Supporting Information for:

Label-free Profiling of 48-206 Single-Cell Proteomes per Day Enabled by a Multiplexed Nanoflow Liquid Chromatography System

Author List

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Supporting Information
Figures

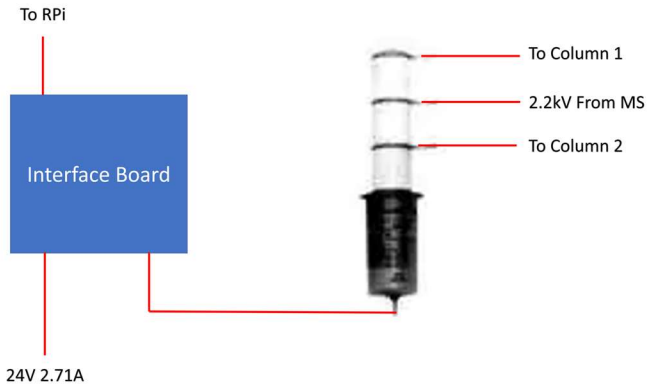

Figure S1. Schematic for High Voltage Switch. The Interface board receives a signal from the raspberry pi, activating a 24V 2.71A power supply, which triggers the bipolar high voltage switch. The middle post of the high voltage switch is connected to the high voltage output of the mass spectrometer and the other two posts are each connected to a column.

