Turner Lab Notebook

Callum Malcolm

Contents

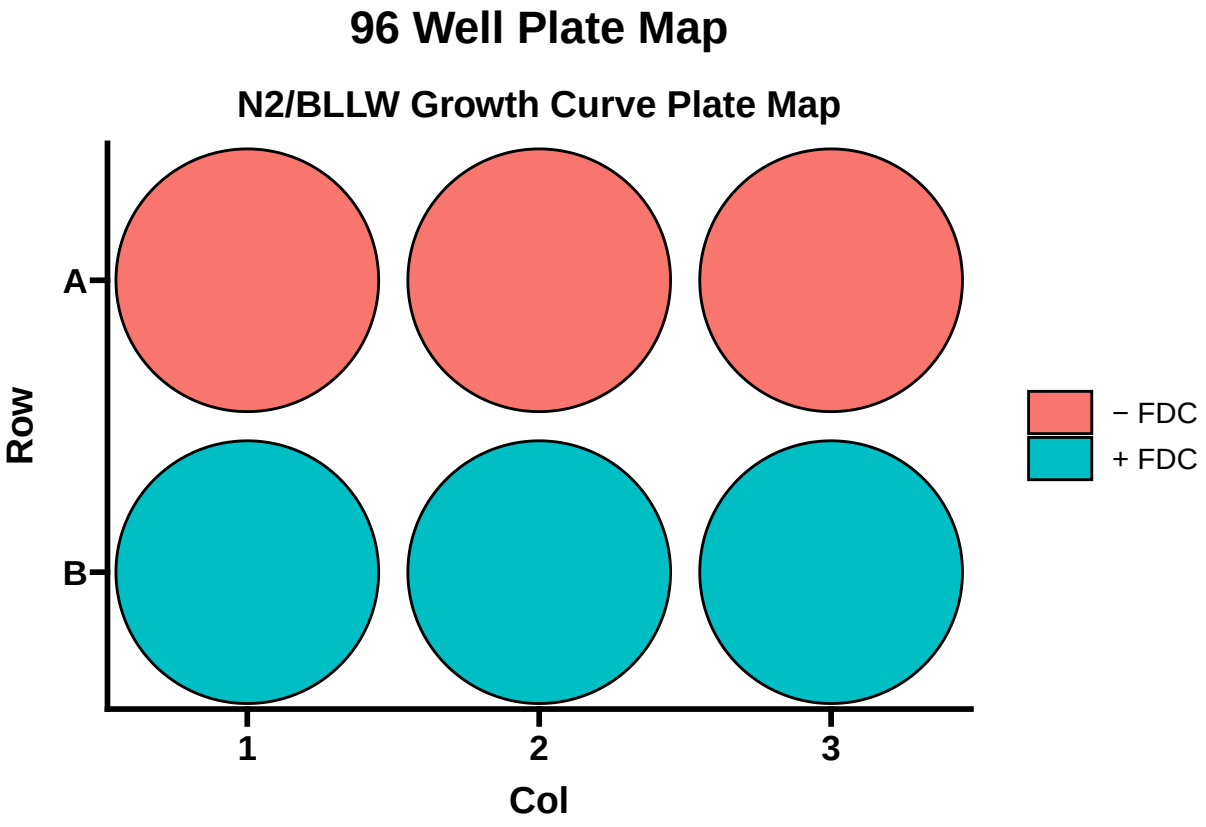
Wednesday 15-03-2023

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 0.25x10^6cells/mL



Thursday 16-03-2023

N2/BLLW Growth Curve Day 1

- Cells gently mixed
- 30 μL 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 μL 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 μL 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)

Monday 27-03-2023

Split Cells

- Split cell lines N2/RBL1/JIRE
- Dirty split 1:3

Wednesday 29-03-2023

Split Cells

- Split by Chris Steel.

