Supplemental Information

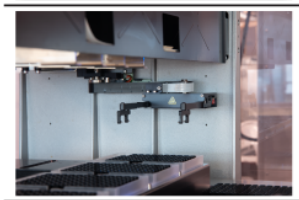
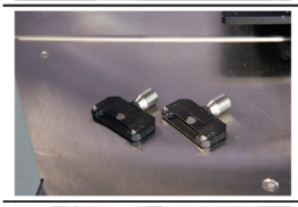
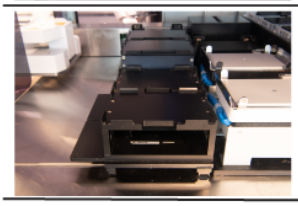
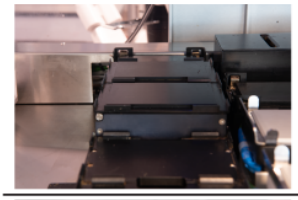
Table S1. Resources

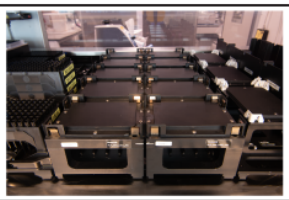
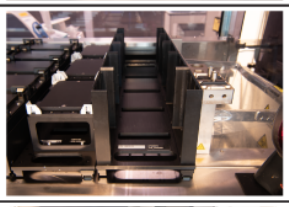
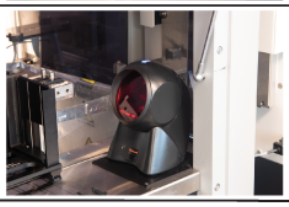
Identifier	Reagent/Material Name	Source	Description
Cell Culture			
Cat#85851	mTeSR™1 Medium	STEMCELL Technologies	Media
Cat#05876	mTeSR™1 Medium Without Phenol Red	STEMCELL Technologies	Media
Cat#15070063	Penicillin-Streptomycin	Thermo Fischer Scientific	Media
Cat#72305	Y-27632 (Dihydrochloride)	STEMCELL Technologies	ROCK Inhibitor
Cat #356231; Lot # 5292003, Lot # 9021357	Matrigel Growth Factor Reduced Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free	Corning	Matrigel
Cat#11039021	DMEM/F12 (1:1) 1X	Thermo Fischer Scientific	DMEM/F-12
Cat#14190144	Dulbecco's phosphate-buffered saline (DPBS) (1X)	Thermo Fischer Scientific	DPBS
Cat#A1110501	StemPro™ Accutase™ Cell Dissociation Reagent	Thermo Fischer Scientific	Accutase
Cat#201252-100	Reservoir, single cavity, polypropylene, 290 mL, 8 row base geometry, 44 mm height, 25/pk	Agilent	Media/Reagent reservoir
Cat#200856-100	Lids, universal, clear, polystyrene, 100/pk	Agilent	Reservoir and microplate lids
Cat#203852-100	Reservoir, 2 column, polypropylene, 146 mL/column, 292 mL maximum, pyramid base geometries, 44 mm height, 25/pk	Agilent	2-column reservoir
Cat#202061-100	Storage/reaction microplate, 24-well, polypropylene, 10 mL/square well, round bottoms, 44 mm height, 25/pk	Agilent	24-well microplate
Cat#383721	Vi-CELL™ XR Sample Vials	Beckman Coulter	Cell counter sample tubes
Cat#731196	Vi-Cell XR Series Cell Viability Analyzer	Beckman Coulter	Cell counter

