APPLICATION NOTE

Effectiveness of CRISPR-Cas9 using pools of Dharmacon Edit-R synthetic crRNAs in high-content analysis screening experiments.

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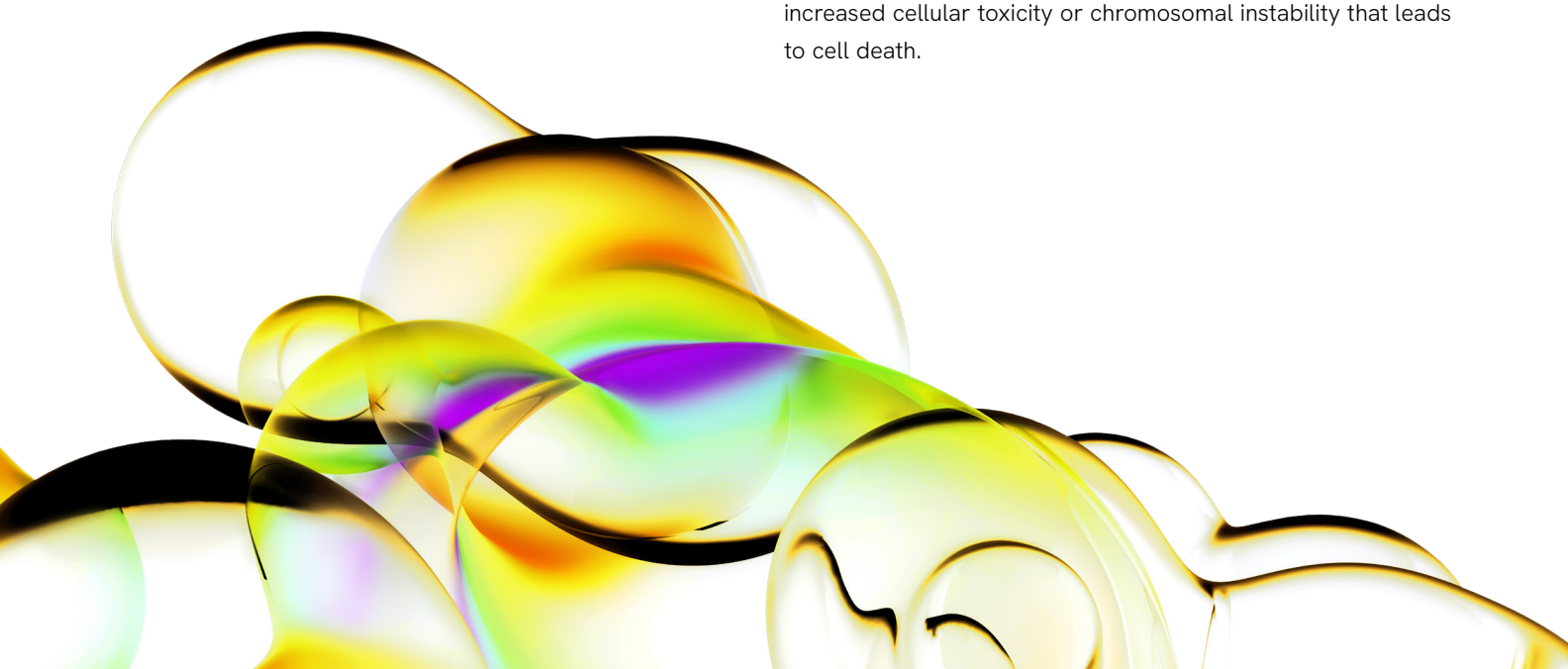
Introduction

Gene knockout using the CRISPR-Cas9 system has emerged as a powerful technology for loss-of-function screening. Although screening using pooled lentiviral sgRNA libraries is a powerful way of discovering gene function[1,2], arrayed screening expands the types of phenotypic readouts from simple population enrichment or depletion to complex multiparametric high-content imaging and morphological assays. Chemical synthesis of guide RNAs for CRISPR-Cas9 gene editing allows for accurate and rapid production of CRISPR libraries and enables screening in an arrayed, one-gene-per-well fashion. Several high-throughput arrayed screens using synthetic crRNA as the CRISPR guide RNA have been published[3-7]. These screens were performed using a single crRNA per well with multiple unique crRNAs per gene for more data points, and therefore increased confidence in hit identification.

