N2 - Drug Pressure Plan

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Day-2 - Friday 19-05-2023

1. Seed Feeders Note: Add 2ml of $4.8x10^4$ feeder cells / mL cell suspension to each well of 9x 6-well plates

Day 0 - Satuday 20-05-2023

1. Count N2-Barcode cells and dilute cell suspension to $5x10^5$ cells/ml

2. Add 1mL of cell suspension/well
   Note: Thermofisher indicates that $1.2x10^6$ cells in a 6 well plate is "confluent" and recommends seeding density of $0.3x10^6$. Seeding $0.5x10^6$ means that after 2 days if dividing normally cells should be close to confluent while adding in extra cells to support those in higher concentrations of drug.
3. Make 1μM working stock of each drug
   - Doxorubicin: 1μL 10mM stock in 10mL media
   - Methotrexate: 1μL 10mM stock in 10mL media
   - Vincristine: 333.33μL 30mM stock in 10mL media

Plate Maps

Doxorubicin Dilutions **Note:** Drug will be diluted by 1/2 when added to well
Doxorubicin Working Stock (WS) = 1μM

Drug	Drug ID	Dilution volume	Media Volume	Dil 1[Drug]	[Final Drug]
DMSO	DMSO	1 μL	9999μL	0	0
Doxorubicin	Dox 1	600 μL (from WS)	9700 μL	600nm	300nm
Doxorubicin	Dox 2	5000 μL (from Dox 1)	5000 μL	300nm	150nm
Doxorubicin	Dox 3	5000 μL (from Dox 2)	5000 μL	150nm	75nm
Doxorubicin	Dox 4	5000 μL (from Dox 3)	5000 μL	75nm	37.5nm
Doxorubicin	Dox 5	5000 μL (from Dox 4)	5000 μL	37.5nm	18.75nm

Doxorubicin Plates

Dox Plate 1

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	$5x10^5$	1 mL	DMSO	1mL	0
A2	$5x10^5$	1 mL	DMSO	1mL	0
A3	$5x10^5$	1 mL	DMSO	1mL	0
B1	$5x10^5$	1 mL	Dox 1	1mL	300nm
B2	$5x10^5$	1 mL	Dox 1	1mL	300nm
B3	$5x10^5$	1 mL	Dox 1	1mL	300nm

Dox Plate 2

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	$5x10^5$	1 mL	Dox 2	1mL	150nm
A2	$5x10^5$	1 mL	Dox 2	1mL	150nm
A3	$5x10^5$	1 mL	Dox 2	1mL	150nm
B1	$5x10^5$	1 mL	Dox 3	1mL	75nm
B2	$5x10^5$	1 mL	Dox 3	1mL	75nm
B3	$5x10^5$	1 mL	Dox 3	1mL	75nm

Dox Plate 3

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	$5x10^5$	1 mL	Dox 4	1mL	37.5nm
A2	$5x10^5$	1 mL	Dox 4	1mL	37.5nm
A3	$5x10^5$	1 mL	Dox 4	1mL	37.5nm
B1	$5x10^5$	1 mL	Dox 5	1mL	18.75nm
B2	$5x10^5$	1 mL	Dox 5	1mL	18.75nm
B3	$5x10^5$	1 mL	Dox 5	1mL	18.75nm

Methotrexate Dilutions **Note:** Drug will be diluted by 1/2 when added to well Methotrexate Working Stock = 1μM

Drug	Drug ID	Dilution volume	Media Volume	Dil 1[Drug]	[Final Drug]
DMSO	DMSO	1 μL	9999μL	0	0
Methotrexate	Meth 1	200 μL (from WS)	9800 μL	200nm	100nm
Methotrexate	Meth 2	5000 μL (from Meth 1)	5000 μL	100nm	50nm
Methotrexate	Meth 3	5000 μL (from Meth 2)	5000 μL	40nm	20nm
Methotrexate	Meth 4	5000 μL (from Meth 3)	5000 μL	20nm	10nm
Methotrexate	Meth 5	5000 μL (from Meth 4)	5000 μL	10nm	5nm

Methotrexate Plates

Meth Plate 1

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	5x10^5	1 mL	DMSO	1mL	0
A2	5x10^5	1 mL	DMSO	1mL	0
A3	5x10^5	1 mL	DMSO	1mL	0
B1	5x10^5	1 mL	Meth 1	1mL	100nm
B2	5x10^5	1 mL	Meth 1	1mL	100nm
B3	5x10^5	1 mL	Meth 1	1mL	100nm

Meth Plate 2

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	5x10^5	1 mL	Meth 2	1mL	50nm
A2	5x10^5	1 mL	Meth 2	1mL	50nm
A3	5x10^5	1 mL	Meth 2	1mL	50nm
B1	5x10^5	1 mL	Meth 3	1mL	20nm
B2	5x10^5	1 mL	Meth 3	1mL	20nm
B3	5x10^5	1 mL	Meth 3	1mL	20nm

Meth Plate 3

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	5x10^5	1 mL	Meth 4	1mL	10nm
A2	5x10^5	1 mL	Meth 4	1mL	10nm
A3	5x10^5	1 mL	Meth 4	1mL	10nm
B1	5x10^5	1 mL	Meth 5	1mL	5nm
B2	5x10^5	1 mL	Meth 5	1mL	5nm
B3	5x10^5	1 mL	Meth 5	1mL	5nm

