 6.10. Transfer the cuvettes to the electroporator. Press the **Start** button on the touchscreen to
317 initiate electroporation. Directly after electroporation, add 500 µL of the culture medium to the
318 cuvette (100 µL if using a microcuvette).
319
320 6.11. Gently transfer the cells to the prepared plate and return to the tissue culture incubator.
321
322 6.12. For procedures employing an AAV6 donor template, add the vector immediately after the
323 cells have been transferred to a fibronectin-coated plate at a concentration of 5,000 vectors/cell.
324
325 6.13. After 6–18 h, prepare fresh medium and perform a medium change as described in
326 section 5. For this medium change, remove only 80%–90% of the medium.
327
328 NOTE: Avoid drawing up the medium from the base of the well.
329
330 6.14. Analyze the cells by flow cytometry after 2 or more days (see Section 8 for details).
331
332 6.15. Continue to perform medium changes every 2 days, as described in section 5, until the
333 experimental end point is reached.
334
335 NOTE: Editing rates of CRISPR/Cas9 using this method rely highly on the targeting efficiency of
336 the designed sgRNA. Editing rates up to 95% have been observed with an efficient guide[15].
337 Guidelines for sgRNA design have been previously detailed elsewhere[19,20].
338
339 **7. Transducing cultured HSPCs with lentiviral vector**
340
341 NOTE: Initiate cultures with sufficient numbers of cells, to perform this at the desired
342 experimental time point.
343
344 7.1 Generate and titer lentiviral vector (depending on the experimental goals). Thaw lentiviral
345 vector on ice using two controls—a guide sequence that is not found anywhere in the genome
346 and one that targets a safe harbor locus, such as Rosa26.
347
348 7.2 Perform a medium change (as in section 5); then, mix and transfer the HSPCs for
349 transduction into a tube. Collect 10 µL of the cells and count using Türks' solution at a dilution of
350 1:2 with a hemocytometer.
351
352 NOTE: Cells can be transduced immediately following plating. However, cells are typically

353 cultured for 1–3 weeks before transduction.
354
355 7.3 Replate the required dose of cells for transduction (typically 100,000 cells per 96-well
356 plate). Separately, plate un-transduced negative control cells.
357
358 NOTE: If using negative surface charged plates, transfer the cells to fibronectin-coated plates for
359 lentiviral vector transduction.
360
361 7.4 Add lentiviral vector to each well of cells: add 20 transduction units per cell to achieve
362 ~30% transduction efficiency. However, determine the lentiviral vector dose empirically,
363 depending on the experimental requirements.
364
365 NOTE: Ensure lentiviral vector is disposed of according to institutional guidelines.
366
367 7.5 Return the cells to the tissue culture incubator for 6 h. After, perform a medium change
368 as described in section 5.
369
370 NOTE: The supernatant contains live virus. Dispose of according to institutional guidelines.
371
372 7.6 Analyze the cells by flow cytometry (e.g., for GFP expression) after 2 days or more. See
373 section 8 for details.
374
375 7.7 Continue to perform medium changes every 2 days, as described in section 5, until the
376 experimental end point is reached.
377
378 **8. Flow cytometric analysis of HSPC cultures**
379
380 8.1. Prepare a concentrated cultured *ex vivo* HSC antibody mix in PBS containing 2% FBS (**Table**
381 **4**). Store the mixture at 4 °C in the dark for up to 1 month.
382
383 NOTE: Work in a tissue culture hood with the lights off when using dye-conjugated antibodies.
384
385 8.2. Add 2 µL of concentrated antibody mix to 50 µL of cells. Incubate for 30 min at 4 °C in the
386 dark. Alongside this sample staining, prepare appropriate staining control samples for
387 compensation and gating.
388
389 8.3. Add 200–1,000 µL of PBS containing 2% FBS and centrifuge at 450 × *g* for 5 min at 4 °C.
390 Remove as much supernatant as possible and resuspend in 100–500 µL of PBS containing 2% FBS
391 and 0.5 µg/mL PI.
392
393 8.4. Set up the flow cytometer and record at least 10,000 live cells per samples.
394
395 NOTE: Flow cytometers should be operated by a trained scientist. Users must contact their local
396 FACS facility to discuss this analysis if they are not experienced in flow cytometry.