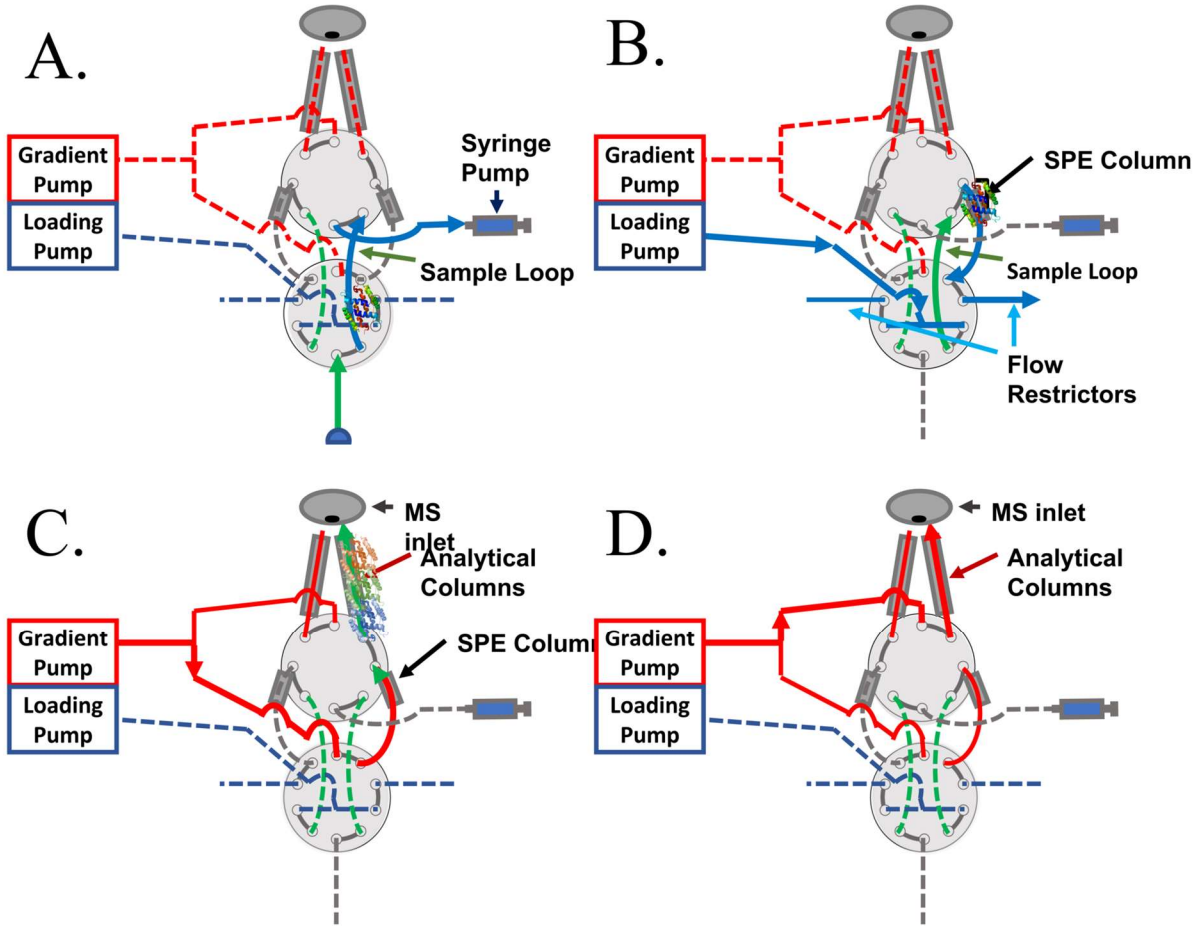

Figure. S2. Process for analyzing a sample using the multiplexed nanoLC system. (A) The lyophilized sample in a nanowell is reconstituted on the chip in 0.1% formic acid. The remaining time in the 15-minute cycle is spent washing the sample well to maximize analyte recovery and aspirating the sample into the sample loop. The sample's path is colored in green, and the end location of the sample is indicated by the protein depiction. (B) The next cycle is spent loading the sample from the sample loop onto the head of the SPE column. The split flow in blue passes through the flow restrictor. (C) The next cycle is spent analyzing the sample after re-equilibrating the column using the loading buffer left in the SPE loop. (D) The final cycle is spent regenerating the column by washing the column with another gradient and re-equilibrating. The split flow elutes the next sample.

