

Turner Lab Notebook

Callum Malcolm

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Wednesday 15-03-2023

Cell Culture

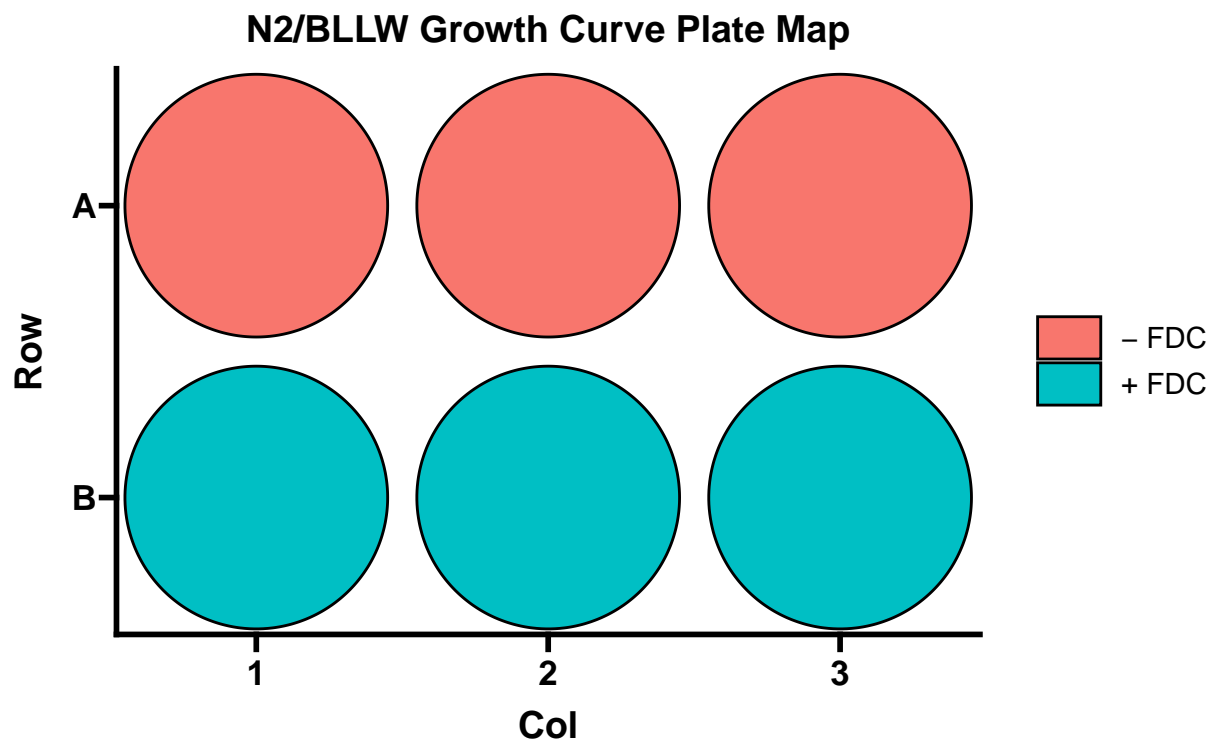
- Given N2/RBL1/BLLW PDX line from Chris Steele

N2 Growth Curve

Looking to compare N2 growth on and off feeders

1. Seeded 2mL of N2/BLLW cell suspension at 0.25×10^6 cells/mL

96 Well Plate Map



Thursday 16-03-2023

N2/BLLW Growth Curve Day 1

- Cells gently mixed
- 30 μ L cell suspension taken and counted
- Only counted 1 square and multiplied by 4

Cell Line	FDC Status	Count	Cell Number
N2	+ FDC	25	2000000
N2	+ FDC	26	2080000
N2	+ FDC	12	960000
N2	- FDC	15	1200000
N2	- FDC	14	1120000
N2	- FDC	6	480000
BLLW	+ FDC	6	480000
BLLW	+ FDC	6	480000
BLLW	+ FDC	5	400000
BLLW	- FDC	3	240000
BLLW	- FDC	3	240000
BLLW	- FDC	6	480000

