1. Purpose

This document provides the procedures for characterizing new cell lines on the CytoSort Array to determine optimal seeding density for imaging single cells and isolating single-cell colonies into 96-well plates using the AIR System.

2. Procedure

2.1. Materials

2.1.1. Cell type of interest
2.1.2. Appropriate cell culture media
2.1.3. FBS
2.1.4. Pen/Strep
2.1.5. PBS (Ca-/Mg-)
2.1.6. 0.25% Trypsin-EDTA
2.1.7. Sterile pipettes
2.1.8. Sterile tubes
2.1.9. T-75 flasks
2.1.10. Trypan blue
2.1.11. Countess slides
2.1.12. 100 Quad Array
2.1.13. 200 Quad Array
2.1.14. 96-well Plates

2.2. Prepare the CytoSort Array (100 Quad & 200 Quad)

2.2.1. Pre-warm sterile PBS (Ca-/Mg-) to 37°C (no less than 30 minutes).
2.2.2. Add 1mL of pre-warmed PBS to each reservoir and wash for 3 minutes.
2.2.3. Aspirate, and repeat above wash two additional times.
2.2.4. Aspirate the PBS wash and add 1mL of appropriate cell culture media to each reservoir to be seeded. Incubate prepared array in a 37C incubator until cell seeding (at least 5 minutes).

- Note: array can be stored at 4C, but do not allow the array to go dry, as this will cause bubbles in the microwells.

2.3. Counting Cell Suspension

2.3.1. Dissociate cells per appropriate dissociation protocol and ensure that a single cell suspension is achieved prior to cell counting.

- Note: for clumpy cells, a filtration step through a sterile filter may increase single cell number.

2.3.2. Take 2 x 20uL aliquots of the cell suspension into two microcentrifuge tubes for counting.

2.3.3. To each of the aliquots, add an equal part trypan blue (20uL) and load 10uL onto one side of a countess slide or hemacytometer. Record the counts in Table 1 below and calculate the number of viable cells/flask using the equation below:

Table 1. Cell Count using Countess

	Cell Count	Viability
Count 1		
Count 2		
Average:		

Viable Cells/flask= Average count x total volume =

2.3.4. Prepare a dilute cell suspension concentration of $3.2x10^4$ cells/mL using the concentration equations below. Create a stock of 6mL of cell suspension at this concentration of $3.2x10^4$ cells/mL.

2.3.5. From the stock of 6mL cell suspension at a concentration of $3.2x10^4$ cells/mL, serially dilute each concentration below in Table 2 to seed the array. Take 3mL from T1 and add into T2 with an additional 3mL complete media to create the next dilution. Repeat this process with the next dilution by adding 3mL T2 and an additional 3mL complete media into T3 to create the next dilution. Do this for all dilutions.

2.4. Determining Seeding Volume for 100Q & 200Q Arrays

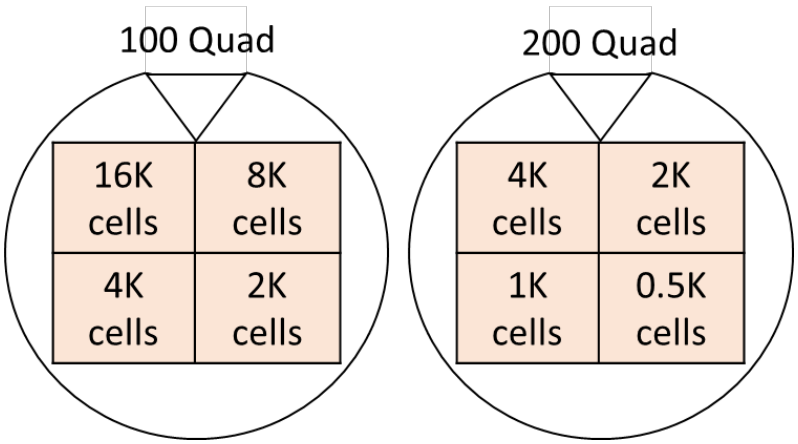
Table 2. Dilution for Array

Tube #	Cells per Reservoir	Cells to Raft Ratio: 100Q	Cells to Raft Ratio: 200Q	Concentration (cells/mL)	Cell Volume (6 mL)	Media Volume for (6 mL)
T1	**16000**	2.5:1		$3.2x10^4$		
T2	**8000**	1.25:1		$1.6x10^4$	3mL (T1)	3mL
T3	**4000**	0.63:1	2.5:1	$0.8x10^4$	3mL (T2)	3mL
T4	**2000**	0.31:1	1.25:1	$0.4x10^4$	3mL (T3)	3mL
T5	**1000**		0.63:1	$0.2x10^4$	3mL (T4)	3mL
T6	**500**		0.31:1	$0.1x10^4$	3mL (T5)	3mL

2.5. Seeding the Array (100 Quad & 200 Quad)

2.5.1. Seed the 100Q and 200Q per diagram below with 0.5mL of appropriate cell suspension per reservoir.

2.5.2. Incubate the array and allow cells to settle and adhere for no less than 4 hours prior to initial scan on the AIR system.