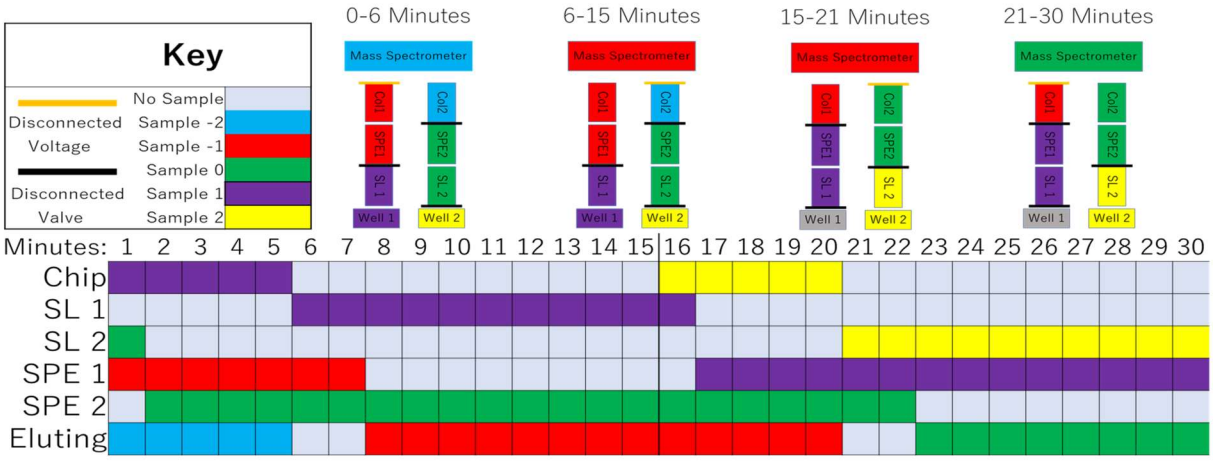

Figure S3. Multiplexing for 15-minute cycles. (Top) Simplified diagram of multiplexing. While a sample (Sample 1, purple) is loaded into the sample loop (SL), the previous sample (Sample 0, green) is transferred to the SPE. The sample before that (Sample -1, red) is making its way to the MS through the LC column, and the sample before that (Sample -2, blue) is finishing its elution from the column. Once the elution of Sample -2 is completed, the voltage is switched to connect the left column (Col1) and disconnect the right column to avoid interference. The valve then switches, pushing each sample to the next step of the process, and another sample (Sample 2, yellow) is loaded into the sample loop. (Bottom) Timing for multiplexing of the autosampler for 15-minute cycles. Each colored box shows which of the 5 samples is in each location as the workflow progresses.

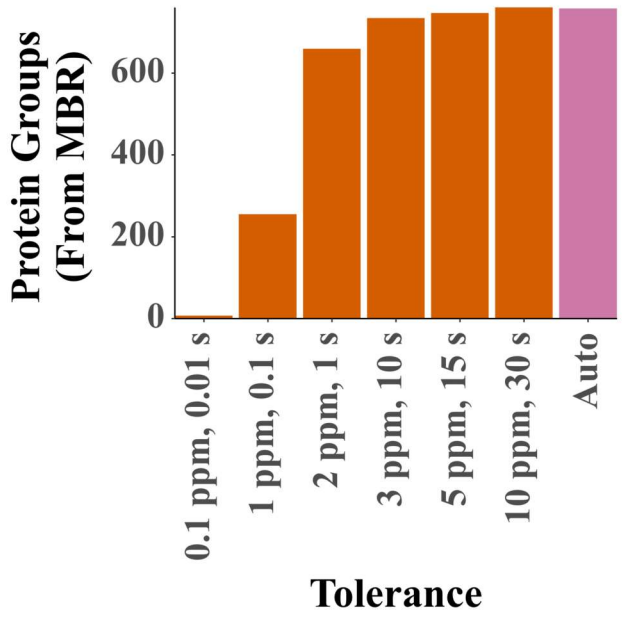

Figure S4. MBR protein identifications for different mapping parameters for 15-minute cycles, where 'Auto' allows both the mass tolerance and retention time to be automatically calculated by Proteome Discoverer.