Friday 17-03-2023

N2/BLLW Growth Curve Day 2

- Cells gently mixed
- 30 μ L cell suspension taken and counted
- Only counted 1 square and multiplied by 4

Cell Line	FDC Status	Count	Cell Number
N2	+ FDC	36	2880000
N2	+ FDC	28	2240000
N2	+ FDC	38	3040000
N2	- FDC	61	4880000
N2	- FDC	38	3040000
N2	- FDC	16	1280000
BLLW	+ FDC	3	240000
BLLW	+ FDC	5	400000
BLLW	+ FDC	5	400000
BLLW	- FDC	1	80000
BLLW	- FDC	0	0
BLLW	- FDC	1	80000

Split Cells

Cell Line	Cells/mL	Cells/mL Seeded	CS volume	Final Volume	Seeded Cells/mL
N2	8.8x10 ⁵	3x10 ⁵ .	6.8 mL	12 mL	3x10 ⁵
RBL1	8.6x10 ⁵	3x10 ⁵ .	11.6 mL	12 mL	5x10 ⁵

Saturday 18-03-2023

N2/BLLW Growth Curve Day 3

- Counted by Chris

Sunday 18-03-2023

N2/BLLW Growth Curve Day 4

- Counted by Chris

Monday 20-03-2023

N2 Growth Curve Day 5

- BLLW were all dead, experiment stopped
- Cells gently mixed
- 30 μ L cell suspension taken and counted
- Only counted 1 square and multiplied by 4

Cell Line	FDC Status	Count	Cell Number
N2	+ FDC	146	11680000
N2	+ FDC	112	8960000
N2	+ FDC	132	10560000
N2	- FDC	112	8960000
N2	- FDC	155	12400000
N2	- FDC	141	11280000

Split Cells

- Split cell lines N2/RBL1/JIRE
- Dirty split 1:2

Seed BLLW

- Given BLLW aliquot from Chris Steel
- Thawed and resuspended in 12 mL media
- Added to T75 pre-seeded with FDC cells (from Chris Steel)

Tuesday 21-03-2023

Large Infection in TC

- N2 Growth Curve stopped

Friday 24-03-2023

Seed Cells - N2/RBL1/JIRE

- Vials given by Chris Steel
- Thawed and resuspended in 12 mL media
- Added to T75 pre-seeded with FDC cells (from Chris Steel)

Tuesday 02-05-2023

Seeding Cells - N2/RBL1/RAMOS

1. Thawed N2, RBL1, and RAMOS into T75 flasks

Note: N2 and RBL1 flasks seeded with feeders

Wednesday 03-05-2023

Split Cells

Cell Line	Cells/mL	Cells/mL Seeded	CS volume	Final Volume	Last day split	P number
N2	8.93×10^5	3×10^5 .	6.71 mL	12 mL	Tues 02-05-23	2
RBL1	7.73×10^5	3×10^5 .	7.76 mL	12 mL	Tues 02-05-23	2

2. Expanded N2-Barcodes to T175

3. Seeded feeders into 96-well

- a. Diluted feeders to 3.2×10^4 cells/mL
- b. Add 100 μ L cell suspension to each well

