APPLICATION NOTE

Target essentiality assay

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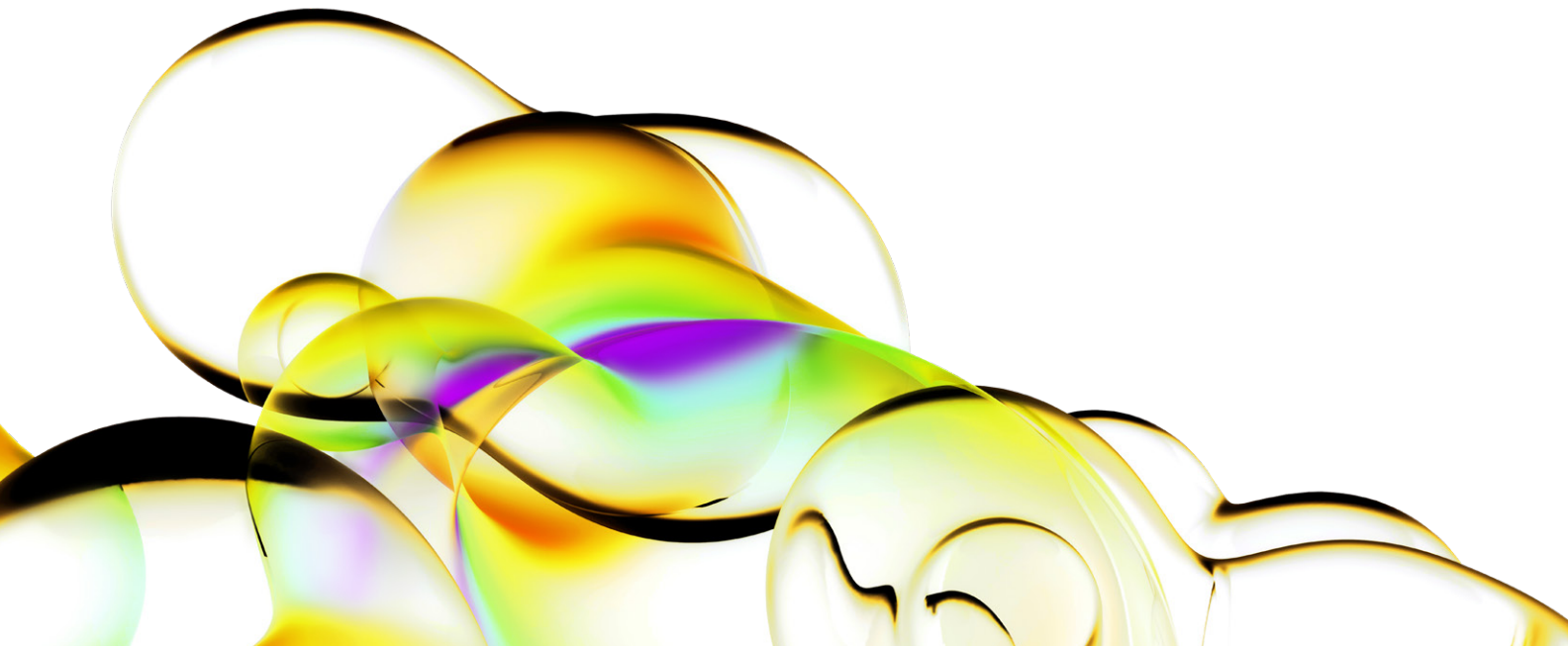
Introduction

Target selection is a crucial step in the successful development of therapeutics. Clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 editing of specific loci offers an alternative method to RNA interference and small molecule inhibitors for determining whether a cell line is dependent on a specific gene product for proliferation or survival. Utilising CRISPR-Cas9 gene editing, we developed an assay to analyse 10s–100s of clones for target gene disruption. Following target gene disruption by CRISPR-Cas-9, cell lines were single cell diluted and clones were analysed for target gene disruption by a restriction digest based assay, which allowed assessment of the presence of editing at the Cas9 cut site, and fragment length analysis, which discriminated between in-frame and frame shift indels. Revvity's target essentiality assay clearly indicates whether or not a specific gene is essential for survival and/or proliferation in a given cell line. Such data can aid the development of more robust cancer therapeutics by increasing confidence in target selection.