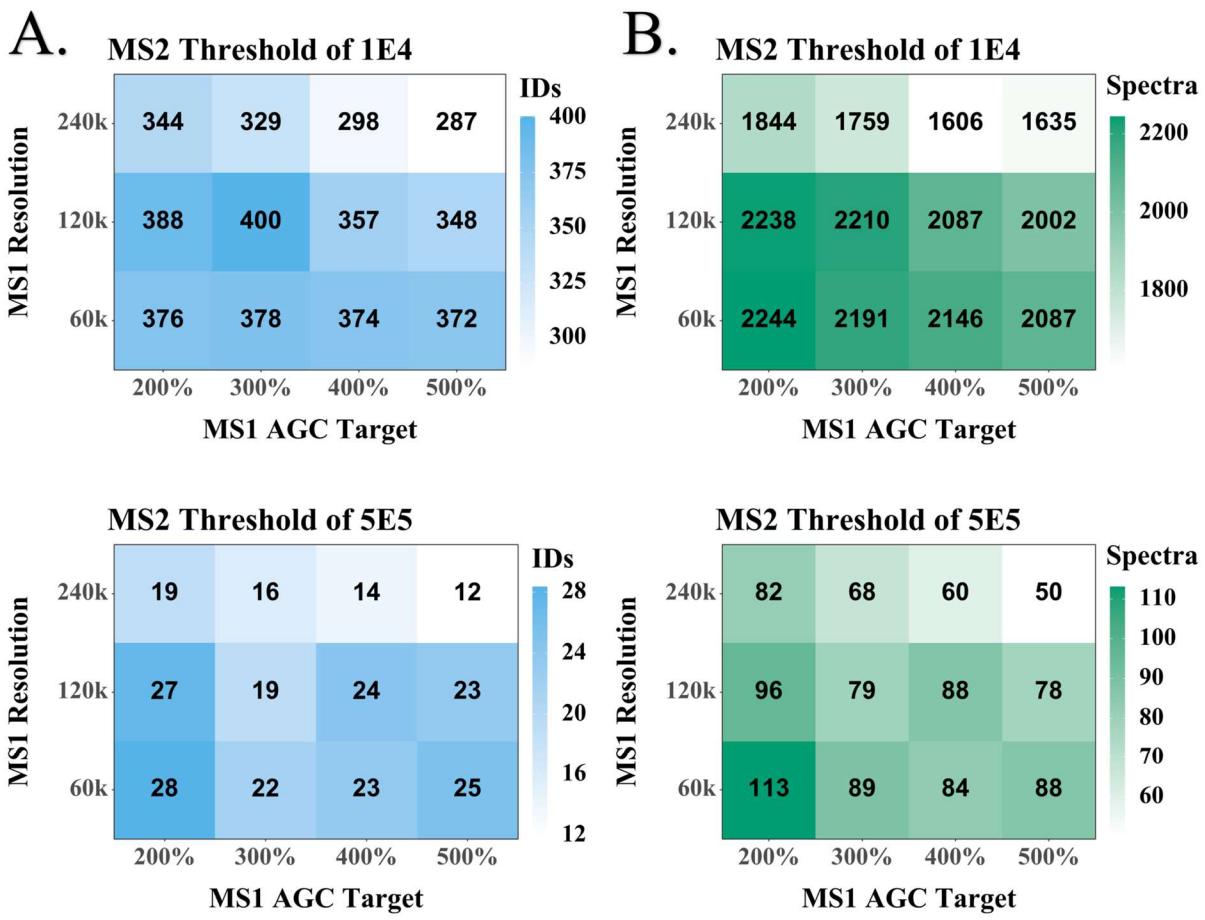

Figure S5. (A) 3D matrix of mass spectrometer settings for MS2-based identifications only. The shading indicates the average number of proteins identified by MS2. Note the different scales for the different MS2 thresholds. (B) The number of MS2 spectra for each set of MS acquisition settings.

