Supplemental Information

Direct Comparison of Four Hematopoietic Differentiation Methods from Human Induced Pluripotent Stem Cells

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Figure S1. Improved 2D-Multistep method resulted in production of multi-lineage functional hematopoietic progenitors, related to Figure 1. A-F: Representative light microscopy images of colonies formed from Day16 harvested suspension cells following culture in clonogenic colony forming unit (CFU) assays, demonstrating presence of erythroid progenitors (A-B), myeloid progenitors (C-D), megakaryocytic progenitors (E), and multi-lineage progenitors (F). Scale bars indicate a length of 100μm.
Figure S2. Time course of suspension and adherent cell production for the 2D-multistep method, related to Figure 1. 
Representative data showing cell generation kinetics for suspension cells (solid line) and adherent cells (dotted line).

A: The frequency of CFU per 1×10⁶ CD34+ cells in methocult or megacult media at different time points during hematopoietic differentiation.

B: The percentage of live cells positive for progenitor cell marker CD34, hematopoietic cell markers CD43, CD235a, CD45, CD33, and CD41a, and endothelial cell markers KDR and CD31 over time. Each graph shows three lines, representing percentage of live cells expressing each single cell surface marker (color-coded as per legend), and co-expressing both surface markers (in black) in the adherent and suspension cells.
Figure S3. Further comparison of 4 methods of iPSC hematopoietic differentiation, related to Figure 2. A: Number of CFU per $10^6$ CD34+ cells ($n = 9-74$ replicates, 6-9 iPSC lines per group). B-F: Independent replicates from two iPSC lines (circles, squares) for live cells (B), percentage of CD34+ progenitors (C), CD34+ progenitors per $10^6$ iPSC (D), CFU per $10^6$ iPSC (E), and CFU per $10^6$ CD34+ cells (F), with mean indicated by a horizontal line ($n = 1-21$ replicates per iPSC line). G-I: Percentage of cells expressing CD235a (G), CD45 (H), and CD33 (I) with each iPSC line shown as separate aligned points (2-7 iPSC lines per method, $n = 1-9$ replicates per iPSC line). Method indicated by color: 3D-simple (3DS, light blue), 3D-multistep (3DM, dark blue), 2D-simple (2DS, coral) and 2D-multistep (2DM, dark red). Mean as a horizontal line shown with standard error of the mean. Statistical significance determined by ordinary one-way analysis of variance (ANOVA), with post-hoc Tukey’s analysis across methods, as shown below plots (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$). Abbreviations: CFU, colony forming unit.
Figure S4. 3D-simple method recapitulates disease phenotype, related to Figure 4. The observed increase in total CFU per 10^6 CD34+ cells derived using the 3D-simple method were due to an increased number of multi-lineage and erythroid-lineage CFU. Generated from 2 WT (n = 11-36 replicates) and 2 DS-Tri21-derived iPSC lines (n = 29-36 replicates). Each point indicates an independent replicate, with mean indicated by a horizontal line. Statistical significance by t-tests indicated below graphs, with bars positioned to indicate the methods compared (* = p<0.05; ** = p<0.01). Abbreviations: see Figure 4.
### Supplemental Tables

| Day | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|-----|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|
| Reagent | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A |
| RPMI (%) | 95 | 95 | 90 | 95 | 90 | + | + | + | + | + | + | + | + | + | + | + |
| KO8 (%) | 5 | 5 | 10 | 5 | 10 | 100 | 100 | 100 | 100 | 100 | + | + | + | + | + | + |
| IMDM (%) | 74 | 74 | 74 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| HAMS-F12 (%) | 24 | 24 | 24 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| N-2 supp (%) | 0.5 | 0.5 | 0.5 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| B-27 supp (%) | 1 | 1 | 1 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| BSA (%) | 0.5 | 0.5 | 0.5 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| MTG (mM) | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | + | + | + | + | + | + |
| MTG (mM) | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | + | + | + | + | + | + |
| VEGF (ng/ml) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | + | + | + | + | + | + |
| bFGF (ng/ml) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | + | + | + | + | + | + |
| SCF (ng/ml) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | + | + | + | + | + | + |
| Flt3L (ng/ml) | 25 | 25 | 25 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TPO (ng/ml) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | + | + | + | + | + | + |
| IL-6 (ng/ml) | 10 | 10 | 10 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| EPO (U/ml) | 0.5 | 0.5 | 0.5 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| FICZ (μM) | 0.2 | 0.2 | 0.2 | + | + | + | + | + | + | + | + | + | + | + | + | + |
| CHIR (μM) | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |

| Table S1: Variants of the original 2D-multistep method, refer to Table 1. | | | | | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Variant | B differs from A | F differs from A | B & F differ from A | | | | | | | | | | | | | | |
| | No media change, add bFGF directly | No media change, no [high] bFGF | Extended Wnt signaling | | | | | | | | | | | | | | |
| | No media change, add FICZ and cyto directly | | | | | | | | | | | | | | | |
| | Fewer media top ups add FICZ directly on day 10 | | | | | | | | | | | | | | | |
| | Fewer media top ups no EPO, no FICZ | | | | | | | | | | | | | | | |

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**Abbreviations:** See Table 1; CHIR, CHIR99021.

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**Note:** The table compares variants of the original Smith et al. method (A), improved 2D-multistep method presented herein (B), and Leung et al. HSPC modification of Smith method (F). B and F: Variations of the Smith et al. protocol, with an extension of Wnt activation beyond day 2, no increase in bFGF concentration on day 3, and fewer media top ups after day 7. See Table 1 for additional explanation.
| Calculated number                                                                 | 2D-multistep | 2D-simple | 3D-multistep | 3D-simple |
|----------------------------------------------------------------------------------|--------------|-----------|--------------|-----------|
| Days with complete media changes                                                 | 3            | 3         | 3            | 1         |
| Days with reagents added directly to media in wells                              | 8            | -         | -            | -         |
| Hours hands-on / well from Day 0 to Harvest                                        | 0.39         | 0.26      | 0.26         | 0.09      |
| Wells to produce $1 \times 10^6$ CD34+ cells                                      | 2.1          | 105       | 26           | 135       |
| Wells to produce $1 \times 10^6$ CFU                                             | 125          | 591       | 2,625        | 560       |
| Total hours hands-on to produce $1 \times 10^6$ CD34+ cells (calculated as the proportion of an 11 hour clock) | 0.8 (0.3 hours) | 27 (11 hours) | 6.7 (2.7 hours) | 11.5 (4.7 hours) |
| Total hours hands-on to produce $1 \times 10^6$ CFU                               | 48           | 151       | 669          | 48        |

**Table S2: Total hands-on-time required for each method to produce the same number of target cells, related to Table 3.** Comparison of the number of days and hours of hands-on-time per well required for each method from Day 0 to harvest. Calculation of the number of wells required to produce $1 \times 10^6$ CD34+ progenitor cells or functional hematopoietic progenitors as measured by CFU. Calculation of the total number of hours of hands-on time required for each method to produce $1 \times 10^6$ CD34+ cells or CFU. For the purpose of the Graphical Abstract, relative number of hours to produce $1 \times 10^6$ CD34+ cells calculated as the proportion of an 11 hour clock. **Abbreviations:** CFU, colony forming unit.
| Gene                                      | Symbol | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|-------------------------------------------|--------|------------------------|------------------------|
| Hemoglobin alpha 1                        | HBA1   | CGGTCAACTTCAAGCTCCTAAG | CCGCCCACCTCAGACTTTTATT |
| Hemoglobin beta                           | HBB    | TGGCCTGGCTCACCTGGACAA  | GCAGCTTAGTGACTTTTGGGC  |
| Hemoglobin epsilon 1                      | HBE1   | ATGGACAACCTCAAGCCCGCC  | GGCAATGGCGACAGACAGACA  |
| Hemoglobin gamma                          | HBG    | CTTCAAGCTTGGGAAATGT    | GCAGAAATAAAGCTATCTTTGAA |
| Hemoglobin zeta                           | HBZ    | GATCTCCACGCAGCGAGCAC   | GGTCACGGCGAGATGAGG     |
| Hypoxanthine guanine phosphor-ribosyltransferase 1 | HPRT   | GCCAGTCAAAGGGGACAT     | CCTGACCAAGGAAAGCAAG    |
| Glyceraldehyde 3-phosphate dehydrogenase  | GAPDH  | TGCCACCAACGTCTTACGC    | GCATGGAAGTTGGCTATGAG   |
| Abelson murine leukemia viral oncogene homolog 1 | ABL    | TGGGATAAAGCTCAAGCTAACTAAAGGT | CCATTGGTTGGGGCTTCACACATT |