Cat#P96-1.5H-N	96 well glass bottom plate with high performance #1.5 cover glass	Cellvis	96-well plate
Cat#657185	Multi-well pates for suspension culture, 6 well, clear, lid with condensation rings, sterile	Greiner Bio-One	6-well plate
Cat#235940	1000 µL Conductive Filter Tips	Hamilton Company	1000 µL Hamilton tips with filter
Cat#235985	300 µL Nested Conductive Tips	Hamilton Company	300 µL Hamilton tips without filter
Flow Cytometry			
Cat#85851	mTeSR™1 Medium	STEMCELL Technologies	Media
Cat#15070063	Penicillin-Streptomycin	Thermo Fischer Scientific	Media
Cat#A1110501	StemPro Accutase Cell Dissociation Reagent	Thermo Fischer Scientific	Accutase
Cat#14190144	Dulbecco's phosphate-buffered saline (DPBS) (1X)	Thermo Fischer Scientific	DPBS
Cat#CLS430052	15 mL centrifuge tubes	Corning	
Cat#554655	Fixation Buffer	BD Biosciences	BD Cytofix™
Cat#10828010	KnockOut™ Serum Replacement	Thermo Fischer Scientific	KSO
Cat#41640	Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	DMSO
Cat#AB0620	Tubes, 0.2 mL, flat cap	Thermo Fischer Scientific	250 µl PCR tubes
Cat#561300	Alexa Fluor® 647 Mouse anti-Human Nanog Clone N31-355	BD Biosciences	
Cat#560796	Alexa Fluor® 647 Mouse anti-SSEA-4 Clone MC813-70 (RUO)	BD Biosciences	
Cat#560329	Alexa Fluor® 647 Mouse anti-Oct3/4 Clone 40/Oct-3 (RUO)	BD Biosciences	
Cat#565571	Alexa Fluor® 647 Mouse IgG1 κ Isotype Control Clone MOPC-21 (RUO)	BD Biosciences	
Cat#560803	Alexa Fluor® 647 Mouse IgG3, κ Isotype Control Clone J606 (RUO)	BD Biosciences	
Cat#50-121-5315	Bovine Albumin Fraction V (7.5% solution)	Gibco	BSA
Cat#9002-93-1	Triton™ X-100	Sigma-Aldrich	
Cat#554723	BD Perm/Wash™ Perm/Wash™ Buffer	BD Biosciences	BD Perm/Wash™
Cat#CO9766	CytoFLEX S V4-B2-Y4-R3 Flow Cytometer (13 Detectors, 4 Lasers)	Beckman Coulter	Flow cytometer
	FlowJo version 10.2	Treestar	Flow cytometry analysis software

Cat#08-772-5	96-Well, Non-Treated, U-Shaped-Bottom Microplate	Thermo Fischer Scientific	
Cytogenetic Analysis			
Cat#9381M10	25cm² Rectangular Canted Neck Cell Culture Flask with Vented Cap	Corning	
Cat#85851	mTeSR™1 Medium	STEMCELL Technologies	Media
Cat#15070063	Penicillin-Streptomycin	Thermo Fischer Scientific	Media
Cat#P7793	Parafilm M	Sigma-Aldrich	
Cat#356231	Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free	Corning	Matrigel
High Resolution 3D Imaging			
Cat#C10046; Lot#1813792 (5X final concentration); Lot#1853335 and #1900978 (3X final concentration)	CellMask™ Deep Red Plasma Membrane Stain	Thermo Fischer Scientific	CMDR plasma membrane stain
Cat#R37605	NucBlue™ Live ReadyProbes™ Reagent	Thermo Fischer Scientific	Nuclear DNA stain

Table S2: Robotic Components

Component	Description
	**STAR Deck:** Workplace within the enclosure of the Hamilton Microlab STAR
	**Internal Swivel Arm Plate (iSwap) Handler:** Plate handler used to move plates, lids, racks and reservoirs
	**Compressed O-Ring Expansion (CO-RE) Grippers:** A plate, resrvoir and lid handling tool consisting of 2 paddles picked up by 2 channels
	**1-2. Media Position:** Positions on deck used to stage reservoirs
	**3-4. Tilt Module:** Positions on deck used to tilt plates between 0-10 degrees
	**6-7. Cold Plate Air-Cooled (CPAC) Elements:** Heating and cooling blocks for plates and reservoirs
	**10. 1000µL Conductive Filter Tips Carrier:** Filterd tips infused with carbon for capacitive liquid level detection, each rack has 96 tips
	**11. 300µL Nested Conductive Tips Racks:** Unfiltered tips nested in 5 racks, each rack has 96 tips