However, the pooling of several crRNAs targeting the same gene into a single reagent can simplify the screening process. This approach reduces the number of total wells, which decreases the required amounts of transfection and assay reagents, robotic manipulation, cell culture plates, tips, and storage space. Having several crRNAs to guide the editing of the same gene at different positions could potentially result in more thorough functional gene knockout, therefore leading to a more robust phenotype that would improve hit identification. However, there are several potential concerns for using crRNA pools. Guide RNA off-targets may be additive, causing phenotypic false positive or false negative effects on the phenotype. Multiple genomic DNA breaks may also cause increased cellular toxicity or chromosomal instability that leads to cell death.



Previously we used a cell cycle reporter cell line to perform an arrayed synthetic crRNA screen targeting 169 genes with four individual crRNAs per gene with high content analysis (HCA) to identify genes that regulate the cell cycle[7]. Here we used the same assay to evaluate the phenotypic potency and potential for increased cell death when screening with crRNA pools compared to individuals. We initially tested three genes from the Dharmacon Edit-R Human Cell Cycle Regulation library that were identified as hits in the multiparametric HCA *assay, CCND2, MDM2* and *MYC*, as well as a negative control in the assay, *PPIB*, transfecting four individual crRNAs plus the pool of four. Next, we examined the effect on cell viability when each gene's four crRNAs are pooled for the entire cell cycle library of 169 genes. Finally, we compare the hits identified from the individual arrayed crRNA screen performed in Strezoska et al., to the screen performed with pools of crRNAs.

Results

Comparing the phenotypic effect caused by a pool of four crRNAs targeting previously identified cell cycle hits—proof of concept

To test the functionality of crRNA pools, we used the established method from Strezoska et al.[7]. In this method, the G1S-CPPM-Cas9 cell line stably expressing Cas9 was used in the multiparametric cell cycle phenotypic readout. For the initial proof of concept experiment, medium to strong genes hits were chosen from the cell cycle screen, and included *CCND2* (medium), *MYC* (medium), and *MDM2* (strong). Additionally, we included a *PPIB*-targeting crRNA pool as a negative control for the cell cycle assay phenotype, since it results in double-strand breaks caused by Cas9 but has no relevance in our biological assay. In the initial experiment, each individual crRNA or pools of four crRNAs were complexed with tracrRNA and transfected in triplicate into the G1S-CPPM-Cas9 cell line. Following the method described in the paper, after 72 hours, cells were stained with propidium iodide and Hoechst, and each well was analyzed by the High Content Screening System.

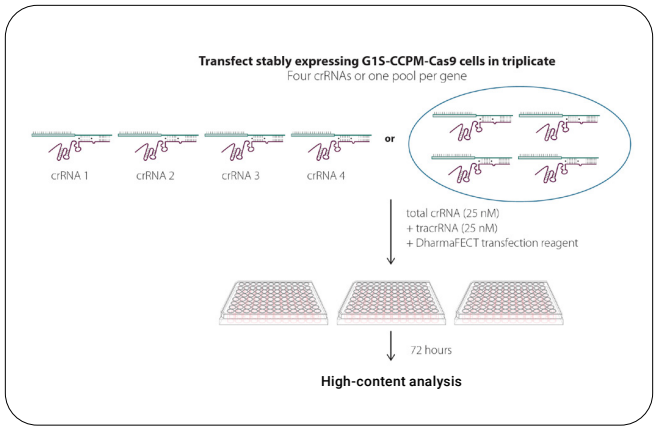


Figure 1: Experimental setup for pooling four crRNAs for phenotypic analysis. Four individual crRNAs or a pool of four crRNAs with tracrRNA were transfected for each gene with into the G1S-CPPM-Cas9 cell line using DharmaFECT 4 transfection reagent. 72 hours after transfections, cells were analyzed by high-content analysis.

