Electronic Supplemental Information

Microfluidics for the Detection of Minimal Residual Disease in Acute Myeloid Leukemia Patients using Circulating Leukemic Cells Selected from Blood

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Table of Contents page #

Activating the microfluidic devices with cleavable oligonucleotide linkers and cell selection mAbs 2
Pre-SCT clinical characteristics 2
Pre-SCT leukemia associated phenotypes (LAPs) 3
CD33, CD34, and CD117 antigen co-expression in AML cell lines 4
Immunophenotyping cells isolated from the anti-CD33 device for Pt #2's apparent "left shift" at day 84 4
References 7

List of Figures

Figure S1. MFC analysis of CD33, CD34, and CD117 expression in three AML cell lines (U937, HL60, and KG-1) 4
Figure S3. Fluorescence images of the cells released from the anti-CD33 device for Pt #2 – day 84 4
Figure S4. The percent change between samples in the CLC and aberrant(-) subpopulations for all patients 6

List of Tables

Table S1. Pre-transplant clinical characteristics of Pts #1-#5 2
Table S2. LAP profiles for Pts #1-#5 as determined by MFC analysis. The percentage of leukemic blasts with markers relevant to this study (CD7, CD56, and the isolation antigens) are shown along with full LAP panels 3
Table S3. CLC counts and aberrant(-) cell counts for Pts #1-5 throughout post-SCT longitudinal tracking 5
Table S4. Aberrant(+) and aberrant(-) cell counts for healthy donors 6

Surface activation of the microfluidic devices and attachment of cleavable oligonucleotide linkers with mAbs. Immediately prior to oligonucleotide immobilization, assembled devices were UV/O_3 activated (22 mW/cm^2, 15 min) in an activation chamber equipped with a quartz, low pressure Hg lamp to generate -COOH groups on the microfluidic surfaces.[1,2] To react the oligonucleotide's 5'-amino group with the activated surfaces, devices were incubated for 15 min at room temperature with a solution of 20 mg/mL EDC and 2 mg/mL NHS in 100 mM MES buffer, after which the devices were dried with an air-filled syringe and incubated overnight at 4°C with 40 µM oligonucleotide in PBS buffer. For mAb immobilization, devices were air-dried, washed with 100 µL PBS (50 µL/min) and the 3'-disulfide group was reduced using 300 mM DTT in 200 mM carbonate buffer adjusted to pH 9 (400 µL, 20 µL/min) to produce maleimide-reactive sulfhydryl groups. Devices were then immediately air-dried, washed with 100 µL PBS (50 µL/min), air-dried again, flooded with freshly prepared maleimide-labeled mAbs and incubated overnight at 4°C for 2 h at 25°C. As described previously,[3] all mAbs (0.5 mg/mL) were azide-purified with a Zeba column, reacted with sulfo-SMCC for 45 min and again purified with a Zeba column. Finished devices were either used within 48 h or air-dried, flooded with 2X protein stabilizing cocktail in PBS and used within 3 weeks.

Table S1. Clinical characteristics of Pts #1-5.

Characteristic	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5
Age	52 / Female	36 / Female	68 / Female	67 / Male	54 / Female
Cytogenetics	Diploid	Diploid	Diploid	Complex, del(5q)	Diploid
Molecular	NPM1+ FLT3-ITD+	CEBPa+ (silent mutation)	CEBPa+ (heterozygous)	None	NPM1+ DNMT3A+
Risk (ELN)	Intermediate-I	Intermediate-I	Favorable	Adverse	Favorable
Aberrant marker	CD7 (51%)	CD7 (50%)	CD7 (na)	CD56 (34%)	CD56 (52%)
Conditioning	MAC Bu/Flu/ATG	MAC Bu/Cy/ATG	RIC Bu/Flu/ATG	RIC Bu/Flu/ATG	MAC Bu/Flu/ATG
Pre-SCT test day	-11	-20	-12	-24	-30
Pre-SCT test results	MFC negative NPM1(2753/10000)	6% phenotypically normal blasts	6% phenotypically normal blasts	7/20 abnormal metaphases	NPM1(27/10000)
Pre-SCT status	Relapsed AML, CR(p)	Relapsed AML, CR(p)	Relapsed AML, CR(p)	De novo AML, CR	De novo AML, CR
Donor gender	Male	Male	Male	Female	Female
HLA match	10/10	10/10	10/10	10/10	10/10

All pre-SCT tests were performed on a bone marrow biopsy. Aberrant marker percentages are the proportion of leukemic blasts positive for the marker by MFC. Abbreviations: ELN (European LeukemiaNew Risk); na (not available); MAC (myeloablative conditioning); RIC (reduced-intensity conditioning); Bu (busulfan); Flu (fludarabine); ATG (anti-thymocyte globulin); Cy (cyclophosphoamide); CR (complete remission); CR(p) (CR but incomplete recovery of platelet counts).