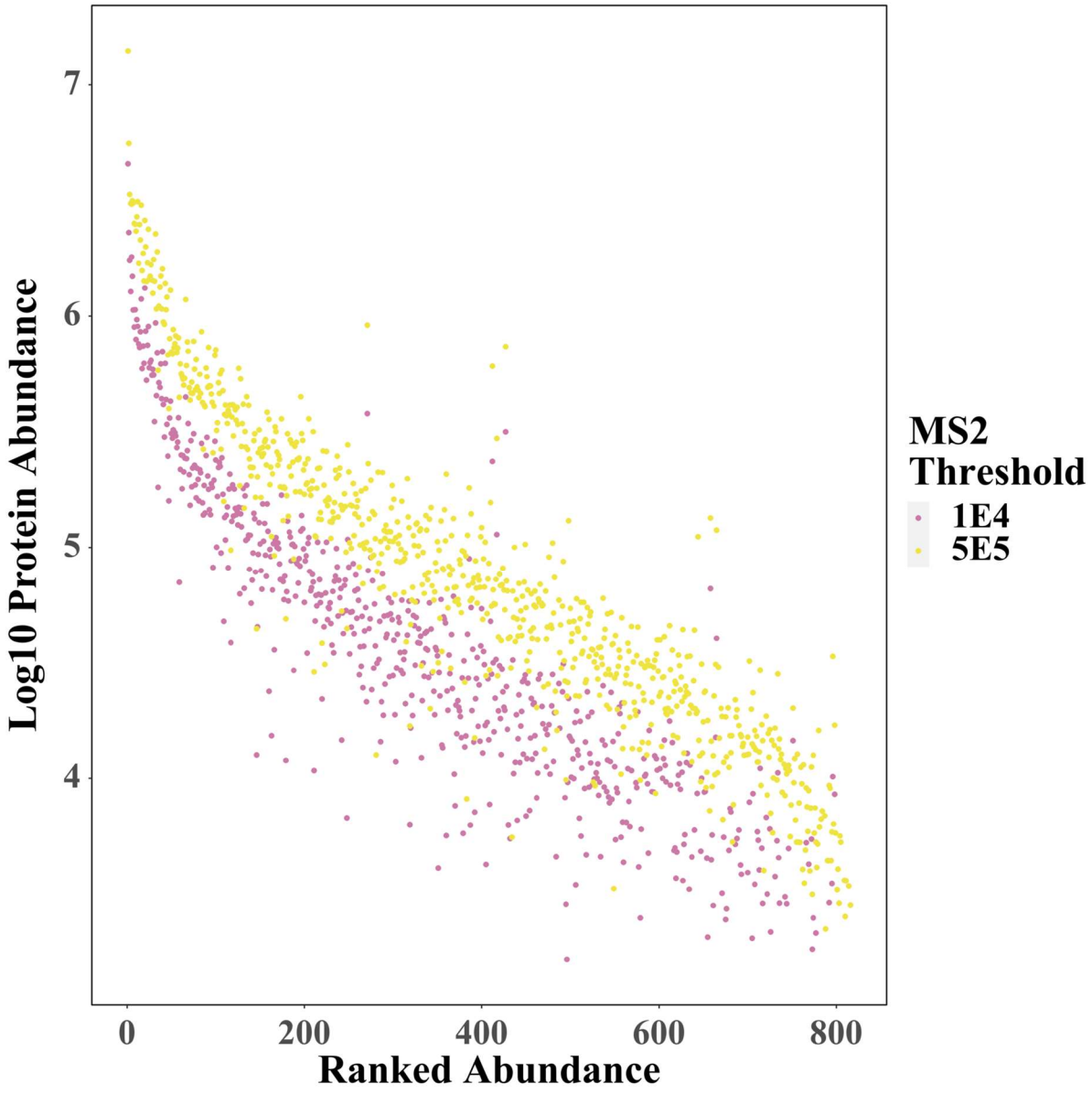

Figure S6. Quantification effects resulting from relying on MBR for identification. The conditions with the highest number of identifications for each threshold were used to create protein abundance curves based on ranked abundances derived from analysis of 10 ng aliquots of HeLa digest. Protein abundances are log10 transformed. The lowest abundance proteins were identified using 'high-threshold' settings.