Table S3: Primers used for quantitative Real Time PCR (qRT-PCR), related to Experimental Procedures. Primers for detection of globin genes, and HPRT, GAPDH, and ABL housekeeper genes.
Supplemental Experimental Procedures

Cell culture.
All iPSC lines were generated from wildtype, Down syndrome (DS) or β-thalassemia human fibroblasts using an episcopal non-integrating vector as previously reported by us (Briggs et al. 2013) with informed consent of patients under the approval of the University of Queensland Human Research Ethics Committee, QLD, Australia (HREC 2008001651) and work reported herein was carried out under the approval of the St Vincent’s Hospital Human Research Ethics Committee, VIC, Australia (HREC LNR/14/SVH/147). All lines were maintained feeder-free on 35mm tissue culture dishes coated with extra cellular matrix (ECM, E1270, Sigma Aldrich, Castle Hill, NSW, Australia) in mTESR1 serum-free medium (05850, StemCell Technologies, Vancouver, Canada). Medium was replaced daily and cultures were passaged every 7 days, manually or using 1mg/mL collagenase IV (17104019, Thermo Fisher Scientific, North Ryde, NSW, Australia) as per mTESR1 manufacturer’s recommendations. Classic karyotyping was frequently performed to ensure stability during long term culture (Cytogenetics Department, St Vincent’s Hospital, Darlinghurst, Sydney, Australia).

Hematopoietic differentiation of iPSC.
For plating of iPSC in embryoid body (EB, 3D method) or monolayer (2D method) cultures, clumps of iPSC were formed as per the passaging protocol and plated in mTESR1 as described below. The EB simple-step differentiation (3D-simple) method was adapted from Lapillonne et al. 2010. Embryoid bodies were generated by suspending clumps from 3 – 5 large iPSC colonies in mTESR1 in Corning non-treated 35mm culture dishes (CLS430588, Sigma Aldrich) for 24 hours. On day 0 EB were transferred into 2ml of Iscove’s Modified Dulbecco’s Medium (IMDM, 12440053, Thermo Fisher Scientific) with 3% normal human plasma (NHP, Red Cross, Alexandria, NSW, Australia), bone morphogenetic protein 4 (BMP4) 10ng/ml, vascular endothelial growth factor A165 (VEGF) 5ng/ml, and 100ng/ml each of stem cell factor (SCF), thrombopoietin (TPO), FLT3 ligand (FLT3L), and incubated in 5% oxygen, 5% carbon dioxide and 95% humidity in tissue culture incubators for 8-12 days, with time points selected based on time courses in the original publication showing high CD34+ and CD45+ percentage expression. All suspension cells were harvested and EB dissociated into single cells using 0.4U/ml collagenase B (1108807001, Roche Diagnostics, Castle Hill, NSW Australia) followed by Cell Dissociation Buffer (13151014, Thermo Fisher Scientific, North Ryde, NSW, Australia) as per manufacturer’s instructions, then resuspended in IMDM for cell and viability determination.