Pre-SCT clinical characteristics. The clinical characteristics of Pts #1-5 prior to allogeneic SCT are available in **Table S1**. For Pt #4, CLCs had a cytogenetic abnormality (complex karyotype, deletion of the long arm of chromosome 5, adverse risk).[4] Pts #1 and #5 had common NPM1 mutations (found in ~30% of all AMLs). NPM1 mutations are associated with a low risk of relapse (Pt #5) unless there is a concomitant FLT3-ITD mutation,[5] which was observed in Pt #1, that stimulates proliferation and inhibits apoptosis.[6] CEBPa mutations, present in Pt #3, disrupt differentiation and are associated with a favorable risk (Pt #2's CEBPa mutation did not encode an amino acid change). DNMT3A mutations, detected in Pt #5, have been suggested to impart a high risk of relapse, but the ELN has not incorporated DNMT3A into their risk assessment (**Table S1**).[7-9] These gDNA mutations have been suggested as therapeutic targets.[5,10-13]

While Pts #1-3 had intermediate or favorable risk of relapse, these patients presented with cases of relapsed AML prior to SCT, which is an absolute indication to proceed with SCT.[14] Pts #4-5 had high risk for disease relapse and were referred for SCT (**Table S1**). All patients had ideal (10/10) human leukocyte antigen (HLA) matches with their donors, decreasing the likelihood of graft-versus-host disease, and responded to chemotherapy and achieved CR. Pts #1, #4, and #5 were eligible for additional MRD tracking by FISH (5×10^{-2} sensitivity), PCR (10^{-4} sensitivity), or MFC (10^{-3} – 10^{-4} sensitivity). These results showed that all three of the patients were MRD(+) pre-

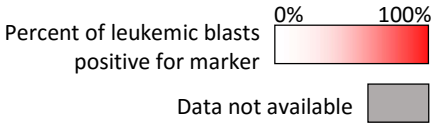
SCT while technically in CR. Pts #1, #2, and #5 were treated with intense myeloablative conditioning, and Pts #3 and #4 received reduced intensity conditioning (RIC) due to their age. RIC therapy is less strenuous and retains higher disease burden at SCT, relying on the GvL effect to combat MRD but has a higher relapse risk.[15]

Pre-SCT leukemia associated phenotypes (LAPs). Each patient's AML was profiled by MFC to identify unique aberrant markers that could be used for post-SCT MRD tracking. "Immature cells" were first gated by low CD45 signal and low side scatter[16] and aberrant marker expression was assessed (**Table S2**). A patient-specific aberrant marker was selected based on the following criteria: (i) The aberrant marker was expressed on as large a proportion of blasts as possible; and (ii) normal blood components did not co-express the aberrant marker and CD33, CD34, or CD117. For example, Pt #5 had 89% CD64(+) leukemic blasts, but normal monocytes that co-expressed CD33 and CD64. For typical MFC analyses, monocytes are gated out of the blast subpopulations, but in the microfluidic assay, monocytes are extracted in the anti-CD33 device along with CLCs and would present false-positives based on positive staining for CD45 and the CD64 aberrant marker. Instead, CD56 was chosen as the aberrant marker for this study, which was expressed on 52% of CLCs and would not generate false positives arising from monocytes. While the presented study is limited by applicable aberrant markers for these reasons, we are currently developing a microfluidic flow cytometry system to identify CLCs isolated in the microfluidic devices. By incorporating side scatter measurements into this system, normal blood cells that co-express the isolation and aberrant marker can be reliably discerned from CLCs as performed in conventional MFC analysis.