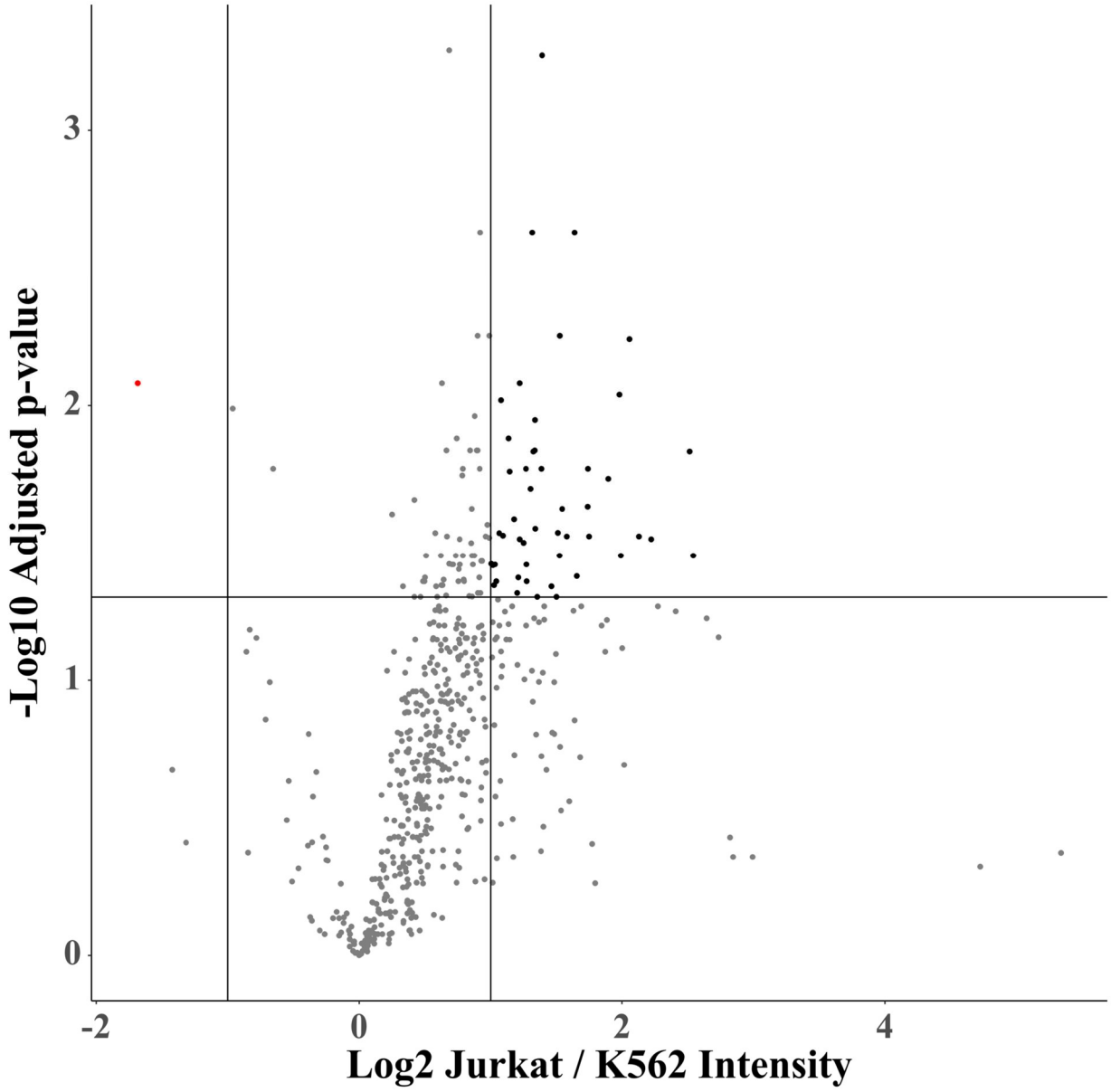

Figure S7. Volcano plot of protein expression in Jurkat vs K562 Cells. Values further to the right are enriched in Jurkat cells and those farther to the left in K562 cells. The black dots indicate proteins that have an absolute fold change of 2 or greater with a p value < 0.05.