Friday 31-03-2023

Split Cells

- Split cell lines N2/RBL1/JIRE
- Dirty split 1:4

Monday 03-04-2023

Large Infection in TC

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)

Seed EC50

DNW - N2/JIRE/RBL1
- Vin/Dactolisib

Wednesday 05-04-2023

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)

Thursday 06-04-2023

Collect EC50 Plate

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- Plate ID: EC50_230619_Plate1
- DNW
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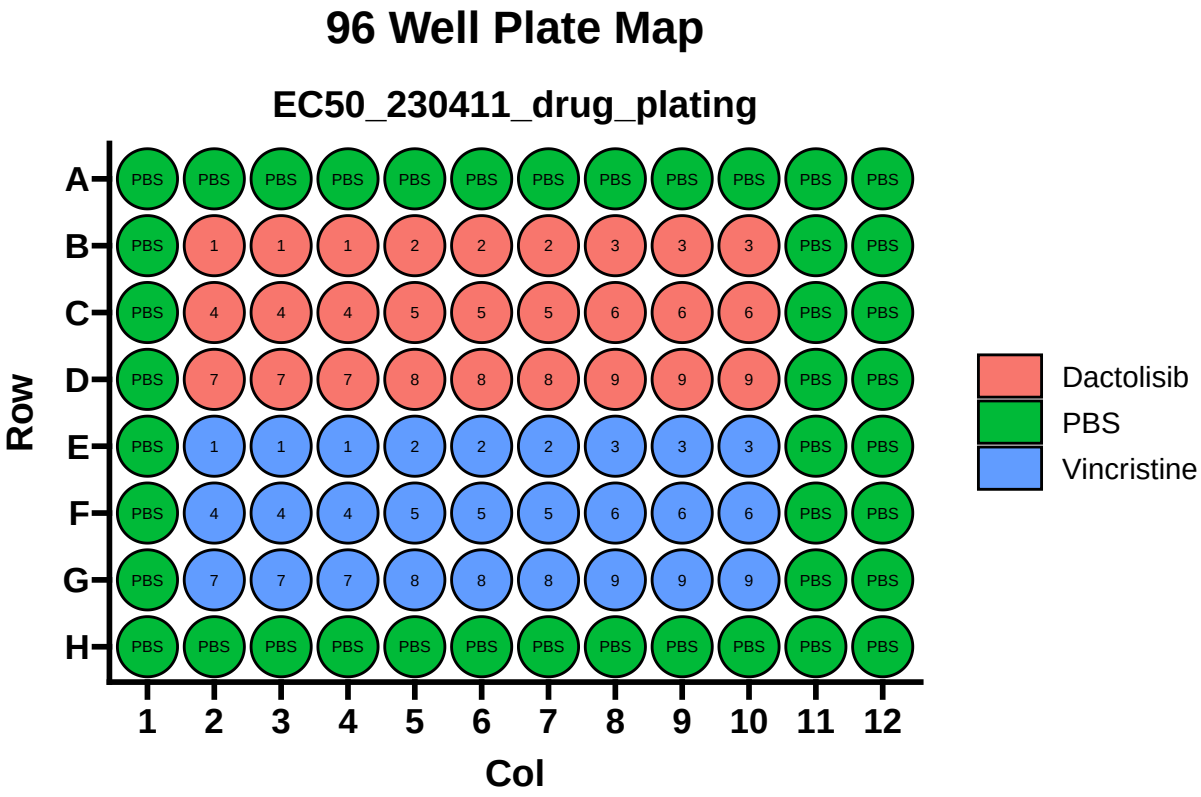
Tuesday 11-04-2023

Seed EC50_230411



Drug Dilutions

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

Drug	Dilution	Concentration	Units	Starting_Concentration
Vincristine	1	30	nM	30000
Vincristine	2	3	nM	3000
Vincristine	3	0.3	nM	300
Vincristine	4	0.03	nM	30
Vincristine	5	0.003	nM	3
Vincristine	6	0.0003	nM	0.3
Vincristine	7	3e-05	nM	0.03
Vincristine	8	3e-06	nM	0.003
Vincristine	9	3e-07	nM	0.0003

- 3 plates seeded
  - Plate 1: N2

  - Plate 2: JIRE
  - Plate 3: RBL1

Wednesday 12-04-2023

Cell Culture

- Thaw RBL1

Side Population - N2_230412

- Hoescht
  - 2.5 / 2.75 / 3
  - DNW

Tuesday 02-05-2023

Seeding Cells - N2/RBL1/RAMOS

1. Thawed N2, RBL1, and RAMOS into T75 flasks
   Note: N2 and RBL1 flaskes 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 100μL cell suspension to each well