Thursday 04-05-2023

Monday 19-06-2023

Split Cells

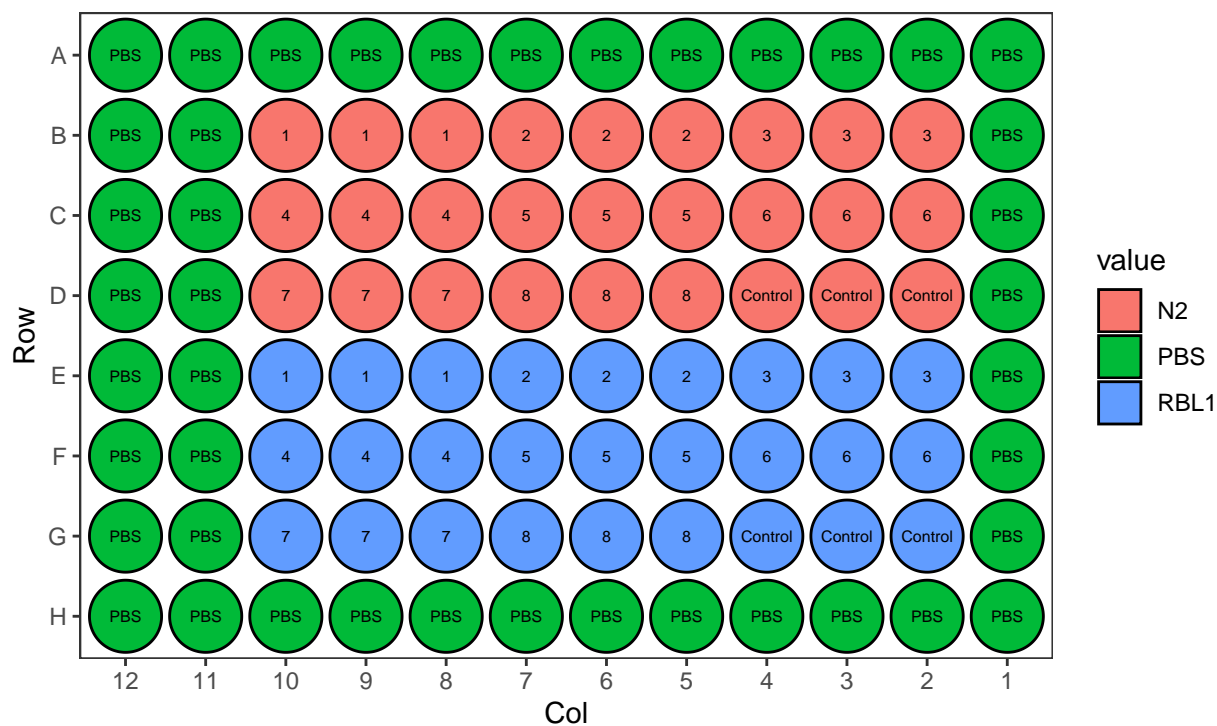
- Split N2/RBL1/JIRE
- Added 10 mL media to BLLW
- * Lots of dead cells observed

Seed EC50

- Plate ID: EC50_230619_Plate1
- Cylophosphamide (Stock: 20mM) EC50 for N2 and RBL1

96 Well Plate Map

EC50_230619_plate1



Drug Dilutions

Drug	Dilution	Concentration	Units	Starting_Concentration
Cyclophosphamide	1	20	uM	20000
Cyclophosphamide	2	2	uM	2000
Cyclophosphamide	3	0.2	uM	200
Cyclophosphamide	4	0.02	uM	20
Cyclophosphamide	5	0.002	uM	2
Cyclophosphamide	6	0.0002	uM	0.2
Cyclophosphamide	7	2e-05	uM	0.02
Cyclophosphamide	8	2e-06	uM	0.002
Cyclophosphamide	9	2e-07	uM	0.0002

Tuesday 20-06-2023

RNA Extraction for SP Test 3 Sample

- Sample collected:
- Low number of cells so used Pico-Pure Kit
- 1 sample (n = 1)

Note before starting:

- Pre-heat block to 42C
- Pre-make DNase treatment master mix (1.1n)

Component	Volume	MM volume
DNase I Stock	5 μ L	5.5 μ L
DNA Digestion Buffer	35 μ L	38.5 μ L

