Date: 9/30/24

Base editing mutagenesis screen of CDK subpools

General Protocol:

1. Transduce ABE or CBE cells with CDK subpool virus (starting with subpool 1) at MOI ≤ 0.3
    a. Performing this at 39,000X to allow for easy splitting
2. Select for successfully transduced cells with blasticidin
3. Allow editing to occur
4. Take t=0 cell pellet
5. Split cells into different drug treatment conditions (2 per drug in triplicate)
6. Harvest cell pellets when resistant cells grow out
7. Extract gDNA
8. PCR amplify sensor cassettes
9. Sequence

Materials:

- Working with A549 and A2780 cells
    - Initial screen being performed in A549 cells
- Cells expressing ABE8e-NG-P2A-Puro or CBE6-NG-P2A-Puro
- Libraries cloned into Trono-BR (blasticidin-P2A-RFP)

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1. **Transduction and screening** (A549 cells)
    a. All transductions are performed in 6-well plates with 2 million cells/well in a total volume of 3 mL of media + virus. No polybrene is added. Puromycin is maintained at 5 ug/mL.
        i. Virus and cells are not pre-mixed. Cells are plated and then virus is added on top.

Amount of Subpool #1 Virus Added (uL in 3 mL total)	RFP+ %
0	0
50	16.7
75	22.6
100	30.6

        ii. Adding 100 uL of virus to the cells
        iii. Library (subpool #1) = 2,730 gRNAs
        iv. There are going to be 13 conditions (6 drugs*2 concentrations + DMSO), so I'm transducing at 13,000X for each replicate, this equals 36 MM cells transduced per replicate.
        v. To achieve this, I need to transduce 120 MM cells/replicate, or 10x 6-well plates with 2 million cells/well (12 MM cells/plate).
    b. 24-hours post-transduction, lift cells and combine the 10X 6-well plates from each replicate. Replate each replicate in a sufficient number of 15-cm plates (15x 15-cm plates/replicate).
        i. **Take a small split from each replicate (and plate in 6-well plate) to assess the transduction % at 72-hours post-transduction to confirm that MOI is ~0.3.**

ii. Maintain puromycin selection at **5 ug/mL**

iii. **Introduce blasticidin selection at 10 ug/mL**

c. **At 72-hours post-transduction, assess the unselected split from each replicate via flow for MOI (RFP+%) calculation.**

i. Note: CBE screen had measured transduction efficiency of 35-38% (slightly higher than expected)

d. 4-days post-transduction, re-plate the cells at ≥2000X representation and continue blasticidin selection.

i. **Note: For ABE screen, ending up changing media with fresh blasticidin- and puromycin-containing media at this time-point because of insufficient cell number.**

e. After 7-days post-transduction, take multiple t=0 cell pellets for each replicate and plate out 13x 1000X 15-cm plates for each replicate. **At this point, also freeze down stocks of the leftover screen cells to allow for a "restart" if anything goes wrong in the screen, or to test different compounds in the future.**

i. **Additionally, run flow at this time-point to check for the RFP+ % of the cells that I'm screening.**

f. After allowing cells to adhere overnight, replace media with 20 mL media containing drug at specified concentration or DMSO (1%) to each plate. **At this point, do not add puromycin or blasticidin to media (**don't want additional cytotoxicity from these).

i. This is performed in 2 steps: (1) add 10 mL of media to cells, (2) add an additional 10 mL of 2X drug containing media to cells.

g. **Every 3 days, replace media with fresh media containing drugs. If necessary, passage cells, maintaining 1000X representation, and taking a gDNA pellet at this time-point.**

i. During passage, take and record cell count for each sample.

ii. **NOTE:** Continue to replate DMSO-containing cells and taking cell pellets until the final plate grows out.

Screening Notes/Modifications:

h. **At day 7 post-treatment in the CBE screen,** drug was removed from the cells that failed to grow out (cells with no gDNA pellet taken at day 14 post-transduction time-point)

i. Initially plated cells in drug at 10/14 split

ii. And then removed drug on 10/15

i. **At day 3 post-treatment (10/18) in first split of ABE screen, concentration of SY-5609 was reduced to 500 nM because of excessive cell death observed for an LC50.**