Component	Description
	**12-13. Plate Park Positions:** Positions on deck used to stage plates and their lids
	**14-15. 24 Well Microplate Stages:** Positions on deck used to stage 24-well microplates and their lids
	**16. Plate Stacker Carrier** 5 columns of stacking carriers that hold up to 5, 6- or 96-well plates each
	**17. Barcode Reader:** Orbit presentation laser barcode scanner used for plate and lid barcode scanning
	**19. Celigo Cytometer:** Bench-top, micro-well plate based, brightfield and fluorescent imaging system used for cell counting and growth tracking
	**20. Agilent Microplate Centrifuge:** Robot-accessable automated centrifuge for microplates
	**21. Cytomat 24:** Automated incubator with plate hotel, shuttle system with 360 plate storage capacity. Kept at 37°C, 5% CO_2 and 85% relative humidity
	**22. Cytomat 6002:** Automated refrigerator at 4°C with plate and reservoir hotel, shuttle system and capacity for 105 plates and 20 reservoirs

Supplementary Experimental Procedures

Calibration, priming/ initialization steps

Preventative maintenance methods are run daily and weekly. These methods initialize and check the movements of the arm that carries the Internal Swivel Arm Plate Handler (iSwap) and channels, in addition to checking the movements of the carried components. Channel tightness is verified with both positive and negative timed pressure tests and concludes with the testing of each channel's capacitive liquid level detection sensor. Failure in these tests result in a re-test; if failures continue, service is called for maintenance. Semi-annual service consists of the inspection of belts, springs, and cables, as well as cleaning, replacement and lubrication of rails and spindles. Stop disks, O-rings, and washers are replaced twice a year. Finally, tip eject cycles are reset for tracking O-ring life.

Daily Workflow

The three crucial workflows (as outlined in **Figure 2**) and associate methods for (1) Plate Maintenance, (2) Passaging, and (3) Matrigel Coating of plates are listed and described in detail below. Each method is described and followed by a table containing the approximate timing, robotic equipment needed, and key action parameters (diagramed in **Figure 3**) relevant to each method.

Supplementary Workflow: Thawing

Frozen vials of source cells are thawed according to previously published cell culture methods (WTC Cell Culture v1.7, https://www.allencell.org). Adaptations to the automated method start after the manual resuspension of the cell pellet. Cell suspension is transferred to the 24-well microplate and seeded onto Matrigel-coated 6-well plates using the Microlab STAR platform and associated seeding protocols described here (Method 2.8 as described).

Supplementary Workflow: Flow Cytometry Stemness Marker Staining

Cells were harvested after 10 passages at 70-85% confluency and fixed with BD CytoFix Fixation Buffer (BD Biosciences) at 4°C for 30 min. Fixed cells were frozen at -80°C in KnockOut Serum Replacement (Thermo Fischer Scientific) with 10% dimethyl sulfoxide (Sigma Aldrich) for later processing in batches. Prior to staining for flow cytometry, cells were thawed, washed with 2% Bovine Albumin Fraction V (BSA, 7.5% Solution, Thermo Fischer Scientific) in Dulbecco's Phosphate-Buffered Saline (DPBS, Thermo Fischer Scientific) and split in half. For intracellular staining, one half of the cells were permeabilized using 0.1% Triton X-100 (Sigma Aldrich) and 2% BSA in DPBS for 30 min at RT. These cells were subsequently stained for transcription factors with anti—Nanog Alexa Fluor® 647, anti—Oct3/4 Alexa Fluor® 647, and Mouse IgG1 κ Isotype Control Alexa Fluor® 647 (all BD Biosciences), followed by a wash with BD Perm/Wash buffer (BD Bioscience). The other half of the cells were stained for stemness surface markers using anti-SSEA-4 Alexa Fluor 647 and Mouse IgG3 k Isotype Control Alexa Fluor® 647 (all BD Biosciences) for 30 min at RT and then washed with 2% BSA in DPBS. Finally, both groups of cells were washed with 2% BSA in DPBS before a resuspension in 2% BSA in DPBS for flow cytometry acquisition. Flow cytometry was performed with a CytoFLEX S V4-B2-Y4-R3 Flow Cytometer (Beckman Coulter) equipped with 405-, 488-, 561-, and 637-nm lasers. Analysis was performed using FlowJo version 10.2 (Treestar). Further details for flow cytometry reagents and consumables can be found in **Table S1**. Cells were distinguished from debris using side-scatter versus forward-scatter area, and subsequently sorted using forward scatter and side scatter (height vs. width) to isolate singlets. To create positive staining thresholds, isotype controls were used Positive gates for the reagents of interest were set to include 1-2% of isotype control cells.