RNA Extraction Pico-Pure Protocol

1. Extract cells with 100 μ L of Extraction Buffer (XB)
 - Resuspend the cell pellet gently by pipetting
 - DO NOT VORTEX
2. Incubate at 42C for 30min
3. Centrifuge sample at 3000xg for two minutes
4. Collect supernatant
 - Can stop here and freeze RNA at 70C
5. Pre-condition the RNA Purification Column
 - Add 250 μ L Conditioning Buffer onto the purification column filter membrane
 - Incubate RNA Purification Column with CB for 5min at RT
 - Centrifuge purification column at 16000xg for 1 min
6. Pipette 100 μ L of 70% Ethanol (EtOH) into cell extract
 - Mix well by pipetting
 - DO NOT VORTEX
7. Add cell extract to column
8. Centrifuge column for 2 minutes at 100 x g and then 16000 x g for 30s
9. Pipette 100 μ L Wash Buffer 1 (W1) into the purification column and centrifuge for 1min at 8000 x g
10. Add 40 μ L of DNase treatment master mix to purification tube
 - Add directly onto membrane
11. Incubate at RT for 15 min
12. Pipette 40 μ L Wash Buffer 1 (W1) onto purification column membrane and centrifuge at 8000 x g for 15 seconds
13. Pipette 100 μ L Wash Buffer 1 (W2) into purification column membrane and centrifuge for one minute at 8000 x g
14. Pipette 100 μ L Wash Buffer 1 (W2) into purification column membrane and centrifuge for two minutes at 16000 x g
 - Check if any wash buffer remains in column and centrifuge again at 16000 x g for one minute to clear all the liquid
15. Transfer purification column to new 0.5mL tube
 - Insert purification column/0.5mL assembly into 1.5mL tube for centrifugation
16. Pipette EB directly onto membrane of the purification column
 - Gently touch tip to surface of membrane to ensure maximum absorption of EB into membrane
 - Use 11 μ L to 30 μ L EB
17. Incubate purification column for one minute at room temperature

18. Centrifuge column for one minute at 1000 x g to distribute EB in column
19. Centrifuge column for one minute at 16000 x g to elute RNA
20. Started PCR and completed Tapestation

Tapestation_230620

High-Sensitivity RNA ScreenTape Protocol

Results

- Inconclusive, proceeded with PCR

Reverse Transcription

Notes Before Starting:

- Label PCR strip tubes
- Thaw RT reagents
- Uses WILDseq specific RT primer
- Dilute RT primer to 2 μ M
 - Do this in a PCR hood
 - **IMPORTANT** Do not mix primers and carefully note which RT primer is added to which sample
- Make RT and ExoI master mixes (MM) before starting
 - MM amounts are 1.1n

Protocol:

Note: Used “rt” protocol in callum folder on thermocycler for heating steps

1. In PCR strip tubes diluted 1-5ug RNA in a total volume of 10 μ L of RNase/DNase-free water
2. Added 1 ul of RT primer
3. Added 1 ul of dNTPs
4. Denatured at 65 C for 5 mins in PCR machine
5. Added straight to ice for 2 mins
6. Spun to get liquid to bottom of tube
7. Prepared master mix of RT enzyme and buffers (Made prior to starting)

Component	Volume	MM volume
5x SSIV Buffer	4 μ L	4.4 μ L
SSIV RT	1 μ L	1.1 μ L
100 mM DTT	1 μ L	1.1 μ L
RNase Out	1 μ L	1.1 μ L

8. Added 7 μ L of RT MM prepared above to each sample and mix
 - Spun briefly to get liquid to bottom of the tube
9. In PCR Machine: incubated at 53C for 10 mins followed by 80C for 10 mins
10. Added 3 μ L of ExoI MM to each sample

Component	Volume	MM volume
Thermolabile Exonuclease I	1 μL	1.1 μL
NEBuffer r3.1*	2 μL	2.2 μL

- Most PCR buffers are compatible
11. In PCR Machine: heat at 37C for 4 mins followed by 80C for 1 min
 - Spin briefly to get liquid to bottom of the tube
 12. Add 1 μL of RNase H and incubate at 37C for 20 mins
 13. Dilute final cDNA 1 in 2 with DNase/RNase-free water

PCR Amplification of cDNA

1. Prepare mastermix of below reagents at 1.1x the number of samples

Component	Volume	MM volume
Q5 Master Mix	25 μL	
P5_R1_fwd (100 μM)	2.5 μL	
P7_rev	2.5 μL	
DNase/RNase H20	13.5 μL	
Q5 polymerase	0.5 μL	
Total Master Mix volume	40 μL	
cDNA from above	10 μL	

2. Perform PCR using the following parameters:

Steps	Time
Step 1: 98C	30s
Step 2: 98C	10s
Step 3: 61C	30s
Step 4: 72C	30s
Step 5: 72C	2 min
Step 6: 12C	Hold