Drug	Stock Concentration	LC50 Concentration (nM)	uL of stock to add to 60 mL for LC50	LC90 Concentration (nM)	uL of stock to add to 60 mL for LC90
KI-CDK9d-32	1 mM **(make this stock)**	100	6	1,000	60
KI-CDK9d-32N	10 mM	1,250	7.5	5,000	30
KB-0742	10 mM	1,500	9	5,000	30
SY-5609	1 mM	1,000 **ABE Split #1 (10/18): Changed to 500 nM (30 uL)**	60	2,500	150
Senexin B	1 mM	2,000	120	15,000	900
SEL120	1 mM	4,000	240	8,000	480

DMSO	N/A	1%	600		

BLACK = CBE SCREEN

BLUE = ABE SCREEN

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	30 Initiated CBE transduction	**1** Expand cells to 15-cm and start blast selection	**2**	**3** Run flow to check MOI	**4** Split cells, maintaining blast + puro	**5**
6	**7** Take a t=0 cell pellet and plate cells for drug administration Initiate ABE transduction	**8** Add drugs to cells (morning) Expand cells to 15-cm and start blast selection	**9**	**10** Run flow to check MOI	**11** Add fresh media with blast + puro **CBE split 1**	**12**
13	**14**	**15**	**16**	**17**	**18**	**19**

	Take a t=0 cell pellet and plate cells for drug administration **CBE split 2**	Add drugs to cells (morning); CBE drug removed for some cells		**CBE split 3**	**ABE split 1**	
20 **CBE split 4**	**21** **ABE split 2**	**22**	**23** **CBE split 5 (FINAL)**	**24** **ABE split 3**	**25**	**26**
27 **ABE split 4**	**28**	**29**	**30** **ABE split 5 (FINAL)**	**31**	**1**	**2**

Notes/Total Cell counts for CBE SCREEN:

SPREADSHEET LINK:

https://docs.google.com/spreadsheets/d/1PXt-WJ6nyy6XlWEH0L4u2eVn7cbCC3KLu1X_s-hVLls/edit?usp=sharing

- Maintained 10 ug/mL blasticidin and 5 ug/mL puromycin UNTIL day 8, when drug is applied
- 2 cell counts taken for each replicate (that's why there are multiple numbers)
- The below table is how many cells are **plated on that day (includes gDNA pellet to keep track of total cell number):**

Date	Time Post-Transduction	Antibiotic	# plates/ replicate	**TOTAL CELL COUNT** Rep 1	**TOTAL CELL COUNT** Rep 2	**TOTAL CELL COUNT** Rep 3	**REPLATED CELL COUNT** Rep 1	**REPLATED CELL COUNT** Rep 2	**REPLATED CELL COUNT** Rep 3	**RFP + %**
9/30/24	0	Puro	10x 6-well (2M cells/well)	120 MM	120 MM	120 MM				**~35%**
10/1/24	1	Puro;blast	15x 15-cm plates	No count	No count	No count				
10/4/24	4	Puro; blast	15x 15-cm plates	177 MM; 201 MM	184 MM; 190 MM	187 MM; 186 MM	70 MM	70 MM	70 MM	
10/4/24	4		gDNA pellet	120 MM	120 MM	120 MM				
10/7/24	7	Puro; blast	13x 15-cm plates	113 MM; 123 MM	121 MM; 127 MM	117 MM; 111 MM	36 MM	36 MM	36 MM	**83%+**
10/7/24	7		4x gDNA pellet (15 mL falcon)	3 MM x 4	3 MM x 4	3 MM x 4				
10/7/24	7		Leftover gDNA pellet	~40-50 MM	~40-50 MM	~40-50 MM				

			(50 mL falcon)							

Notes/Total Cell counts for ABE SCREEN:

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Date	Time Post-Transduction	Antibiotic	# plates/ replicate	**TOTAL CELL COUNT** Rep 1	**TOTAL CELL COUNT** Rep 2	**TOTAL CELL COUNT** Rep 3	**REPLATED CELL COUNT** Rep 1	**REPLATED CELL COUNT** Rep 2	**REPLATED CELL COUNT** Rep 3	**RFP + %**
10/7/24	0	Puro	6 and 2/3 6-well (2M cells/well)	80 MM	80 MM	80 MM				**~33.5 %**
10/8/24	1	Puro;blast	15x 15-cm plates	No count	No count	No count				
10/14/24	7	Puro; blast	13x 15-cm plates				36 MM	36 MM	36 MM	
10/14/24	7		4x gDNA pellet (15 mL falcon)							
10/14/24	7		Leftover gDNA pellet (50 mL falcon)							