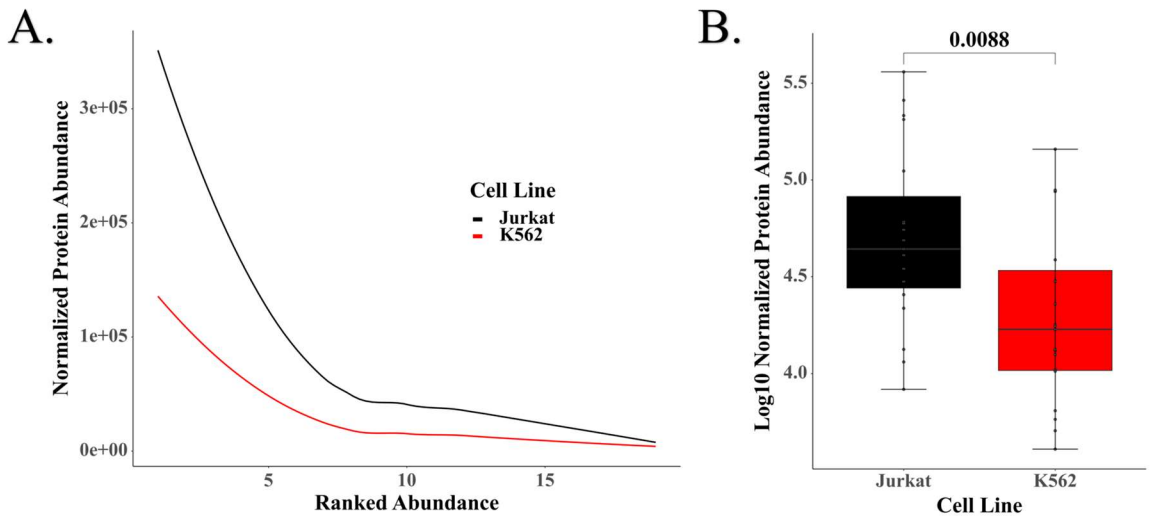

Figure S8. Upregulated mitochondrial proteins only in Jurkat cells compared to K562 cells. **(A)** The protein abundances of 36 upregulated mitochondrial proteins (categorized by DAVID). Proteins were ranked according to the normalized abundances in Jurkat cells. **(B)** Box and whisker plot of log10-transformed protein abundances for the two cell lines. Significance was calculated using a paired t-test.

Table S1. LC Gradient for 7-minute Cycles

Start Time	Mobile B
0	10%
3	20%
4.3	45%
4.5	75%
4.7	75%
4.9	8%

Table S2. LC Gradient for 10-minute Cycles

Start Time	Mobile B
0	10%
6	20%
7	45%
7.3	80%
7.8	80%
8	50%
8.3	50%
8.5	80%
8.8	80%
8.9	8%

Table S3. LC Gradient for 15-minute Cycles

Start Time	Mobile B
0	10%
9	20%
10.5	45%
11	80%
11.75	80%
12	60%
12.25	60%
12.75	70%
13	70%
13.25	80%
13.5	80%
14	8%

Table S4. LC Gradient for 30-minute Cycles

Start Time	Mobile B
0	10%
24	20%
25.5	45%
26	80%
27	80%
27.5	8%

Table S5. MS Settings

	SINGLE-CELL AND SINGLE-CELL-SIZED ALIQUOTS (200 PG)	10NG LIBRARY
ESI VOLTAGE	2200 V	2200 V
MAXIMUM MS1 INJECTION TIME	502 ms	118 ms
MS1 SCAN RANGE	375-1575	375-1575
MS1 AGC TARGET	300% or as specified	300%
MS1 RESOLUTION	120k or as specified	120k
MAXIMUM MS2 INJECTION TIME	246 ms	118 ms
MS2 AGC TARGET	150%	100%
MS2 RESOLUTION	60k	15k
DYNAMIC EXCLUSION WINDOW	30 s	30 s
PRECURSOR INTENSITY THRESHOLD FOR MS2 SELECTION	1E4 or as specified	5.00E+03