20-25 cycles of steps 2-4

Ran overnight and left at 12C

Wednesday 21-06-2023

Tapestation_230619_SPTTest3_cDNA

[High-Sensitivity DNA ScreenTape Protocol](#)

[Results](#)

* Did not work, bands present but faint and not at 200bp

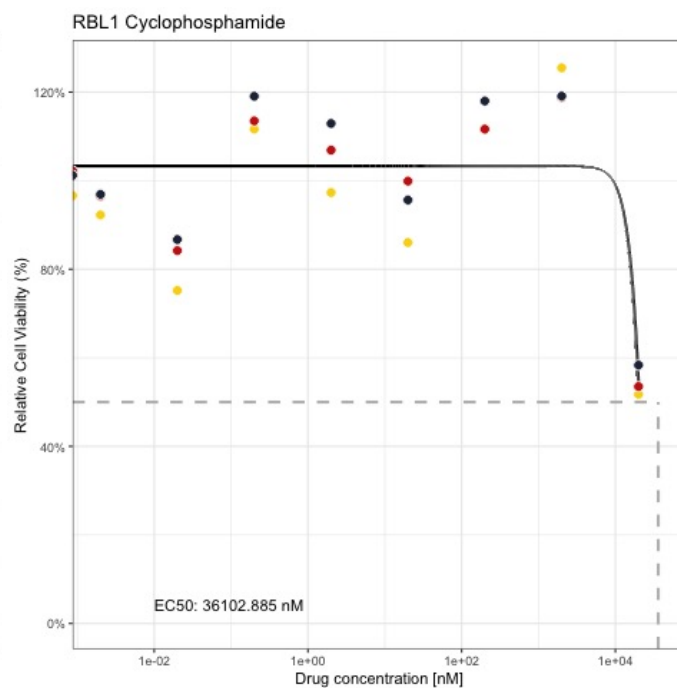
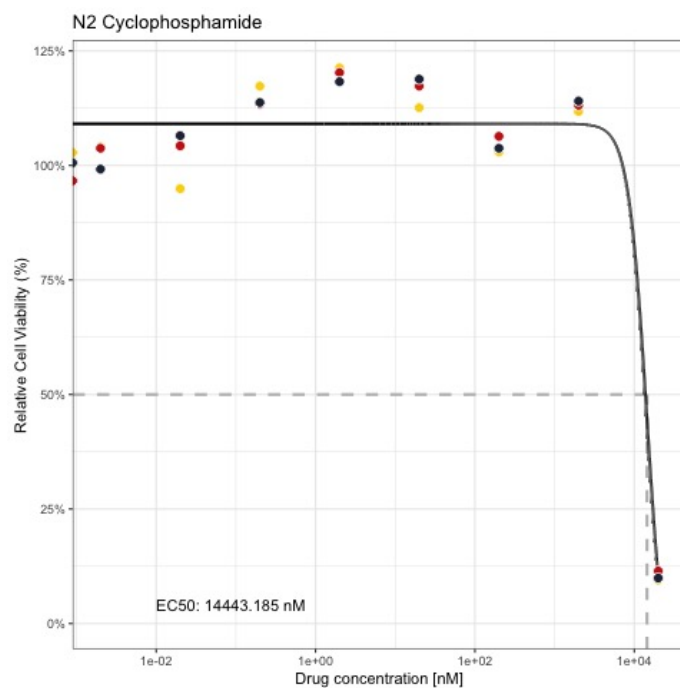
* Potential solutions:

- Repeat PCR with increased cycles
- Repeat RNA extration with trizol protocol (Anna)