8.5. Export the data in FCS format and analyze the data using appropriate flow cytometry analysis software. See **Figure 2** for the standard gating strategy used here.

REPRESENTATIVE RESULTS:

For the FACS purification of HSCs, we expect that within the c-Kit-enriched bone marrow, ~0.2% of the cells are the $CD150^{+}CD34^{-}c\text{-}Kit^{+}Sca1^{+}Lineage^{-}$ population for young (8–12-week-old) C57BL/6 mice (**Figure 1**). However, it is likely that transgenic mice or mice of different ages display differing HSC frequencies. After 4 weeks of culture, we expect the $CD201^{+}CD150^{+}c\text{-}Kit^{+}Sca1^{+}Lineage^{-}$ fraction to be ~10% (**Figure 2**). These results are similar for cell cultures initiated from c-Kit enriched bone marrow or FACS purified HSCs. Following transduction with a GFP-expressing lentiviral vector (at 20 transduction units/cell), we expect ~30% GFP^{+} cells (**Figure 3**).

When confluent, we expect the cultures to be at ~2 million cells/mL. Within the culture, we expect to see mainly small round cells, although it is normal to see a small frequency of larger round (megakaryocyte-like) cells. Within the cell cultures initiated from c-Kit-enriched bone marrow, some initial cell death is expected[14]. Approximately 50% cell death is expected within the first 24–48 h, before the first medium changes are performed. Initial dead cell debris in these cultures likely comes from cell death within the cultures rather than bone debris (from the crushed bones), since the c-Kit-enrichment step should deplete such bone debris. Cell numbers, however, return to 80%–100% of the seeded cell numbers after 1 week (see **Table 5** for expected cell density values). If poor results are seen, we recommend troubleshooting the protocol (**Table 6**). In this case, it can be most simple to batch-test reagents using the c-Kit-enriched bone marrow protocol (**Section 3**), as this avoids complications associated with FACS-sorting. Note that the representative results are based on the use of reagents and equipment detailed in the **Table of Materials**; similar results may be achieved using reagents from different vendors, however, validation (and titration) of new reagents is likely to be necessary.

FIGURE AND TABLE LEGENDS:

Figure 1: Gating strategy for FACS purification of HSCs from c-Kit-enriched bone marrow. Sequential gating used to identify $CD150^{+}CD34^{-}c\text{-}Kit^{+}Sca1^{+}Lineage^{-}$ cells from c-Kit-enriched bone marrow. Abbreviations: FACS = fluorescence-activated cell sorting; HSCs = hematopoietic stem cells; SSC-A = side scatter-peak area; FSC-A = forward scatter-peak area; FSC-W = forward scatter-peak width; FSC-H = forward scatter-peak height; SSC-H = side scatter-peak height; PI = propidium iodide; PE = phycoerythrin; APC = allophycocyanin; FITC = fluorescein isothiocyanate.

Figure 2: Gating strategy for flow cytometric analysis of cultured HSPCs. Sequential gating used to identify $CD201^{+}CD150^{+}c\text{-}Kit^{+}Sca1^{+}Lineage^{-}$ cells from HSPC cultures. Abbreviations: HSPCs = hematopoietic stem progenitor cells; SSC-A = side scatter-peak area; FSC-A = forward scatter-peak area; FSC-W = forward scatter-peak width; FSC-H = forward scatter-peak height; SSC-H = side scatter-peak height; PI = propidium iodide; PE = phycoerythrin; APC = allophycocyanin.