2.6. Imaging on CellRaft AIR® System

2.6.1. After cells have adhered (up to 18 hours post-seeding), scan the 100Q and 200Q arrays on the AIR System (time zero)

2.6.2. Scan the arrays every 24 hours post-seeding until each array has reached the appropriate confluency for isolation.

2.6.3. The 100Q Array should be imaged for 2-3 days before isolation. Cells should divide in the microwells on a raft until reaching approximately 8 cells/raft. This will be the optimal confluency for isolation of the 100Q.

2.6.4. The 200Q Array should be imaged for 3-4 days before isolation. Cells should divide in the microwells on a raft until reaching approximately 16 cells/raft. This will be the optimal confluency for isolation of the 200Q.

2.6.5. Between imaging, monitor each array for single cell and colony growth using Off-the AIR-software.

2.7. Isolation on CellRaft AIR System

2.7.1. Using Off the AIR software, map the desired number of 96-well plates for isolation between the 100Q and 200Q Array (ideally one full plate each).

2.7.2. Add 100uL of appropriate collection media to each reservoir of the 96 well plate for isolation.

2.7.3. Perform isolation using the AIR system.

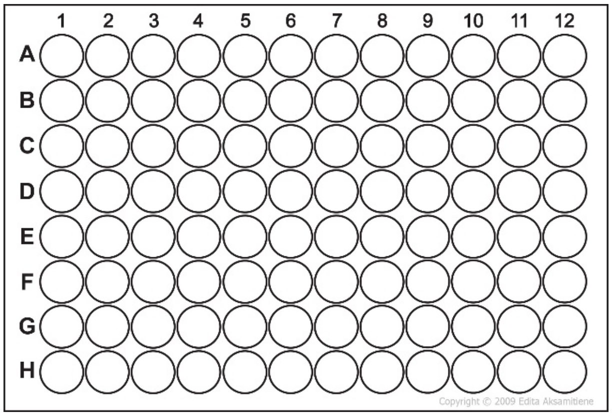
2.7.4. Record the number of wells that contain a single raft.

2.7.5. 2-3 days post-isolation, an additional 100uL of media can be added to feed cells if desired.

- Note: Do NOT perform a full media exchange until the cells have grown off the rafts, or the rafts can be aspirated and lost.

2.7.6. Record outgrowth off the Array and monitor cell growth for at least 1 week.

2.7.7. Once colonies have reached desired size, they can be passaged as normal.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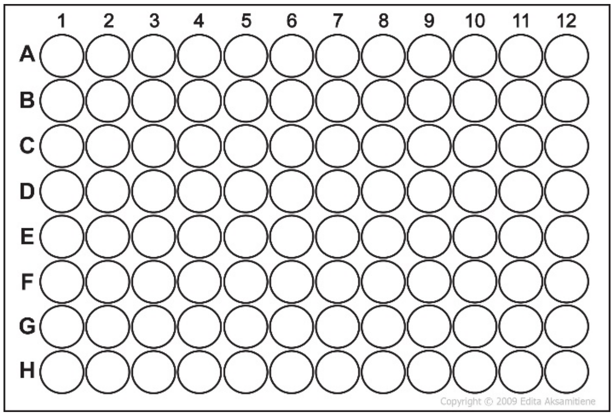
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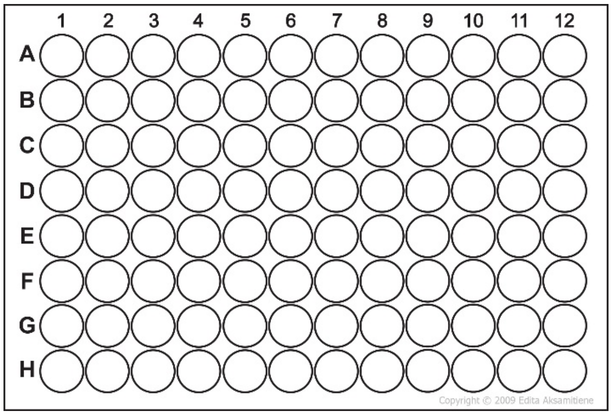
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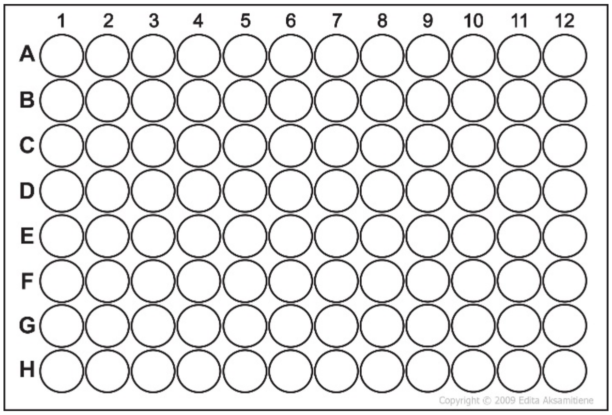
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