Thursday 22-06-2023

Collect EC50 Plate

- Plate ID: EC50_230619_Plate1



Friday 23-06-23

Split Cells

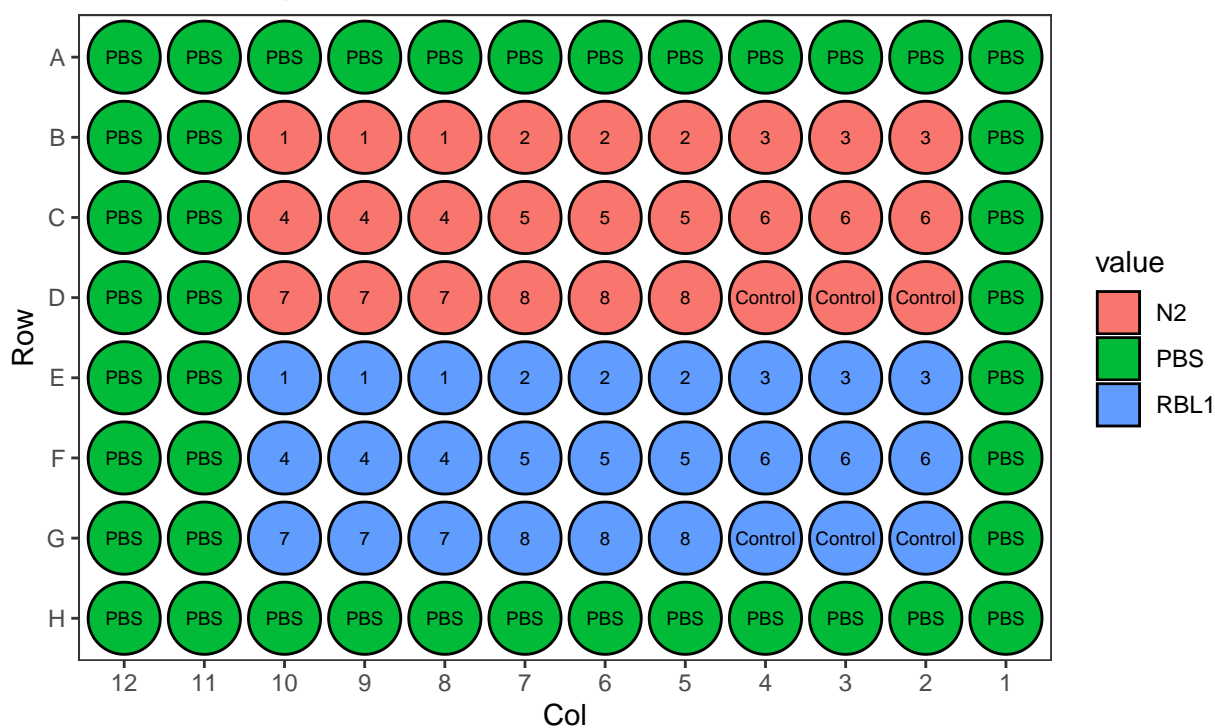
1. Threw out BLLW (all dead)
2. Split JIRE 1/2
3. Split N2/RBL1 1/4