Leukemic associated phenotype	Antigen	Proportion of leukemic blasts				
		Pt #1	Pt #2	Pt #3*	Pt #4	Pt #5
Pan-leukocyte	CD45	100%	100%	(+)	100%	100%
Normal myeloid markers (Selection antigen)	CD33	91%	34%	(+)	70%	99%
	CD34	4%	69%	(-)	57%	1%
	CD117	72%	71%	(+)	28%	6%
Lineage infidelity	CD7	51%	50%	(+)	6%	3%
Asynchronous	CD56	5%	1%	(-)	34%	52%
Lineage infidelity	CD2	1%	5%	(-)	9%	0%
	CD3	1%	2%		1%	0%
	CD5	1%	2%	(-)	3%	0%
	CD10	2%	3%	(-)	0%	2%
	CD19	8%	1%	(-)	1%	3%
	CD20	1%	2%		0%	0%
Asynchronous	CD11b	21%	19%	(-)	19%	90%
	CD14	10%	6%	(-)	1%	6%
	CD15	55%	15%		27%	
	CD64	22%	26%	(-)	24%	89%
Leukemic blasts in sample		3%	1%	26%	2%	66%

*Only (+) and (-) status was reported for Pt #3's markers; percentages were not available. A (+) marker status was assigned 100% in the heat map and (-) status was assigned 0%.

Table S2. Leukemic associated phenotypes for Pts #1-5 as determined by MFC analysis. The percentage of CLCs with markers relevant to this study (CD7, CD56, and the isolation antigens) are shown along with full leukemic associated phenotype panels. Refer to legend to interpret the heat map.



CD33, CD34, and CD117 antigen co-expression in AML cell lines. Three AML cell lines were analyzed by MFC to determine co-expression of the CD33, CD34 and CD117 isolation antigens. The U937 cell line[17] was a gift from Dr. Gregory Lizee at MD Anderson and the Kasumi-1 cell line[18] was a gift from Dr. Douglas Graham at the University of Colorado, Denver. The HL60[19] and KG-1[20] cell lines were purchased from the American Type Culture Collection (ATCC) through the Tissue Culture Facility at UNC. The cell lines were immunostained with anti-CD33-APC, anti-CD34-PE-Cy7, anti-CD117-PE antibodies (BD Biosciences) and analyzed by MFC using a MACSQuant Analyzer (Miltenyi Biotec GmbH). Data was analyzed using FlowJo (FlowJo, LLC) and is shown in **Figure S1**. Both the U937 and HL60 cell lines highly expressed the CD33 antigen, while the KG1 cell line showed expression of CD33, CD34, and to a lesser extent CD117. The Kasumi-1 cell line was also tested (data not shown) and expressed both CD33 and CD117 (mean fluorescence intensity of 29× the isotype control and 230× the isotype control, respectively).

Given the diversity of antigen expression and co-expression in the cell lines, we decided to process clinical samples through the 3 microfluidic devices arranged in parallel rather than in a serial fashion.