The EB multistep (3D-multistep) method was adapted from Chou et al. 2012. EB were formed as above and on day 0 were transferred to 5% oxygen (as above) in 2ml Stemline II Hematopoietic Stem Cell Expansion Medium (SLII, S0192, Sigma Aldrich) with 1% Insulin Transferrin-Selenium (ITS, I3146, Sigma Aldrich), BMP4 25ng/ml and VEGF 50ng/ml. On day 2 media and cytokines were replaced including addition of 50ng/ml each of SCF, TPO, FLT3L and 20ng/ml basic fibroblast growth factor (bFGF). On day 4 media and cytokines were replaced without BMP4. Cultures were harvested between days 8-12, based on the original publication. EB were dissociated as above.

The monolayer simple differentiation (2D-simple) method was adapted from Niwa et al. 2011. 3 – 5 large iPSC colonies were divided into clumps and plated onto extra cellular matrix-coated 35mm dishes in mTESR1. When colonies grew to ~500μm in size this was considered as day 0 and media was replaced with 2ml SLII with 1% ITS and BMP4 20ng/ml and incubated in standard tissue culture incubators (5% carbon dioxide and 95% humidity). On day 4 the media was replaced with SLII, 1% ITS, VEGF 40ng/ml and SCF 50ng/ml. On day 6 media was replaced with SLII, 1% ITS, SCF 50ng/ml, TPO 10ng/ml, interleukin-3 (IL3) 50ng/ml, interleukin-6 (IL6) 50ng/ml, and erythropoietin (EPO) 5U/mL. Cells were harvested together between days 8-12, based on the peak frequency of CFU from iPSC reported in the original publication. Suspension cells were collected, and the adherent cells were dissociated into single cells with Dispose (07923, Stem Cell Technologies, Tullamarine, VIC, Australia) as per manufacturer’s instructions. Suspension and adherent were combined for cell count and viability determination.

The monolayer multistep (2D-multistep) differentiation method was adapted from Smith et al. 2013 with additional optimisation to approximately halve the overall cost of culture reagents and to limit media supplementation to six days per week, by replacing some full media changes with media supplementation, and not supplementing media on days 1, 8, and 15, as follows. Cell seeding density was optimised, such that approximately 7% of an 80% confluent iPSC dish was plated as small clumps per well of an extra cellular matrix-coated 6 well dish in mTESR1. This was the equivalent of approximately 6x10^4 viable iPSC per well. Cells were cultured for 48 hours. On day 0 media was replaced with 2ml Roswell Park Memorial Institute medium (RPMI, 11875093, Thermo Fisher Scientific) with 5% KnockOut Serum Replacement (KOSR, 10828010, Thermo Fisher Scientific), BMP4 5ng/ml, VEGF 50ng/ml, Wingless-type MMTV integration site 3A (WNT3a) 25ng/ml, 0.4mM monothioglycerol (M1753, Sigma Aldrich), and 0.3mM 2-phospho-L-ascorbic acid...
(A4034, Sigma Aldrich). On day 2, media was supplemented with bFGF 20ng/ml. On day 4 media was replaced with StemPro-34 SFM Complete Medium (SP34, 10639011, Thermo Fisher Scientific) with glutamax (25030081, Thermo Fisher Scientific) 2mM, VEGF 15ng/ml, bFGF 5ng/ml, 0.4mM monothioglycerol, and 0.3mM 2-phospho-L-ascorbic acid. On day 6 media was replaced with 74% IMDM, 24% Ham’s nutrient medium F-12 (HAMS-F12, 11765054, Thermo Fisher Scientific), 1% B-27 supplement (12587010, Thermo Fisher Scientific), 0.5% N-2 supplement (17502048, Thermo Fisher Scientific), 0.5% Bovine serum albumin (BSA, A3059, Sigma Aldrich), VEGF 50ng/ml, bFGF 100ng/ml, SCF 100ng/ml, FLT3L 25ng/ml, 0.4mM monothioglycerol, and 0.3mM 2-phospho-L-ascorbic acid. On day 7 media was supplemented with TPO 50ng/ml, IL6 10ng/ml, EPO 0.5U/ml and 6-formylindolo(3,2-b)carbazole (FICZ, ab141631, Abcam) 0.2μM. On day 9 wells were topped up with an additional 0.5ml/well of 74% IMDM, 24% HAMS-F12, 1% B-27 supplement, 0.5% N-2 supplement, 0.5% BSA, VEGF 50ng/ml, bFGF 100ng/ml, SCF 100ng/ml, FLT3L 25ng/ml, TPO 50ng/ml, IL6 10ng/ml, EPO 0.5U/ml, FICZ 0.2μM, 0.4mM monothioglycerol, and 0.3mM 2-phospho-L-ascorbic acid. On day 10 media was supplemented with FICZ 0.2μM. On days 11 – 14 and 16 – 18 wells were topped-up daily with 0.5ml/well of fresh day 9 media and supplements. Cultures were harvested on days 9, 12, 16 and 19 for time course studies, with the optimum time point selected as day 16, based on the peak frequency of CFU. For time course studies suspension cells and adherent cells were harvested and analysed separately. Suspension cells were collected directly from cultures, including a gentle wash with 1ml DPBS. Adherent cells were treated with 1.5ml/well Gentel Cell Dissociation Solution to produce single cells, as per manufacturer’s instructions. For all other 2D-multistem data the suspension cells alone were used for analysis due to the robust cellular production with significantly higher percentages of hematopoietic cells compared to the adherent cells.