Vincristine Dilutions **Note:** Drug will be diluted by 1/2 when added to well
Vincristine Working Stock = 1μM

Drug	Drug ID	Dilution Volume	Media Volume	Dil 1[Drug]	[Final Drug]
DMSO	DMSO	1 μL	9999μL	0	0
Vincristine	Vin 1	8 μL (from WS)	9992 μL	8nm	4nm
Vincristine	Vin 2	5000 μL (from Vin 1)	5000 μL	4nm	2nm
Vincristine	Vin 3	5000 μL (from Vin 2)	5000 μL	2nm	1nm
Vincristine	Vin 4	5000 μL (from Vin 3)	5000 μL	1nm	0.5nm
Vincristine	Vin 5	5000 μL (from Vin 4)	5000 μL	0.5nm	0.25nm

Vincristine Plates

Vin Plate 1

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	5x10^5	1 mL	DMSO	1mL	0
A2	5x10^5	1 mL	DMSO	1mL	0
A3	5x10^5	1 mL	DMSO	1mL	0
B1	5x10^5	1 mL	Vin 1	1mL	4nm
B2	5x10^5	1 mL	Vin 1	1mL	4nm
B3	5x10^5	1 mL	Vin 1	1mL	4nm

Vin Plate 2

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	5x10^5	1 mL	Vin 2	1mL	2nm
A2	5x10^5	1 mL	Vin 2	1mL	2nm
A3	5x10^5	1 mL	Vin 2	1mL	2nm
B1	5x10^5	1 mL	Vin 3	1mL	1nm
B2	5x10^5	1 mL	Vin 3	1mL	1nm
B3	5x10^5	1 mL	Vin 3	1mL	1nm

Vin Plate 3

Well ID	Well Cell Count	Well volume	Drug	Drug Volume	[Final Drug]
A1	5x10^5	1 mL	Vin 4	1mL	0.5nm
A2	5x10^5	1 mL	Vin 4	1mL	0.5nm
A3	5x10^5	1 mL	Vin 4	1mL	0.5nm
B1	5x10^5	1 mL	Vin 5	1mL	0.25nm
B2	5x10^5	1 mL	Vin 5	1mL	0.25nm
B3	5x10^5	1 mL	Vin 5	1mL	0.25nm

Day 2 - Monday 22-05-2023

1. Visually check cells for viability
2. Count wells which are growing and split back if overconfluent (>1.0x10^6 cells)
3. Replenish drug media

- Collect and spin down supernatant
  Note Add associated drug media to wells while supernatant is being spun down as cells are still attached to feeders
- Resuspend in associated drug media **Note** Repeat drug dilutions from Day 0 except do not make Dil 1 2x [final]

Day 3 - Tuesday 23-05-2023

1. Seed 9x 6-well feeders
2. Seeding density: Add 2ml of $4.8x10^4$ feeder cells / mL cell suspension to each well

Day 4 - Wednesday 24-05-2023

Collection Day 1

1. Homogenize each well thoroughly

2. Take 1/2 of cell suspension for RNA extraction
   a. Spin down for 5 min at 250 x g
   b. Snap freeze in liquid nitrogen

3. Reseed remaining 1/2 of cell suspension
   a. Count cell suspension
   b. Reseed $0.5x10^6$ cells/well (or remaining amount if less than 500K)

Day 6 - Friday 26-05-2023

1. Visually check cells for viability
2. Count wells which are growing and split back if overconfluent ($>1.0x10^6$ cells)
3. Replenish drug media

- Collect and spin down supernatant
  Note: Add associated drug media to wells while supernatant is being spun down as cells are still attached to feeders
- Resuspend in associated drug media
  Note Repeat drug dilutions from Day 0 except do not make Dil 1 2x [final]

Day 7 - Saturday 27-05-2023

Note: This can be done Friday 1. Seed 9x 6-well feeders 2. Seeding density: Add 2ml of $4.8x10^4$ feeder cells / mL cell suspension to each well

Day 8 - Sunday 28-05-2023

Collection Day 1

1. Homogenize each well thoroughly

2. Take 1/2 of cell suspension for RNA extraction
   a. Spin down for 5 min at 250 x g

b. Snap freeze in liquid nitrogen

3. Reseed remaining 1/2 of cell suspension
   a. Count cell suspension
   b. Reseed $0.5x10^6$ cells/well (or remaining amount if less than 500K)

Day 10 - Tuesday 30-05-2023

1. Visually check cells for viability
2. Count wells which are growing and split back if overconfluent ($>1.0x10^6$ cells)
3. Replenish drug media

- Collect and spin down supernatant
  Note: Add associated drug media to wells while supernatant is being spun down as cells are still attached to feeders
- Resuspend in associated drug media
  Note: Repeat drug dilutions from Day 0 except do not make Dil 1 2x [final]

Day 12 - Thursday 1-06-2023

1. Homogenize each well thoroughly

2. Collect entire cell suspension for RNA extraction
   a. Spin down for 5 min at 250 x g
   b. Snap freeze in liquid nitrogen