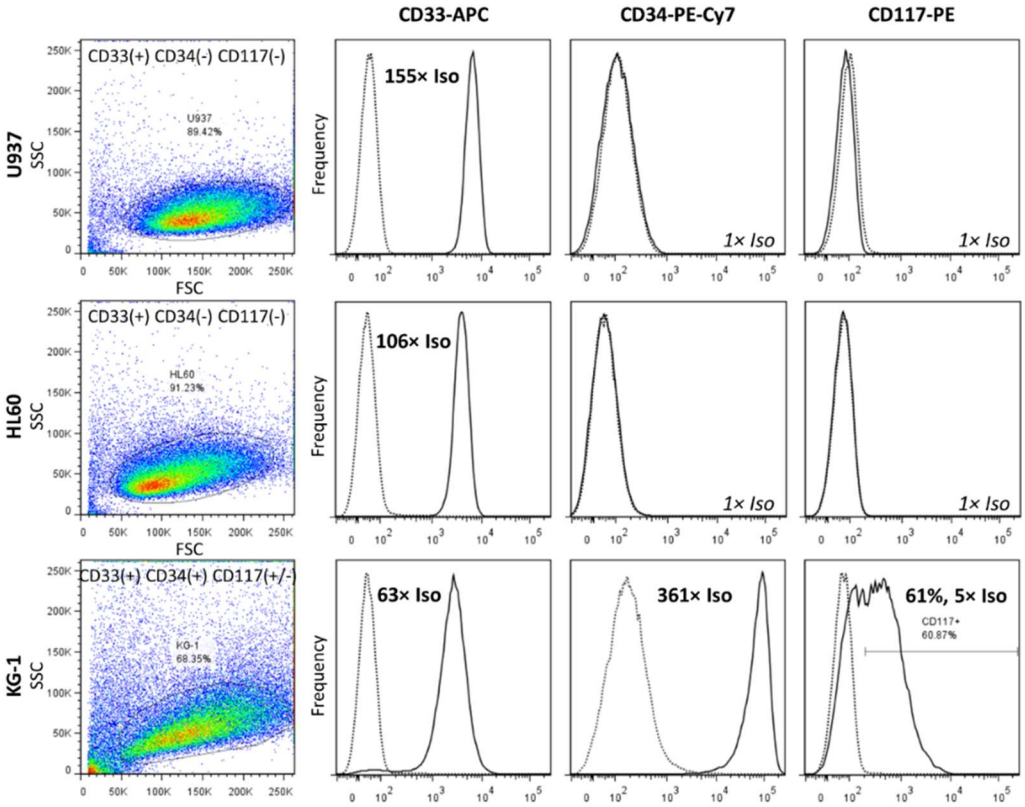


Figure S1. MFC of CD33, CD34, and CD117 expression in U937, HL60, and KG-1 AML cell lines. Iso = isotype control.

Immunophenotyping cells isolated from the anti-CD33 device for Pt #2. Fluorescence images of the cells isolated using the anti-CD33 device for Pt #2's day 84 sample are shown in **Figure S2**. Particle analysis of the DAPI image in **Figure S2A** indicated the well contained 89,492 cells, which showed high agreement with CD45-Cy5 staining (**Figure S2B**). This cell count is likely to be an under-approximation of the true cell count due to imperfect particle analysis. The entire well was scanned to identify CLCs that expressed the CD7-FITC aberrant marker, 99 of which were identified (**Figure S2C-E**).

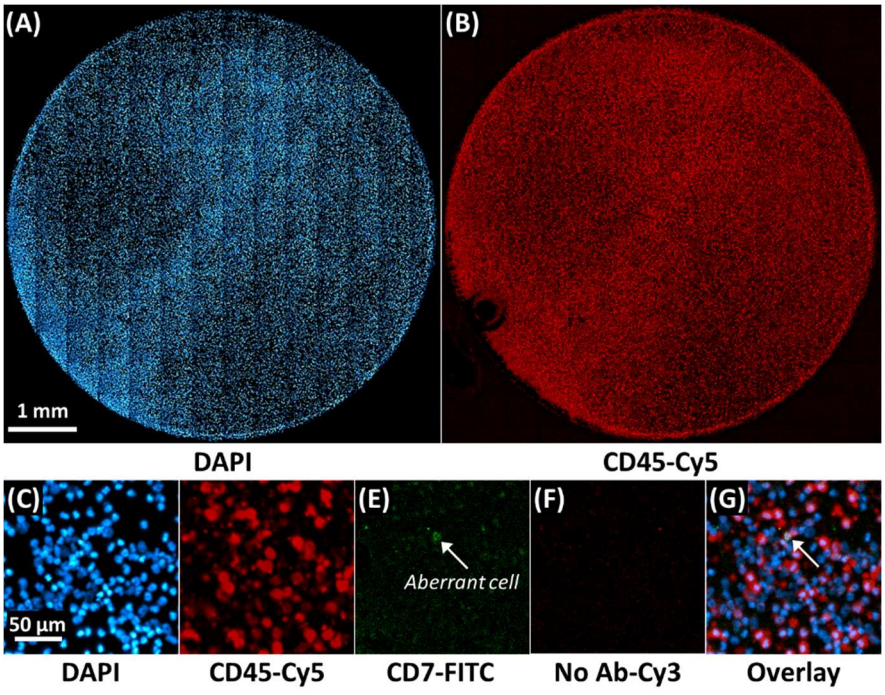
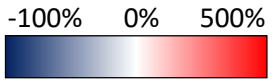


Figure S2. (A,B) DAPI and CD45-Cy5 fluorescence images of the cells secured from the anti-CD33 device for Pt #2 – day 84. These images were stitched from 225 individual images acquired with a 20X objective. The apparently dark portion in the bottom, left corner of panel (B) is an artifact of background subtraction used to produce this figure, but was not present during the identification of aberrant cells. **(C-G)** Zoomed fluorescence panel of a single aberrant, CD7-FITC(+) CLC amidst a large background of aberrant(-) cells. A Cy3-labeled Ab was not used (autofluorescence channel). Scale bar in (A,B) is 1 mm. Scale bar in (C-G) is 50 µm.