Thursday 04-05-2023

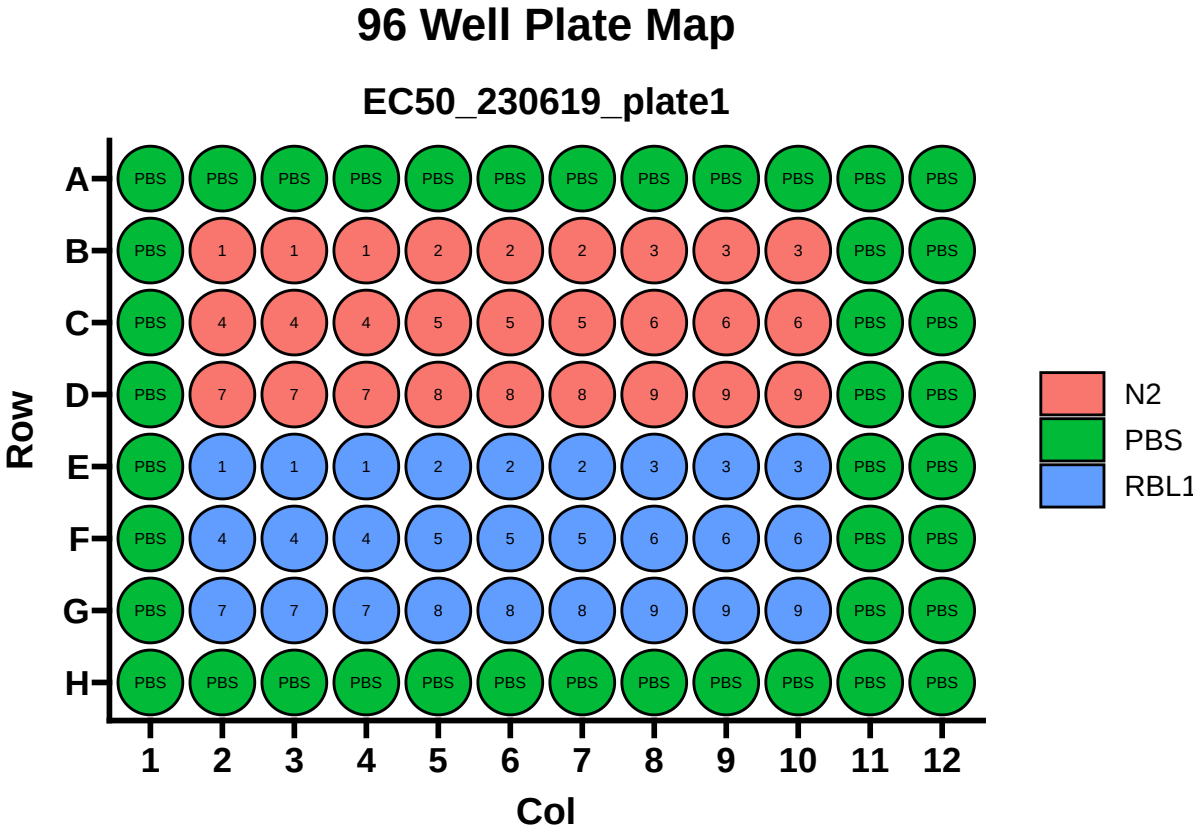
Monday 19-06-2023

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



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

Saturday 20-05-2023

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 μL	5.5 μL
DNA Disgestion Buffer	35 μL	38.5 μL

RNA Extraction Pico-Pure Protocol

1. Extract cells with 100 μL 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 μL 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 μL 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 μL Wash Buffer 1 (W1) into the purification column and centrifuge for 1min at 8000 x g

10. Add 40 μL of DNAse treatment master mix to purification tube

- Add directly onto membrane

11. Incubate at RT for 15 min

12. Pipette 40 μL Wash Buffer 1 (W1) onto purification column membrane and centrifuge at 8000 x g for 15 seconds

13. Pipette 100 μL Wash Buffer 1 (W2) into purification column membrane and centriguge for one minute at 8000 x g

14. Pipette 100 μL Wash Buffer 1 (W2) into purification column membrane and centriguge 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 μL to 30 μL 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 2μM
  - 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 μL 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 μL	4.4 μL
SSIV RT	1 μL	1.1 μL
100 mM DTT	1 μL	1.1 μL

Component	Volume	MM volume
RNAse Out	1 μL	1.1 μL

8. Added 7 μL 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 μL 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 (100μM)	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_SPTest3_cDNA