The number of positive cells was then determined by the number of cells stained with the reagent of interest that fell within the positive gate The commonly used guideline of >85% stemness-marker expression was used to verify stemness of cells (Baghbaderani et al., 2015). Representative histograms for each of the stemness markers resulting from the gating strategy explained above can be found in **Figure S1**.

Table S3. Method and workflow key parameters

Workflow	Method	Timing	Equipment	Parameters
1: Plate Maintenance	Media preparation	~50''	Cytomat 6002, CPAC 1&2, iSwap, CO-RE Grippers	• Media from Cytomat 6002 warmed on CPAC set at 34ºC for 30-40 min, then placed on Media Position 1 • Movements slowed when handling media, reagents, and tissue culture plates to ~¼ default speed of ~40 cm/sec for all steps in this workflow
	96-well plate feeding	14'30''	Cytomat 24, Tilt Module 1, iSwap CO-RE Grippers	• One plate at a time moved from Cytomat 24 to Tilt Module 1; tilted 10º • 300 µL tips on channels 1-3 aspirate media from the plate at 175 µL/sec and dispense 150 µL of new media from channels 4-6 at 75 µL/s • Plate is returned to Cytomat 24, repeats for all 96-well plates
	6-well plate feeding	6'45"	Cytomat 24, Tilt Module 1, iSwap CO-RE Grippers	• One plate at a time moved from Cytomat 24 to Tilt Module 1; tilted 10º • 1,000 µL tips on channels 1-4 (two per well) aspirate media from the plate at 175 µL/sec and dispense 2 mL of media from channels 4-8 (two per well) at 75 µL/sec • Plate is returned to Cytomat 24, repeats for all 6-well plates
	Plate imaging	1'30" to 20'	Cytomat 24, Tilt Module 1, iSwap CO-RE Grippers, Celigo Cytometer	• One plate at a time removed from Cytomat 24, imaged and returned to the Cytomat 24 - all movements performed using the iSwap
	Media storage	3' to 4'30"	Cytomat 6002, CPAC 1&2, CO-RE Grippers	• Media stored in Cytomat 6002 at 4ºC using the iSwap
2: Passaging	Media preparation	8'30"	Cytomat 6002, CPAC 1, iSwap CO-RE Grippers	• DPBS, Accutase, mTeSR1 + ROCK Inhibitor (RI), and the 24-well microplate are staged on the deck from the Cytomat 24 (**Figure 1B**) • mTeSR1 + RI) is mixed in 2-column reservoir on the deck at 300 µL/sec • Accutase is warmed on CPAC 2 at 37ºC • Movements slowed when handling media, reagents and cells culture plates to ~¼ default speed of ~ 40 cm/sec *for all methods in this workflow
	6-well plate incubation	6'	Cytomat 6002, Cyomat 24, iSwap CO-RE Grippers, Barcode reader	• Matrigel-coated 6-well plates moved from 4ºC Cytomat 6002 to 37ºC Cytomat 24 for ≥2 hrs prior to Matrigel removal and seeding
	96-well plate incubation	11'15"	Cytomat 6002, Cyomat 24, iSwap CO-RE Grippers, Barcode reader	• Matrigel-coated 96-well plates moved from 4ºC Cytomat 6002 to 37 ºC for ≥2 hrs prior to Matrigel removal and seeding
	6-well plate disassociation	28'	Cytomat 6002, Cyomat 24, iSwap, CO-RE Grippers, Tilt module 1, CPAC 1&2	• For this method: aspirations 200 µL/sec, dispenses 150 µL/sec • 6-well plate moved from Cytomat 24 and onto Tilt Module 1; tilted 10° and, media removed and each well washed with 2 mL of media • 1 mL of warm Accutase added/well; plate incubated on CPAC 1 at 37ºC for 4 min • Row D of the 24-well microplate (**Figure 3F**) receives 4 mL of DPBS/well prior to cell pooling • Two 1,000 µL tips triturate/well at defined angle, speed and location (**Figure 3A**) • Accutase/DPBS cell suspension from each well transferred to the corresponding well in Row D of 24-well microplate (**Figure 3H**) • 1 mL DPBS added/well and transferred to corresponding well in Row D of 24-well microplate to wash remaining cells from wells • 24-well microplate moved at ~¼ default speed (40 cm/sec) to Vspin Centrifuge using iSwap