Table S6. Differentially Expressed Proteins between Jurkat and K562

GENE SYMBOL	FOLD CHANGE JURKAT/K562	P-VALUE	BENJAMINI	CANCER DRUG TARGET
ACTN4	2.880209	7.45E-05	0.005588	
AKR1C1	5.710127	0.000661	0.014691	
AP1B1	2.047471	0.004966	0.037996	
BLVRB	3.113584	2.03E-05	0.002345	
CALU	5.820006	0.004056	0.035198	
CANX	2.409926	0.000919	0.016975	
CORO1C	2.988908	0.002743	0.029916	
CS	2.133896	0.002466	0.029693	
CYCS	3.343197	0.000827	0.016975	Yes
ETFA	2.299533	0.008507	0.048204	
ETFB	2.616097	0.000868	0.016975	
FERMT3	2.830516	0.009465	0.049818	
FSCN1	2.21221	0.000993	0.017398	
GRPEL1	2.263446	0.001882	0.025904	
H1-5	0.310965	0.000172	0.008298	
HADHA	3.972428	0.003966	0.035198	
HNRNPL	2.416649	0.005127	0.037996	
HSPA1B	2.507084	0.000648	0.014691	
HSPA9	2.315643	0.006092	0.042384	
IGF2BP1	2.523059	0.000605	0.014568	
IMMT	2.852793	0.002262	0.029049	
KPNB1	2.197344	0.000428	0.013186	
KRT19	4.371127	0.002688	0.029916	Yes
LAP3	2.532185	0.002136	0.028053	
LMNA	2.060674	0.006623	0.043734	
MAPK1	2.381642	0.003169	0.031584	
MDH2	2.917288	0.001643	0.023745	

MRPL12	2.036869	0.007433	0.045217	
NAMPT	2.556784	0.009289	0.049818	
OLA1	2.624457	1.86E-06	0.000536	
P4HB	2.092953	0.002368	0.029125	
PABPC1	2.006101	0.00471	0.037814	
PARK7	2.024723	0.005225	0.03823	Yes
PARP1	3.152437	0.005868	0.041871	Yes
PDIA4	2.491672	1.79E-05	0.002345	
PGD	2.331308	0.002968	0.030609	
PHB2	3.336464	0.001532	0.023297	
PPP2R1A	2.111916	0.000232	0.009564	
PRDX6	2.469661	0.001252	0.020097	
PSMB5	2.332254	0.000164	0.008298	Yes, Acute Lymphoblastic Leukemia
PSMD7	4.662143	0.003018	0.030609	
RNH1	3.719258	0.001119	0.018478	
SLC25A3	2.417702	0.006696	0.043734	
SND1	3.944464	0.000206	0.009137	
SSB	2.526782	0.000332	0.011299	
TUFM	2.875236	0.003774	0.035198	
UBE2D3	3.360602	0.002568	0.029916	
XPO1	4.160989	8.94E-05	0.005742	
YARS1	2.754918	0.007743	0.045637	

Table S7: Example Protocol for Loading from a Vial

Tip Location	Action
Waste Vial	Dispense 3800 nL @ 9000 nL/min
In the air	Aspirate 100 nL @ 9000 nL/min
DMSO vial	Aspirate 3800 nL @ 9000 nL/min
Waste Vial	Dispense 3900 nL @ 9000 nL/min
In the air	Aspirate 100 nL @ 9000 nL/min
Sample Vial	Aspirate 1000 nL @ 9000 nL/min
In the air	Aspirate 100 nL @ 9000 nL/min
Buffer A Vial	Aspirate 2600 nL @ 9000 nL/min

Table S8: Example Protocol for Loading from a Chip

Tip Location	Action
Waste Vial	Dispense 3800 nL @ 9000 nL/min
In the air	Aspirate 100 nL @ 9000 nL/min
DMSO vial	Aspirate 3800 nL @ 9000 nL/min
Waste Vial	Dispense 3900 nL @ 9000 nL/min
In the air	Aspirate 100 nL @ 9000 nL/min
Buffer A Vial	*Aspirate 150 nL @ 9000 nL/min*
Sample Well	*Dispense 150 nL @ 9000 nL/min*
Sample Well	*Wait 30 seconds*
Sample Well	*Aspirate 150 nL @ 9000 nL/min*

	Repeat last 4 steps (*in italics*) 7 times to ensure thorough sample recovery
Buffer A Vial	Aspirate 100 nL @ 9000 nL/min
Sample Well	Dispense 100 nL @ 9000 nL/min
Sample Well	Wait 30 seconds
Sample Well	Aspirate 100 nL @ 9000 nL/min
In the air	Aspirate 100 nL @ 9000 nL/min
Buffer A Vial	Aspirate 2600 nL @ 9000 nL/min