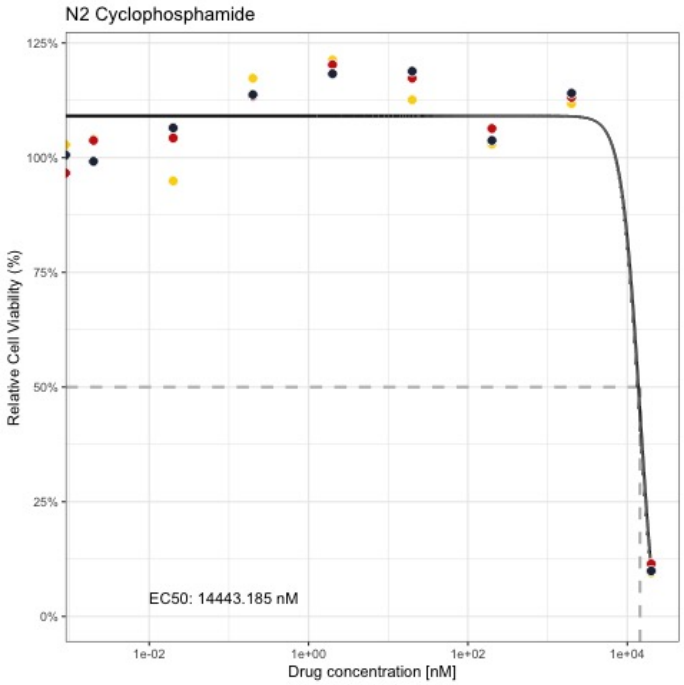
High-Sensitivity DNA ScreenTape Protocol

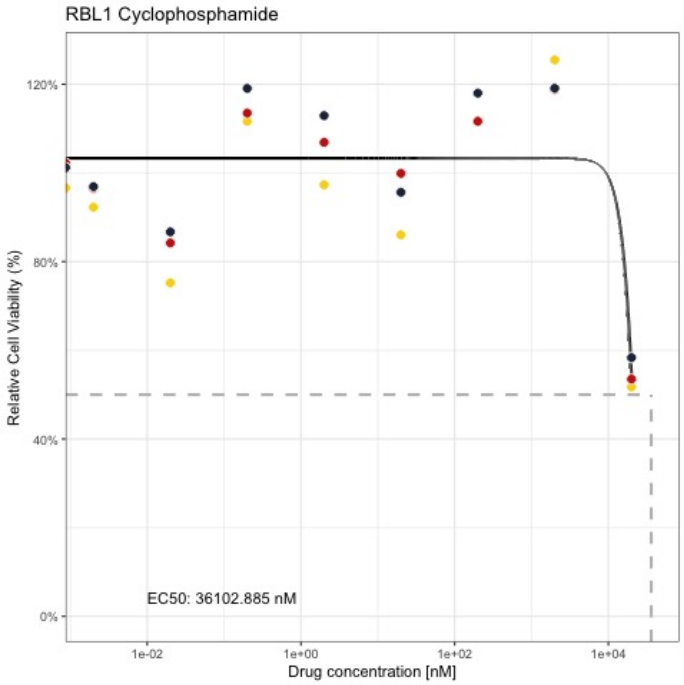
Results
* Did not work, bands present but faint and not at 200bp
* Potential solutions:
- Repeat PCR with increased cycles
- Repeat RNA extration with trizol protocol (Anna)