	Cell centrifugation	3'30"	Vspin Centrifuge	• 24-well microplate centrifuged at 1,000 rpm (RCF = 211 x g) for 3 min.
	Cell seeding preparation	18'58"	Cytomat 6002, iSwap, CO-RE Grippers	• For this method: aspirations 200 µL/sec, dispenses 200 µL/sec, mixes 200 µL/sec • 24-well microplate returned to STAR deck and supernatant is aspirated from defined location to ensure pellet is undisturbed (**Figure 3G**). • To disperse the cell pellet: 2.5 mL mTeSR1 + RI added per well of Row D at different locations within the well (**Figure 3G**) • Cell suspension mixed at bottom of the well to get single cell suspension and dispensed at the top of the well to ensure even distribution of the cells throughout the suspension • 200 µL/well from Row D aspirated and dispensed in Vi-Cell Counting Tubes in another 24-well microplate (**Figure 1, position 15**) on STAR deck; tubes then cell counting step. • 2 mL/well of mTeSR1 + RI added to Row C of 24-well microplate (**Figure 3F**) • 5 mL/well of mTeSR1 + RI added to Row B of 24-well microplate (**Figure 3F**) • Unused DPBS and Accutase returned to the Cytomat 6002 • Calculated volume for the desired final cell concentrations for 6- and 96-well plates is entered by the operator • Calculated volume of cell suspension added to Row C to total 3 mL/well to seed two 6-well plates • Calculated volume of cell suspension added to Row B to total 6 mL/well to seed five 95-well plates
	6-well plate seeding	26'	Tilt module 1, Cyomat 24, iSwap, CO-RE Grippers,	• For this method: aspirations 200 µL/sec, mixes 125 µL/sec, dispenses 125µL/sec • One of two 6-well plates from Cytomat 24 brought to Tilt Module 1 at 10°; all Matrigel is removed and 1 mL/well of mTeSR1 + RI added • Two 1,000 µl tips/well mix (**Figure 3G**) and aspirate from Row C to seed at distinct locations in 6-well plate (**Figure 3B**) • Plate returned to the Cytomat 24; sequence repeats for second plate
	96-well plate seeding	33'45"	Tilt module 1, Cyomat 24, iSwap, CO-RE Grippers,	• For this method: aspirations and mixes 100 µL/sec, dispenses 30 µL/sec along the side of the well (**Figure 3D**) • One of five 96-well plates from Cytomat 24 brought to Tilt Module 1 at 10°; all Matrigel is removed, 1 mL of mTeSR1 + RI aspirated with channels 1-6, 100 µl /well of mTeSR1 + RI added such that each channel dispense 1ten times across six rows of wells • 1,000 µL tips on channels 1-6 mix (**Figure 3G**) and aspirate 1 mL of cell suspension/well from Row B, 100 µL of cell suspension dispensed to rows B-G, columns 2-11, down the side of the well (**Figure 3D**) • Plate returned to the Cytomat 24; sequence repeats for remaining four plates
3: Matrigel coating	Matrigel preparation	8'45"	Cytomat 6002, CPAC 1, iSwap, CO-RE Grippers	• For this method: mix at 250 µL/sec and dispense at 300 µL/sec • Matrigel (MG) and DMEM/F-12 mixed in 2-column reservoir (brought from Cytomat 6002) on CPAC 1 at 4°C. • Final protein concentration of 0.185 mg/mL.
	96-well plate Matrigel coat	30'	Cytomat 6002 CPAC 1&2, iSwap, CO-RE Grippers,	• For this method: aspirations 150 µl/sec and dispenses 125 µl/sec • Five 96-well plates, one at a time, moved from Cytomat 6002 to CPAC 2 at 4°C; 1 mL diluted MG aspirated by 6 channels, then dispensed to the 96-well plate, 100 µL/well (**Figure 3E**) • 96-well plate returned to Cytomat 6002 at 4°C for at least overnight; process repeats for any number of 96-well plates
	6-well plate Matrigel coat	10'30"	Cytomat 6002, CPAC 1&2, iSwap, CO-RE Grippers	• For this method: aspirations and dispenses 200 µl/sec • Two 6-well plates, one at a time, moved from Cytomat 6002 to CPAC 2 at 4°C; four channels aspirate 750 µl diluted MG and two channels/well dispense 1.5 mL/well; aspiration and dispense repeats twice more to coat all six wells (**Figure 3C**) • 6-well plate picked up by CO-RE grippers, swiftly moved around the deck to distribute MG, and returned to Cytomat 6002 at 4°C for at least overnight; process repeats for any number of 6-well plates