Seed EC50

- EC50 Plate ID: EC50_230623_Plate1

96 Well Plate Map

EC50_230623_plate1



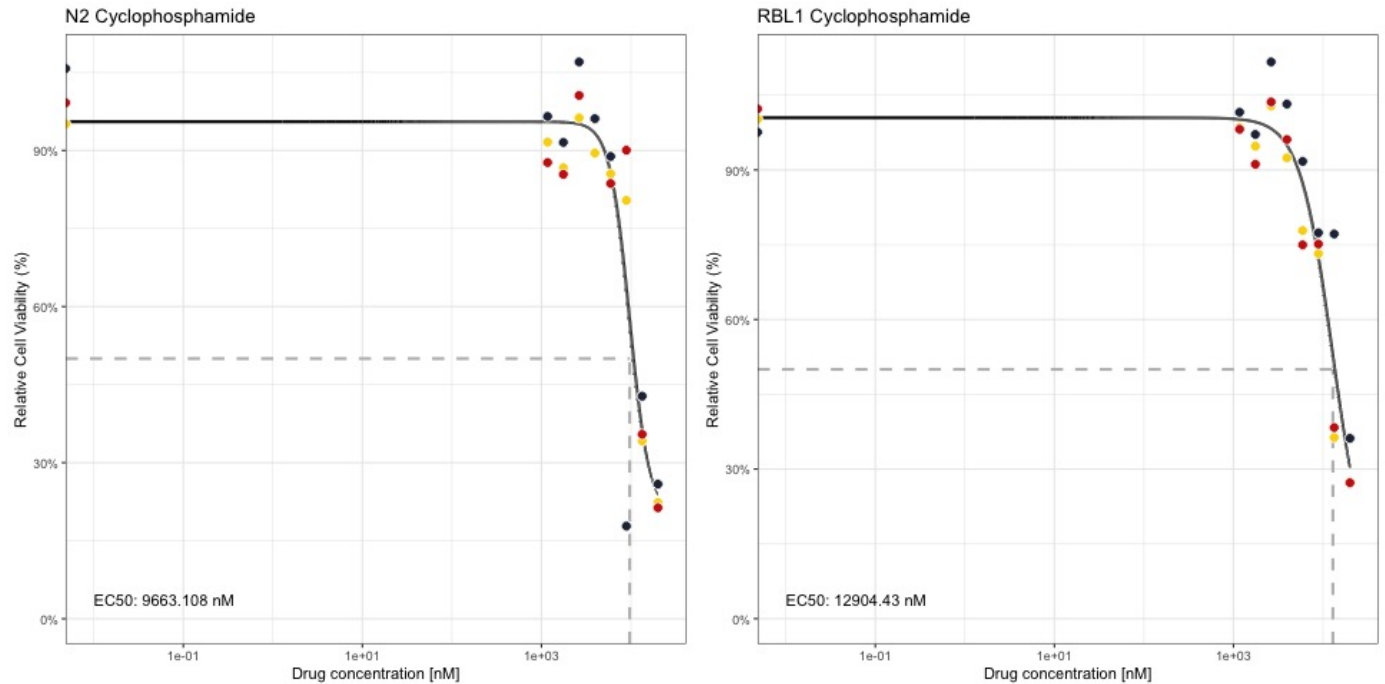
Drug Dilutions

Drug	Dilution	Concentration	Units	Starting_Concentration
Cyclophosphamide	1	20	uM	20000
Cyclophosphamide	2	13.3	uM	13300
Cyclophosphamide	3	8.89	uM	8890
Cyclophosphamide	4	5.93	uM	5930
Cyclophosphamide	5	3.95	uM	3950
Cyclophosphamide	6	2.63	uM	2630
Cyclophosphamide	7	1.76	uM	1760
Cyclophosphamide	8	1.17	uM	1170
Cyclophosphamide	9	0.78	uM	780

Monday 26-06-23

Collect EC50 Plate

- Plate ID: EC50_230623_Plate1



Break for writing MRes Report: 27/06/2023 - 24/07/2023

Monday 24-07-2023

Seeding Cells - RAMOS/FDC

1. Made new media: AR-1

Solution	ID code	Volume	% Total volume
Advanced RMPI	—	500 mL	78%
Glutamax	—	6.5 mL	20%
Pen-Strep	—	6.5 mL	1%
FBS	—	128 mL	1%

2. Seeded Feeders

- 2 vial of FDC cells thawed
- Resuspended in 12 mL of media
- 6 mL of FDC cell suspension were added to 2 separate T75 flasks (Seeding RBL1/BLLW on 25-07-2023)
- Remaining 12 mL of Media were added to T175 (Seeding N2 on 25-07-2023)

3. Seeded Ramos WILDseq Barcode Pool 2

- Thawed a vial of RAMOS pool 2 (previously barcoded DATE)
- Resuspended in 5 mL
- Spun down 90° for 5min
- Resuspended in 12 mL of AR-1 media
- Seeded into T75

Experimental Plans

1. Barcode BLLW/RBL1
2. Set up Drug pressure experiment for RAMOS barcoded cells
 - EC50's for cyclophosphamide and methotrexate need to be completed
 - Drugs to test (EC50):

Drug	Concentration	Replicates
DMSO	1%	3
Doxorubicin	200nM	3
Methotrexate	100nM	3
Vincristine	1nM	3

3. Do Side Population Experiment for N2 Barcoded Cells
 - Grow up sorted side pop to avoid issues with low cell numbers

Tuesday 25-07-2023