All cytokines are human recombinant proteins from R&D Systems (Minneapolis, Minnesota, USA), or Miltenyi Biotec Australia Pty Ltd (Macquarie Park, NSW, Australia).

Calculation of number of cells or CFU produced from 1x10⁶ iPSC.

The number of cells or CFUs produced from 1x10⁶ iPSC were calculated from the average number of iPSC plated per dish per experiment, which were estimated based on previous experience with iPSC colony size and cell counts.

Clonogenic colony forming unit (CFU) assay.

Harvested cells were grown in Methocult Enriched medium (H4435, Stem Cell Technologies) and Megacult-C medium (04973, Stem Cell Technologies), and colonies scored at day 14-16. Following scoring, Methocult colonies of each type were harvested, washed in Dulbecco’s phosphate buffered saline (DPBS, 14190-144, Thermo Fisher Scientific), stored as pellets at -80°C, then used for quantitative PCR (qPCR). Megacult slides were dehydrated, fixed and stained for CD41a expression, and positively stained colonies scored as per the manufacturer’s instructions.

OP9 erythroid differentiation.

Suspension cells harvested following 16 days of hematopoietic differentiation using 2D-multistem method were seeded at 50,000 cells per well onto confluent OP9 cell feeder cells grown on 1% gelatin-coated (G1890-100g, Sigma Aldrich) 24-well plates in α-MEM (12000-022, Thermo Fisher Scientific), 10% fetal calf serum (10099-141, Thermo Fisher Scientific), 100μM monothioglycerol (M1753-100ML, Sigma Aldrich), 100ng/ml SCF (130-096-695, Miltenyi Biotec), 3U/ml EPO (287-TC-500, In Vitro Technologies), 40ng/ml IGF-1 (130-093-885, Miltenyi Biotec), 1μM dexamethasone (D4902-25MG, Sigma Aldrich), for 17 days with half volume media changes every 3-4 days. Hematopoietic cells were then separated from OP9 through a 40μm cell strainer (FAL352340m Invitro technologies).

Flow cytometric analysis.