The experimental parameters measured were:

- Nuclear size (nuclear area)
- Nuclear shape [Nuc1/(Form Factor)]
- Average nuclear intensity per area (Nuc Intensity)
- Coefficient of variance on the nuclear intensity (Nuc Intensity CV)
- Total nuclear intensity (Integrated Intensity)
- Nuclear vs. cytoplasmic EGFP intensity ratio (EGFP Nuc/Cell Intensity)
- PI nuclear intensity (Reference 1 Nuc Intensity)

These experimental parameters were used to classify each cell into a different cellular state:

Classification	Cellular state
irreg	Cell with irregular shaped nuclei due to either multinucleated cells or large nuclei that could not be segmented well
G1	Cell in G1 phase
S+G2	Cell either in S or G2 phase, due to the poor discrimination between the two phases
M+CC	Cell in mitosis or with condensed chromatin (indicative of either aberrant mitosis or early poptosis)
D/A	Cells that are dead or apoptotic
6n+	Cells with multinuclear DNA component

For the initial experiment, we performed three types of transfections:

1. 25 nM of each individual crRNA with 25 nM tracrRNA
2. pools of four crRNAs at 25 nM of each crRNA and 100 nM tracrRNA (pool 1)
3. pools of four crRNAs at 25 nM total crRNA and 25 nM tracrRNA (pool 2)

The data was compiled into a heat map format and with the color intensity corresponding to the number of standard deviations from the non-targeting (NTC) control, with blue as significantly below the NTC average and red as significantly above the NTC average (Figure 2A). When targeting *PPIB*, our additional negative control for the assay, neither the individual crRNAs nor the crRNA pools caused an effect on the phenotype.

