High-Throughput Generation of Knock-Out Cell Line Panels

David Fisher, Caroline Truman, Ben Taylor, Jenna Bradley, Mike Firth and Emanuela Cuomo

Discovery Sciences, IMED Biotech Unit, AstraZeneca, Cambridge, UK

Abstract

The CRISPR/Cas9 system has emerged as an invaluable tool for generating knockout cell lines. Using an easy-to-source guide RNA moiety combined with an RNA-guided endonuclease to generate targeted DNA damage leading to frameshift mutations is now a facile methodology. However, single cell clone generation and validation is still a time consuming, labour-intensive process.

In an effort to adopt a higher throughput, parallelised approach to quickly interrogate whole pathways or cellular processes we looked to repurpose arrayed CRISPR guide RNA screening libraries. Utilising a combination of automation and next generation sequencing we have developed a method for high-throughput generation and validation of panels of knock-out cell lines. Using this technique it is possible to create and validate a large number of clonal genetic knockouts from a single basal cell background in parallel.

Introduction

To evaluate the feasibility of parallel knockout cell line generation we devised a strategy to repurpose an arrayed CRISPR guide RNA library. The library contained pooled crRNAs (4 guides per gene per well) targeting a number of DDR genes (Table 1).

By reverse transfecting a Cas9-expressing cell line we intended to generate pools of cells from which we would subclone to isolate and identify homozygous knockouts.

In order to parallelize the identification, an NGS strategy was employed that identified insertions/deletions (indels) in amplicons spanning the Cas9 cut sites for each gene.

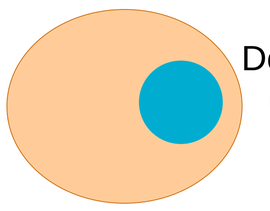
Methods

- A549 cells with a doxycycline-inducible Cas9 cassette stably integrated into the AAVS safe harbour locus were transfected with synthetic Edit-R crRNA:tracrRNA (Dharmacon) using RNAiMAX (Thermo Fisher Scientific)
    - Cas9 expression was induced 72 hours pre-transfection by addition of doxycycline
- 48 hours post-transfection cells were dissociated and single cell sorted using a CellenONE dispenser (Cellenion)
    - 48 cells per gene dispensed
- Cells were screened for clonality using a CellMetric CLD imager (Solentim) and colonies allowed to outgrow
- Replicate plates were generated using a Microlab STAR liquid handler (Hamilton)
- One daughter plate was used to generate samples for NGS analyses whilst remaining plates were cryopreserved

Table 1: 21 DDR Genes in CRISPR Guide Library

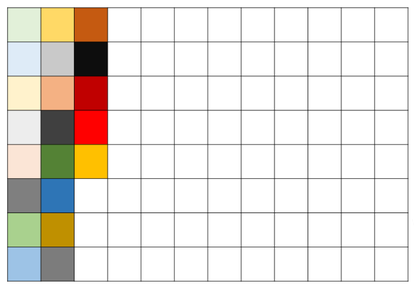
BARD1	CHEK1	RAD51D
BRIP1	FANCA	RAD54L
CHEK2	PALB2	RB1
FANCL	PRKDC	RNASEH2B
PPP2R2A	PTEN	SETD2
ATR	RAD51B	STK11
CDK12	RAD51C	WEE1

Workflow Schematic



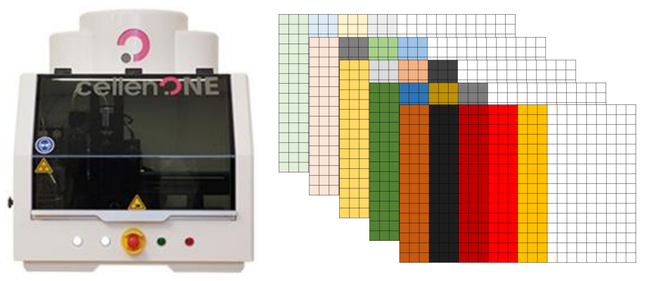
Doxycycline induced Cas9-expressing cell line

Reverse Transfection



Dharmacon Edit-R crRNA pools targeting 21 genes in 96-well plate format

Single Cell Cloning



Clonality Screening

Outgrowth assessment

Plate Replication

NGS **Cryopreserve**

Results

For all genes targeted clonal outgrowth was observed, though survival rates varied (Figure 1 & Table 2).

Figure 1: Clonal Outgrowth

Representative phase contrast image from Cell Metric CLD 2 weeks post-single cell cloning.

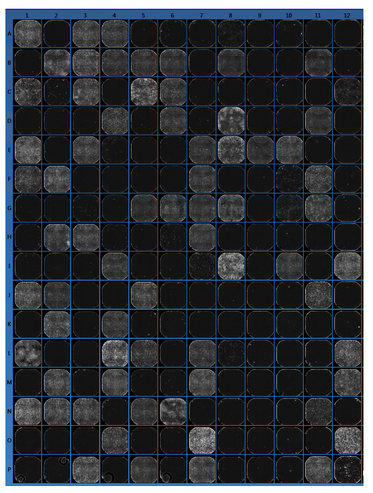


Table 2: Clone Survival Rates

Gene	Total Cells Plated (# potential clones)	Surviving Clones	% clones Survived
BARD1	48	3	6.3
BRIP1	48	26	54.2
CHEK2	16	13	81.3
FANCL	48	26	54.2
PPP2R2A	48	39	81.3
ATR	16	2	12.5
CDK12	48	25	52.1
CHEK1	48	22	45.8
FANCA	48	23	47.9
PALB2	32	17	53.1
PRKDC	48	37	77.1
PTEN	48	42	87.5
RAD51B	48	14	29.2
RAD51C	48	28	58.3
RAD51D	48	37	77.1
RAD54L	48	37	77.1
RB1	48	26	54.2
RNASEH2B	48	35	72.9
SETD2	48	35	72.9
STK11	40	23	57.5
WEE1	48	29	60.4
Average	44	26	57.8%

Homozygous knockout clones were confirmed for all genes assessed by NGS. Of the clones tested, **56%** were found to be homozygous knockouts. However, this likely underestimates the true number of complete knockout cell lines generated, as the NGS methodology cannot determine allelic linkage where indels are generated within different amplicons.

Conclusions

- Arrayed CRISPR screening libraries are suitable for generating knockout cell lines
- By parallelizing the knockout process and utilizing NGS analyses it is possible to generate and identify multiple knockout clones of multiple genes in a much shorter timeframe than traditional methods

Acknowledgements

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