Cells were resuspended in FACS buffer (2% BSA (A3059, Sigma Aldrich), 0.2% sodium azide, DPBS) and stained with a multi-color panel of conjugated human antibodies, including anti-human CD31-PE (555446), CD31-PE-CF594, CD33-PE-Cy7 (333946), CD34-PE (348057), CD38-BV786 (563964), CD41a-BV510 (563250), CD41a-PerCP-Cy5.5 (340931), CD43-BB515 (564543), CD45-APC-H7 (560178), CD45RA-BV510 (563031), CD61-BV650 (564172), CD90-BV421 (562556), CD123-BV650 (563405), CD235a-PE Cy5 (559944), CD235a-PE-Cy7 (563666), CD235a-APC (551336), CD309 (KDR)-Alexa fluoro-647 (560871) (all from BD Biosciences or BioLegend, San Diego, CA, USA). Cell surface markers correspond to the following phenotypes: CD34++ progenitor cells; CD43++ hematopoietic cells; CD35a++ expressed on hematopoietic mesoderm, early stages of primitive hematopoiesis, and erythroid lineage differentiation; CD45++ hematopoietic cells; CD33+ myeloid cells; CD61++ and CD41a++ megakaryocytic cells; CD38 used with CD34, CD45RA, CD90, CD123 identify populations correlating to HSC, and MPP, CMP, MEP, and GMP;
KDR++ marker expressed on mesoderm and epithelial cells; CD31++ epithelial cell marker. Dead cells excluded by DAPI (560947) or fixable viability stain 700 (564997). Samples acquired using a BD LSRII (FacsDIVA 4.0) or BD LSRFortessa X-20 (FACSDiva 8.0), and analyzed by FACSDiva (8.0.1) or FlowJo (10.2).

**Quantitative PCR (qPCR).**
Total RNA was extracted using the miRNeasy Micro Kit (217084, Qiagen, Hilden, Germany). cDNA was generated using the SuperScript® First-Strand Synthesis System for RT-PCR kit (18080051, Thermo Fisher Scientific), and used Platinum SYBR Green qPCR 2x SuperMix-UDG (11733046, Thermo Fisher Scientific). qPCR was performed using the Rotor-Gene Q real-time PCR cycler (Qiagen) as follows: 50°C for 2 mins, 95°C for 2 mins, 40 cycles of 95°C for 30s, 60°C for 30s, 72°C for 60s. Data was analyzed with Rotor-Gene 6 Software (Qiagen) for relative gene expression normalized to housekeeping genes (HPRT, GAPDH and ABL). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA) (Table S3).

**Relative cost.**
Calculated based on the 2018 list price in US dollars and volume required for all reagents per well of culture, adjusted by the average number of CD34+ cells and colony forming unit (CFU) produced per method to determine the number of wells required to produce 1x10^6 target cells, shown relative to the cost for the 2D-multistep method.

**Hands-on-time.**
Calculated for each method based on the number of days and hands-on-time required for complete media changes and for reagents to be added directly to media in wells, to calculate number of hands-on hours per well required from Day 0 to harvest. This was multiplied by the number of wells required to produce 1x10^6 target cells, CD34+ progenitor cells or functional hematopoietic progenitors cells as determined by CFU assay, to calculate the total number of hands-on hours required from Day 0 to harvest to produce 1x10^6 target cells. Total hours were calculated relative to the 2D-multistep method for the Graphical abstract.

**Statistical Analysis.**
Statistical Analysis was performed using GraphPad Prism 7 (GraphPad Software Inc, San Diego, California, USA). Ordinary One-way Analysis of Variance (ANOVA) with post-hoc Tukey’s test was used for analyses of groups with multiple groups, (this incorporates the extension of the Tukey post-hoc test by Kramer, the Tukey-Kramer test, to allow for unequal sample sizes), and unpaired T-tests were used for comparing individual groups. Chi-squared analysis was used for comparison of CFU proportions between wild-type and aberrant hematopoiesis. A p-value of <0.05 was considered statistically significant in all cases.