Table S3. Cell counts for Pts #1-5 throughout post-SCT tracking. Heat maps are the percent change of the total count relative to the previous sample. If MRD(+), the patient's MRD burden was assigned using the quartiles of total CLC counts accumulated from all patients. MRD burden was assigned as low (<29 CLCs/mL), mid (≥29 and <90 CLCs/mL), high (≥90 and <405 CLCs/mL) and very high (≥405 CLCs/mL). na = not available.

Days post-transplant	MRD burden	CLCs / mL blood				Aberrant(-) cells / mL blood				Progression of total counts	
		Total	CD33	CD34	CD117	**Total**	CD33	CD34	CD117		
Pt #1										CD7(+)	CD7(-)
28	Mid	**81**	0	0	81	**403**	232	12	159		
45	High	**90**	1	26	63	**558**	189	28	341		
57	High	**221**	104	14	103	**129**	72	13	44		
71	High	**348**	72	20	256	**207**	69	12	126		
85	Very high	**1761**	469	48	1244	**152**	54	5	93		
95	**Deceased**										
Pt #2										CD7(+)	CD7(-)
-7	Low	**18**	8	0	10	**458**	283	80	95		
14	Very high	**566**	238	77	251	**2812**	764	177	1871		
28	Very high	**1066**	86	116	864	**2470**	740	1166	564		
40	Low	**22**	2	12	8	**98**	38	38	22		
54	High	**102**	12	28	62	**154**	24	80	50		
70	High	**140**	2	1	137	**271**	22	25	224		
84	High	**314**	99	0	215	**89492**	88716	134	642		
98	Very high	**443**	51	372	20	**249**	55	156	38		
118	**Deceased**										
Pt #3										CD7(+)	CD7(-)
-7	Low	**15**	0	11	4	**47**	0	16	31		
13	None	**1**	0	0	1	**67**	22	17	28		
30	Very high	**1430**	1416	13	1	**1301**	1270	31	0		
44	na	**na**	na	11	12	**na**	na	39	319	na	na
55	Very high	**1691**	1640	0	51	**1350**	1021	0	329		
69	Mid	**69**	31	0	38	**109**	21	1	87		
90	None	**0**	0	0	0	**4**	3	1	0		
107	Mid	**29**	28	1	0	**29**	24	5	0		
121	None	**4**	4	0	0	**0**	0	0	0		
137	**Alive**										
Pt #4										CD56(+)	CD56(-)
43	na	**na**	0	na	na	**na**	1573	na	na		
57	Mid	**29**	0	0	29	**2528**	2038	9	481		
71	Low	**11**	0	0	11	**426**	238	52	136		
81	None	**5**	0	0	5	**285**	125	57	103		
95	Mid	**30**	9	21	0	**180**	53	69	58		
144	Mid	**57**	56	0	1	**230**	210	6	14		
186	Mid	**31**	31	0	0	**81**	77	2	2		
214	Low	**27**	0	18	9	**597**	275	225	97		
246	Very high	**405**	405	0	0	**7**	7	0	0		
254	**Alive**										
Pt #5										CD56(+)	CD56(-)
-5	None	**0**	0	0	0	**68**	54	4	10		
12	Mid	**29**	12	0	17	**39**	33	0	6		
28	None	**5**	0	1	4	**146**	115	27	4		
40	None	**2**	0	0	2	**489**	274	175	40		
68	High	**174**	153	10	11	**291**	262	5	24		
85	Very high	**2684**	2663	10	11	**1454**	1310	138	6		
118	Low	**21**	19	0	2	**64**	44	3	17		
146	None	**0**	0	0	0	**4**	4	0	0		
178	**Alive**										



Population progression between samples (% change)

Table S4. Aberrant(+) and non-aberrant(-) cell counts for healthy donors. For healthy donors #1 and #2, 0.5 mL of PB was processed with counts normalized to 1 mL. For healthy donor #3, 1 mL of PB was processed. The threshold for identifying MRD positive was based on aberrant(+) cell counts (3-times the standard deviation; 99% confidence interval) was calculated prior to rounding the cell counts.