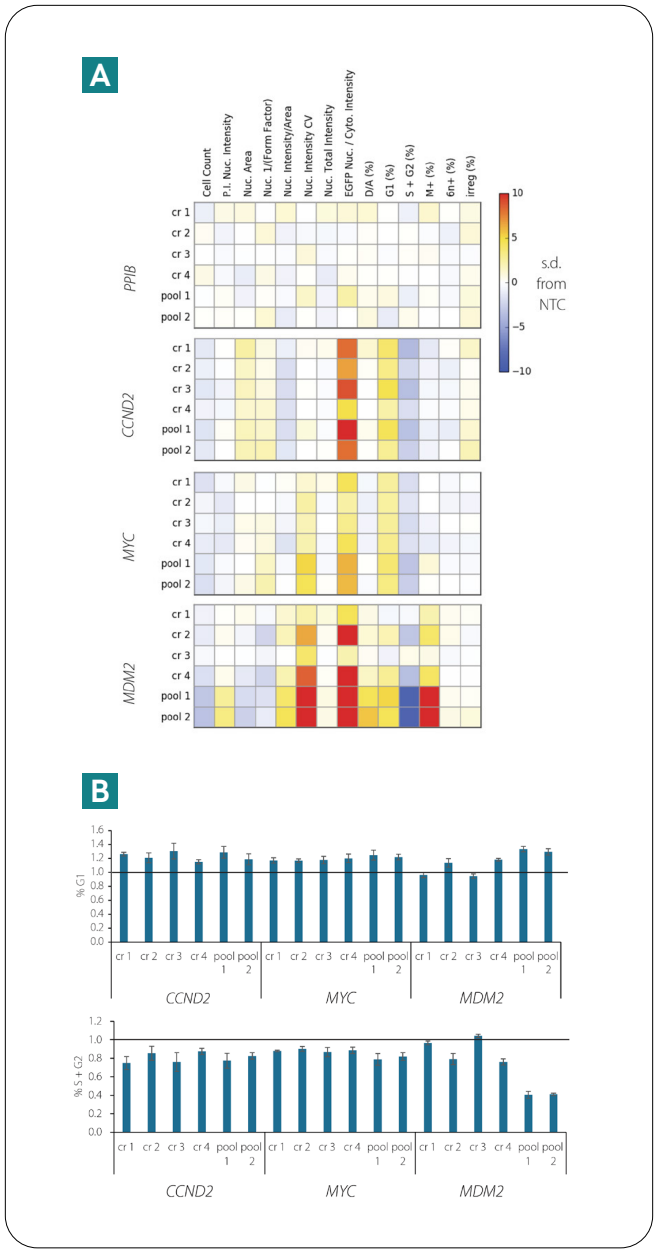


Figure 2: Pools of crRNAs show similar phenotype to individual crRNAs A. Heatmaps depict the standard deviation away from the NTC crRNAs for each high-content analysis parameter. B. The graphs show the percentage of cells in G1 phase (above) or S+G2 phase (below) for each gene when normalized to NTCs (line marked at 1).

For *CCND2*, each of the individual crRNAs caused an increased population of cells in G1 phase and a reduced number in S+G2, but crRNA 1 and 3 had stronger effect than crRNA 2 and 4 (Figure 2B). With the crRNA pools, the phenotype observed was similar to the two strongest crRNAs for *CCND2*. Similar results were seen when targeting *MYC*; the individual crRNAs targeting *MYC* showed a modest increase in the number of cells in G1 phase, and a reduced number in S+G2, while the crRNA pool showed similar phenotypic effect. For *MDM2*, crRNA 2 and 4 had stronger effects than 1 and 3, but the crRNA pool had an additive effect on the phenotype, causing a significantly reduced population in S+G2 phase.

When comparing results from the two different concentrations of the pooled crRNA reagent, we did not observe a negative effect on cell viability at either concentration and we saw no difference in the cell cycle phenotype. Therefore, in further experiments we used the lower total concentration of 25 nM.

Examination of the effect of crRNA pools on cell viability across the entire cell cycle regulation crRNA library

Because our initial experiment was comprised of only four gene targets, our next step was to expand the experiment to a larger gene set to further understand if pooling four crRNAs, which can create four simultaneous double-strand breaks, would have a negative effect on cell viability. We chose to use the Dharmacon Edit-R Human Cell Cycle Regulation library, which includes 169 genes. We pooled the four crRNAs for each individual gene and transfected the G1S-CPPM-Cas9 cell line cells at 25 nM final concentration in triplicate wells. Following the HCA workflow described above, we first looked at the global effect on cell viability by looking at the % dead or apoptotic cells (%D/A). Nine out of 169 genes showed an increase in %D/A cells: *AURKB, TP63, WEE1, CDC20, CHEK1, MDM2, PLK1, RAD51, RBL2* (Figure 3). Except for *RBL2*, the individual crRNAs targeting the same genes had shown an increase of D/A cells by one or more individual crRNAs per target gene[7]. Follow-up work is needed to determine whether the increase of %D/A cells in the *RBL2* pool is a gene specific effect due to increase in editing efficiency or a non-specific effect due to off-targeting.

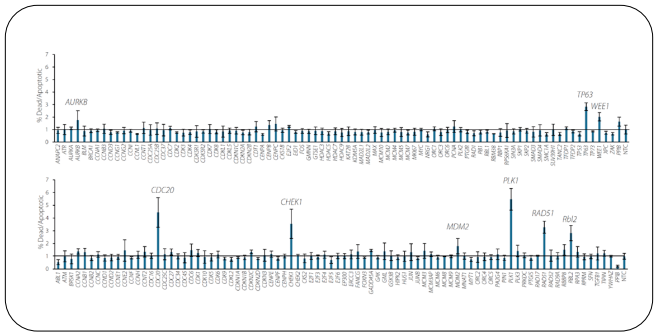


Figure 3: Pools of crRNAs do not increase cell death or apoptosis The graphs show the percentage of dead/apoptotic cells for all 169 genes in the cell cycle regulator synthetic crRNA library.

Comparison of hit identification from an arrayed screen of individual crRNAs or crRNA pools

For hit identification, we used the chi-squared method as described in Strezoska et al. The greater the reduced chi-squared value is for a gene target, the more likely it is a hit. The cutoff for hit significance was chosen as the largest reduced chi-squared value among non-expressed genes (based on RNA-seq data); the threshold was set at > 6 and > 10 for the screen with individual crRNAs and pooled crRNAs, respectively.