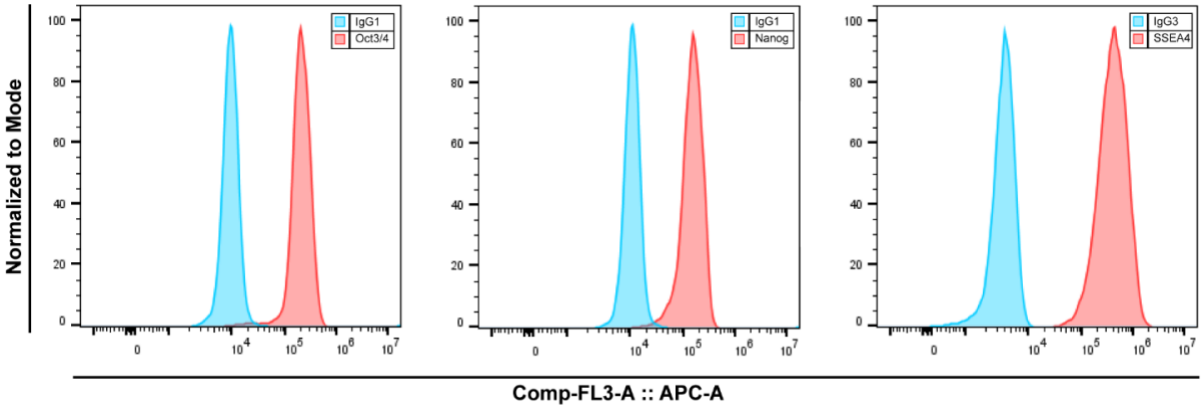


Figure S1. Flow Cytometry Histogram: Example histograms resulting from the gating of pluripotency markers. Isotype control antibodies are in blue; from left to right the pluripotency markers are as follows in red: Oct3/4, Nanog, and SSEA4.