Figure 3: Representative results from lentiviral vector transduction. Representative GFP expression following cultured HSPC transduction with a GFP-expressing lentiviral vector (20

441 transduction units/cell) at 48 h after transduction. Abbreviations: GFP = green fluorescent
442 protein; HSPC = hematopoietic stem progenitor cell; FSC-H = forward scatter-peak height.
443
444 **Table 1: HSC media composition.** Media reagent volumes for 1 mL of complete medium.
445 Abbreviations: PVA = polyvinyl alcohol; SCF = stem cell factor; TPO = thrombopoietin; ITSX =
446 insulin-transferrin-selenium-ethanolamine.
447
448 **Table 2: Biotinylated antibody cocktail.** Antibody volumes for the stock of the biotin antibody
449 cocktail.
450
451 **Table 3: Fresh HSC antibody cocktail.** Antibody volumes for the fresh HSC antibody cocktail.
452 Abbreviations: PE = phycoerythrin; APC = allophycocyanin; FITC = fluorescein isothiocyanate.
453
454 **Table 4: Cultured HSC antibody cocktail (100x).** Antibody volumes for the 100x stock of the
455 cultured HSC antibody cocktail. Abbreviations: PE = phycoerythrin; APC = allophycocyanin; FITC
456 = fluorescein isothiocyanate.
457
458 **Table 5: Expected results.** Expected cell densities and frequencies of c-Kit^{+}Sca1^{+}Lineage^{-} cells
459 following (1) seeding of c-Kit^{+} bone marrow, (2) electroporation, and (3) lentiviral vector
460 transduction based on seeding at 1×10^{6} cells mL^{-1}. Abbreviations: KSL = c-Kit^{+}Sca1^{+}Lineage^{-}; HSPC
461 = hematopoietic step progenitor cell.
462
463 **Table 6: Common troubleshooting issues.** Summary of common issues and suggested
464 troubleshooting.
465
466 **DISCUSSION:**
467 We hope that this protocol provides a useful approach to investigate HSC biology, hematopoiesis,
468 and hematology more generally. Since the initial development of the PVA-based culture method
469 for FACS-purified HSCs[8], the method has been extended. For example, the method has been
470 shown to work with c-Kit enriched with bone marrow and with negative surface charged plates[14].
471 Its compatibility with transduction and electroporation has also been demonstrated[14,15]. The *in*
472 *vivo* validation of these HSC and c-Kit^{+} HSPC cultures can be found in these publications, while
473 readers can refer to other published protocols for *in vivo* transplantation protocols[21]. We do not
474 see major differences in lineage chimerism between fresh and cultured HSPCs following
475 transplantation, however, the reconstitution potency of individual HSCs after *ex vivo* expansion
476 is yet to be determined in detail. The large numbers of HSCs generated by this approach opens
477 up new ways to interrogate HSCs—molecular or biochemical assays that require large cell
478 numbers. Additionally, being able to generate genetically modified HSCs within these culture
479 systems should allow us to further probe the mechanisms regulating HSC activity and the
480 hematopoietic system. For example, these systems are amenable to performing genetic
481 screens[16].
482
483 Mechanistically, we do not yet fully understand why PVA and other polymers can replace serum
484 albumin and support efficient HSC expansion; we believe PVA at least partially replaces serum

albumin through stabilizing cytokines within the media[9]. Additionally, the lack of poorly defined bioactive contaminants found in serum albumin products appears to reduce HSC differentiation. The use of synthetic polymers should also help to reduce batch-to-batch variability and confounding effects associated with these bioactive contaminants when studying HSCs *ex vivo*[22]. There is also still more to be learnt regarding why certain polymers provide better support for HSCs *ex vivo*.

While already powerful, further optimization and characterization of these culture protocols should be possible. In particular, it would be useful to improve the purity of HSCs within these cultures. Additional markers distinguishing the long-term HSC compartment from these cultures would also help track and isolate these cell types. It is also of interest to see whether this system can be eventually used to quantify HSC activity *ex vivo*, without the need for *in vivo* transplantation assays. We also do not yet understand for how long HSCs can be expanded *ex vivo*, although it is certainly longer than 6–8 weeks if maintained properly[8]. Finally, while these cultures provide a useful model to study mouse HSCs, it is also important to develop equivalent culture systems for human HSCs to provide a more tractable system to study human HSC biology and hematopoiesis, and eventually, to generate HSCs for clinical stem cell transplantation therapies.

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DISCLOSURES:

The authors have no conflict of interests.

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