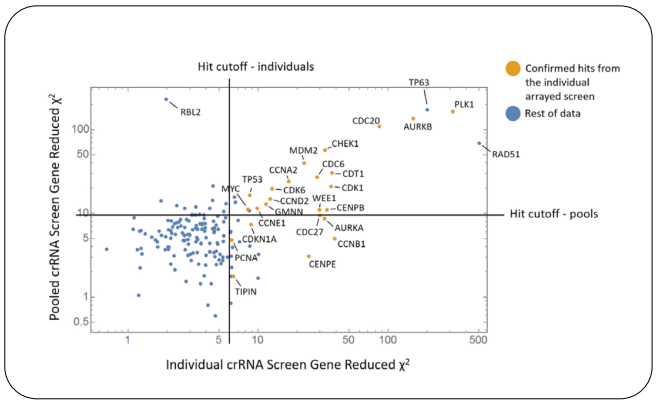


Figure 4: Good correlation between pool of four crRNAs & individual screens identified hits. Chi-squared values from the individual crRNA screen (x-axis) versus the pool of four crRNAs screen (y-axis). Lines within the graph mark the hit cutoffs for each screen.

To compare the results from the individual and pooled crRNA screen, we plotted the chi-squared values from the results against each other (Figure 3). Marked by lines through the plot are the chi-squared values used to signify the cutoffs for hit identification. Genes in the lower left quadrant are non-hits in both screens, while the upper right quadrant are hits in both screens. The genes in the upper left quadrant are hits in the screen using crRNA pools, but not the screen using individual crRNAs, and the genes in the lower right quadrant are hits in the individual crRNA screen, but not the crRNA pool screen. 22 of the 40 gene hits identified with the individual crRNA screen were also identified in the crRNA pool screen. The crRNA pool screen identified > 70% of the validated hits from the screen with individual crRNAs (17 hits out of the 24 identified and reconfirmed hits). In the individual arrayed screen, TP63 and RAD51 each had one crRNA that targeted multiple locations in the genome, which had a toxic effect, so these hits are false positives. Those crRNAs were included in the crRNA pool which showed a similar effect to the single nonspecific crRNA. These crRNAs have since been removed from all collections. Seven hits previously identified and validated in the individual screen were not found in the screen crRNA pools. An additional 14 genes were identified in the crRNA pool screen, but not the crRNA individual screen. Follow-up work is necessary to determine whether these differences represent false positives or false negatives due to differences in functional knockout.

Discussion

Based on the experiments above, we found that pooling crRNAs is as strong as the strongest single crRNAin the pool, or has an additive effect in a phenotypic assay. For genes *CCND2* and *MYC*, we observe that the pools do not increase the phenotype's intensity, but they do for *MDM2*.

We did not observe a major increase of cell toxicity across the cell cycle regulator library when the pool of four crRNAs was used to target the genes for knockout. Only one additional gene out of the 169 genes in the library caused increased cell death/apoptosis that had not been previously identified in the individual screen. This indicates that there is no increased cellular toxicity due to potential additive effect of off- targeting or an additive effect of the multiple DNA breaks at the targeted gene when the pool of four crRNAs is used.

Comparing the individual crRNA screen data with the crRNA pool screen we identified highly similar lists of hits. Of the confirmed hits from the individual screen, 71% were identified by the crRNA pool screen. Even though these screens were run as completely separate experiments, there is a very good correlation between two. However, there were some discrepancies. 14 additional genes were identified in the crRNA pool screen with the hit cutoff method similar to what was used in Strezoska, et al., and seven confirmed hits identified in the individual screen were not identified by the crRNA pool screen. The reduced statistical power of a screen, using one guide RNA reagent per gene instead of four, may account for some of this discrepancy, which also makes it more susceptible to systematic errors. This also suggests there are potential false positives and false negatives associated with the pools of four crRNAs, but this is not a new challenge to researchers carrying out functional genomics screens.