Thursday 22-06-2023

Collect EC50 Plate

- Plate ID: EC50_230619_Plate1





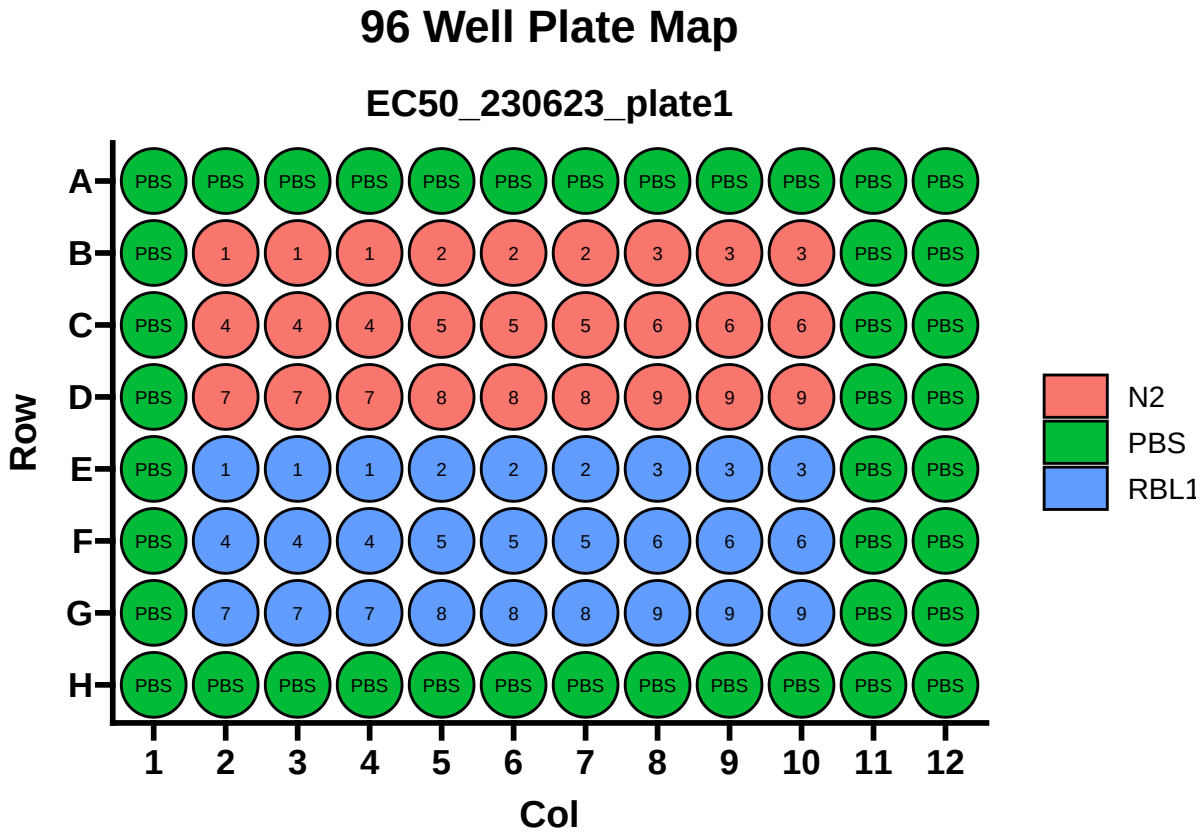
Friday 23-06-23

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



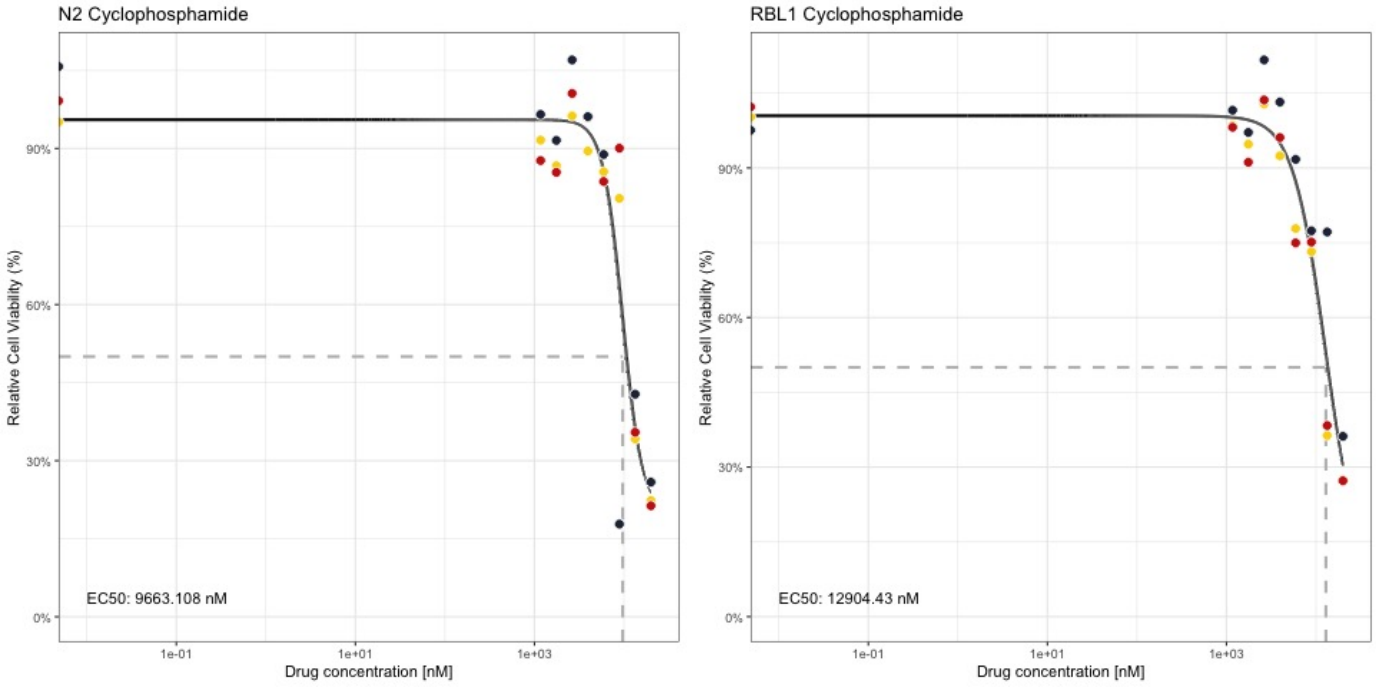
Drug Dilutions

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

Made New Media: AR-1

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 seperate 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 barcorded 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 experiement 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 Experiement for N2 Barcoded Cells

- Grow up sorted side pop to avoid issues with low cell numbers

Tuesday 25-07-2023

Seed Cells - N2-BC4/RBL1/BLLW/RAMOS-BC1

- Got vials from Chris
- Thawed, resuspended in 6 mL AR-1
- Spun down at 90* for 5 min
- Discarded supernatant and resuspended pellet in 12 mL of AR-1
- Added RBL1/BLLW to T75's pre-seeded with FDC (24-07-2023)
- Added RAMOS-BC1 to new T75
  - RAMOS-BC2 from 24-07-2023 was dead
  - Was taken from -80, New RAMOS vial from LN
- Added N2 to T125 seeded with FDC (24-07-2023)

Wednesday 26-07-2023

Cell Culture

- Checked cells, all flasks looked healthy
- Added media
  - N2 - 20ml of AR-1
  - RAMOS - 10 mL of AR-1
  - RBL1 - 10ml of AR-1
  - BLLW - 10ml of AR-1

Thursday 27-07-2023

Split Cells - RAMOS

- Split Ramos 1:2
- Added 10ml of AR-1 to N2
- Plan to split RBL1/BLLW tomorrow

Lucy Drug Randomizer

- Randomized drugs for Lucy Hare mouse experiment

Drug	Identification
Alectinib	A
Erda	B
Vehicle	C
Alectinib + Erda	D

Friday 28-07-2023

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

Friday 06-10-2023

Cell Culture

Made New Media: AR-2

1. Made new media: AR-2

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%