Healthy donor	Aberrant(+) cells / mL blood				Aberrant(-) cells / mL blood			
	Total	CD33	CD34	CD117	**Total**	CD33	CD34	CD117
#1	**6**	4	0	2	**428**	176	34	218
#2	**0**	0	0	0	**158**	52	14	92
#3	**6**	3	1	2	**247**	224	9	14
Mean	**4**	2	0	1	**278**	151	19	108
SD	**3**	2	1	1	**138**	89	13	103
Threshold	-	8	3	5	-	-	-	-

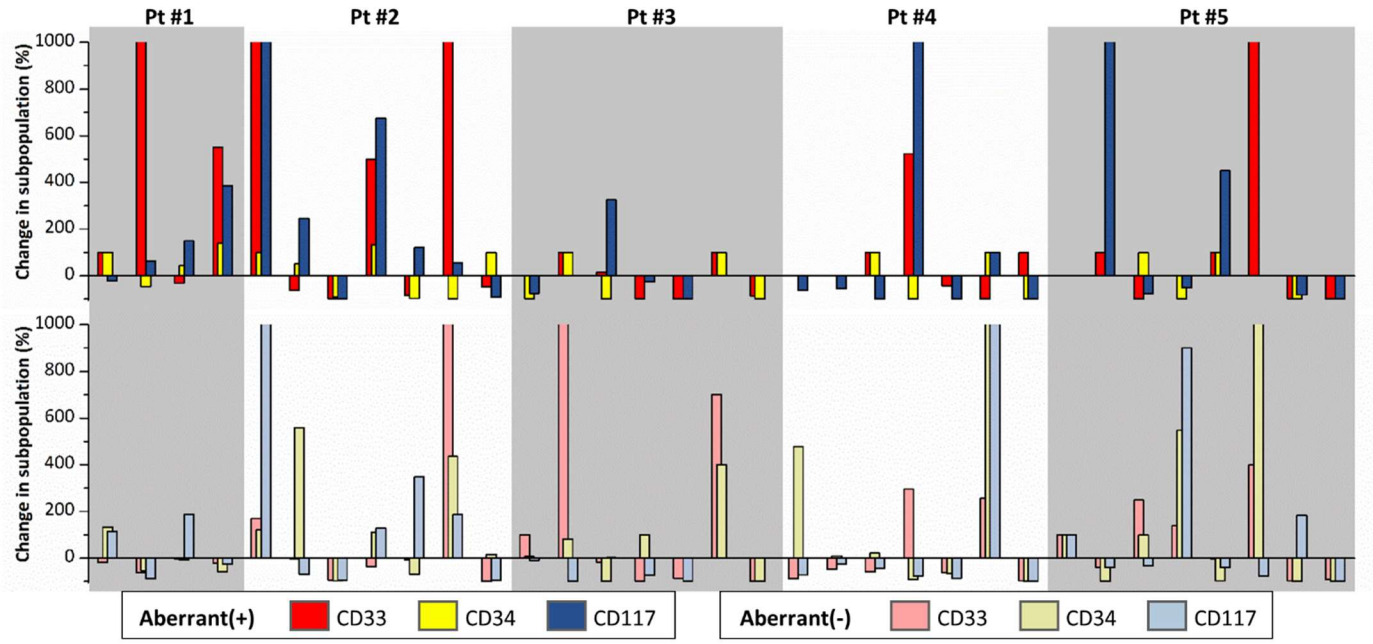


Figure S3. The percent change between samples in the **(top)** aberrant (+) and **(bottom)** aberrant(-) cells. All changes are calculated relative to the previous measurement. Patient data was separated by shading and the x-axis arbitrarily represents subsequent sampling (not scaled with time). We did not observe any apparent trend between the aberrant(+) and the aberrant(-) sets. The maximum decrease in any set was -100% and corresponded to complete loss of cells associated with that set. There is no fundamental limit to increases in the aberrant(+) or aberrant(-) cells, but we did limit the y-axis to +1000% for visualization purposes. Lastly, if a set of cells was present in a sample but was not observed on the previous sample, a change of +100% was assigned.

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