Conclusion

Pooling four crRNAs to the same gene target provides very robust functional knockout in arrayed CRISPR screening experiments. We observe that crRNA pools give a phenotype that is as strong or stronger than any one of the component crRNAs. In looking across results from crRNA pools for each gene in an arrayed library, we do not observe an increase in cell death or apoptosis suggesting that crRNA pools do not have a negative impact on cell viability.

In addition to crRNA pools working successfully in phenotypic screening experiments, this approach also significantly reduces the cost of an experiment. By reducing the number of wells by 75%, the number of consumables is also reduced, including plates, media, lipid reagent, and tips. However, this comes at the cost of reduced statistical power during hit identification and may complicate downstream analysis if validated hits require deconvolution. Similar to siRNA screens, redundancy is important to increase hit confidence, so hits from a screen using crRNA pools should be followed up with the component individual crRNAs to further validate the results. Dharmacon Edit-R crRNA screening libraries are available with four individual crRNAs or one crRNA pool per gene, so there is complete flexibility of format for both a primary screen and follow-up studies.

Materials and methods

Tissue culture: A stably expressing Cas9 cell line was created in the G1S-CPPM U2OS cell line by transducing cells with CAG Cas9 lentivirus. Cells were maintained in RPMI 1640 medium (GE Healthcare Hyclone Cat #SH30096.01) and supplemented with 10% fetal bovine serum (FBS; GE Healthcare Hyclone Cat #SH30071.03), 2 mM L-glutamine (GE Healthcare Hyclone SH3003401), 1 mM sodium pyruvate (GE Healthcare Hyclone Cat #SH30239.01), non-essential amino acids (NEAA) (GE Healthcare Hyclone Cat #SH30238.01) and 10 mM HEPES (GE Healthcare Hyclone Cat #SH30237.01).

Lipid transfection for gene editing: G1S-CPPM U2OS cells stably expressing Cas9 were plated at 2,500 cells/well in a black 96-well plate one day before transfection. Cells were transfected with 25 nM of individual gene targeting crRNA and 25 nM tracrRNA (Dharmacon, Cat #U-002000), or Edit-R crRNA Non-targeting Control #1-4 (Dharmacon, Cat # U-007501-01, U-007501-02, U-007501-03, U-007501-04) using 0.05 µL of DharmaFECT™ 4 Transfection Reagent per well of a 96-well plate (Dharmacon, Cat #T-2004). Pooled crRNA:tracrRNA were transfected at either 25 nM or 100 nM concentration.

Cell cycle assay: To assess cell cycle stage, at 72 hours after transfection, propidium iodide (2 µM) was used to stain apoptotic cells, and Hoechst (6 µM) was used to stain nuclei. Cell plates were incubated for 40 minutes in a 37 °C incubator. Following incubation, cell plates were scanned on the high content screening system.

Cell cycle analysis: The data was analyzed using the reduced chi-squared approach described in Strezoska et al. The chi-squared is directly related to the probability that a gene target is a hit in the screen. The larger the reduced chi-squared, the stronger the hit.

References

1. T. Wang, J. Wei, et al. Genetic screens in human cells using the CRISPR/Cas9 system. Science. 343(6166), 80-84 (2014).
2. O. Shalem, N. Sanjana, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science. 343(6166), 84-87 (2014).
3. E. Anderson, A. Haupt, et al. Systematic analysis of CRISPR-Cas9 mismatch tolerance reveals low levels of off-target activity. J. Biotech. 211, 56-65 (2015).
4. J. F. Hultquist, K. Schumann, et al. A Cas9 ribonucleoprotein platform for functional genetic studies of HIV-host interactions in primary human T cells. Cell Rep. 17(5), 1438-1452 (2016).
5. M. L. Kelley, Z. Strezoska, et al., Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing. J. Biotech. 223, 74-83 (2016).
6. J. Tan, S. Martin, Validation of synthetic CRISPR reagents as a tool for arrayed functional genomic screening. PLoS ONE. 11(12), e0168968 (2016).
7. Z. Strezoska, M. R. Perkett, et al. High-content analysis screening for cell cycle regulators using arrayed synthetic crRNA libraries. J. Biotech. 251, 189-200 (2017).

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