Monday 09-10-2023

Cell Culture

Seeded A20

- Got A20 from Swetha box: 2nd rack in shared -80
- AR-2

Seeded FDC

- Seeded feeders, taken from feeder box, irradiated by Chris
- Will seed N4 PDX tomorrow

Tuesday 10-10-2023

Cell Culture

Seeded N4

- Given by Chris -AR-2
- Seeded onto FDC cells from 09-10
- Future experiments:
  - Barcode PDX
  - EC50: Vincristine/Doxorubicin/Methotrexate/Cyclophosphamide (Active)

Seeded RAMOS-BC

- Seeded barcoded Ramos cells - Pool #4
  - From Chris
  - LN tank 1 - Rack 5 - box 2nd from Bottom
- AR-2
- Future Experiments:
  - Combination Drug pressure: Vin/Dox/Meth

Checked A20

- Look healthy
- ~40% confluency

Wednesday 11-10-2023

Cell Culture

A20 Culture

- ~70% confluent
- Split A20 in half
  - Half in 1xT75 and other half in 1xT175
  - T175 to be frozen down

RAMOS-BC4 Culture

- ~60% confluent
- Spun down at 400xg for 5 min and resuspended in 20ml AR-2

N4 Culture

- Look healthy, attached to feeders with some floaters
- Will add media tomorrow
- Plans:
  - Freeze down aliqouts (2-3x10^6 per vial needed, ideally freeze down 3x vials)
  - Plan barcoding experiment

Thursday 11-10-2023

Cell Culture

Ramos-BC4

- Infected, will need to grab another vial

- Is the Stock infected? Will thaw 2 different pools this time

A20

- 1 x T75 + 1 x T175 both about ~60% confluent
- Media is slughtly turbid, Jamie did a visual check and cells seem clean (A20 have a tendency towards slight turbidity - Jamie)
- Will add 10mL of AR-2 and observe till tomorrow
- If clean:
  - Freeze down 3 vials from T175
  - Set up EC50s with left over cells (Vin/Dox/Meth)
  - Split

N4

- Look healthy, media clean
- About ~40-50% confluent
- Will add 10mL of AR-2 and observe on Friday