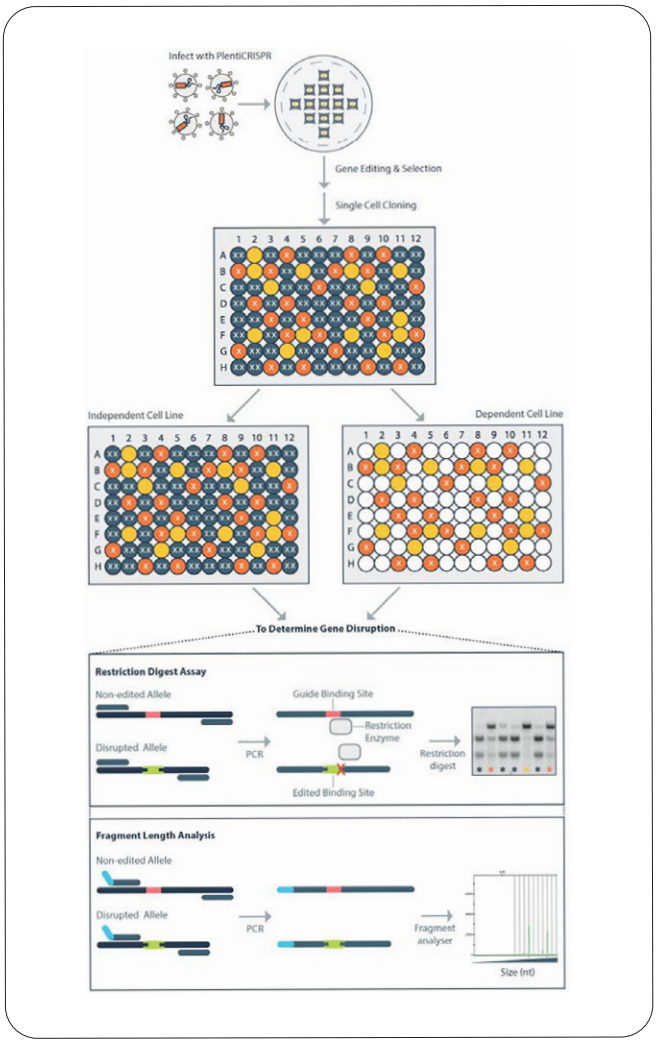


Figure 1: Assay overview

Methods

sgRNA sequence targeting the functional domain of the gene of interest was cloned into pLentiCrispr and lentiviral particles were then produced by transfection of HEK293T cells along with the Virapower packaging system (Life Technologies). One million cells per cell line were infected with virus by spinfection and maintained under selection in the presence of puromycin for at least 14 days.

Cell lines were then plated for single cell dilution in ten 384-well plates per cell line. Growth of colonies from single cells was monitored by imaging with a Cell Metric (Solentim) plate imager every seven days. When colonies had reached sufficient size, they were trypsinised and consolidated into a single 384-well plate for each cell line and lysed in DirectPCR lysis reagent (Viagen).

For restriction digest based assessment of gene editing, the region around the Cas9 cut site targeted by the sgRNA sequence was amplified by PCR, and the PCR products were digested with a restriction enzyme whose recognition site directly covered the Cas9 cut site. Digested PCR products were resolved on agarose gels and assessed for the genotype of disruption of the Cas9 cut site (homozygous edited, heterozygous or unedited).

Fragment length analysis was performed on a portion of colonies by PCR amplification of the same region as for the restriction digest analysis but using a FAM labelled primer. Capillary electrophoresis was performed on PCR fragments (Source Bioscience) and their length analysed using Genemapper 5 software (Applied Biosystems).

Results & discussion

The restriction digest assay allowed determination of the frequency of the different genotypes of editing at the Cas9 cut site, with the expectation being that a cell line that was dependant on the target would show a predominance of clones that retained at least one unedited allele.

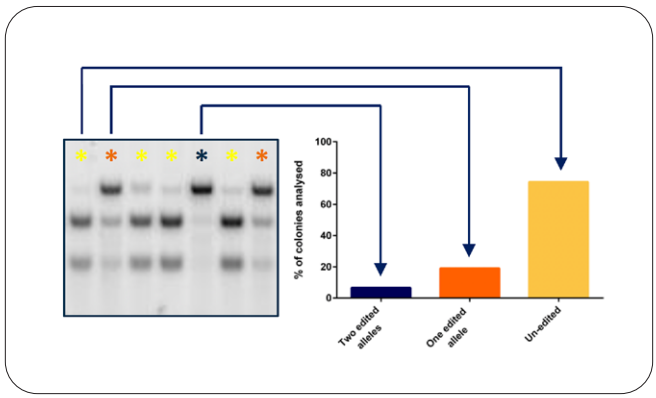


Figure 2: Example of classification of editing genotype by restriction digest assay. Disruption of a restriction site covering the Cas9 cut site targeted by the sgRNA allows determination of the editing genotype of the targeted alleles.

The restriction digest assay allows efficient evaluation of large numbers of colonies but will not distinguish editing events where protein function may not be affected, such as in the case of small in-frame indels. In order to address this we developed a second assay based on the length of PCR fragments covering the targeted region. By comparison to the length of a wild-type fragment, the nature of the indel, regarding whether it is in-frame or not, could be assessed.

By combining the data from both assays, it is possible to evaluate the nature of viable editing events of the target in a specific cell line and hence make a call on target essentiality in that cell line.

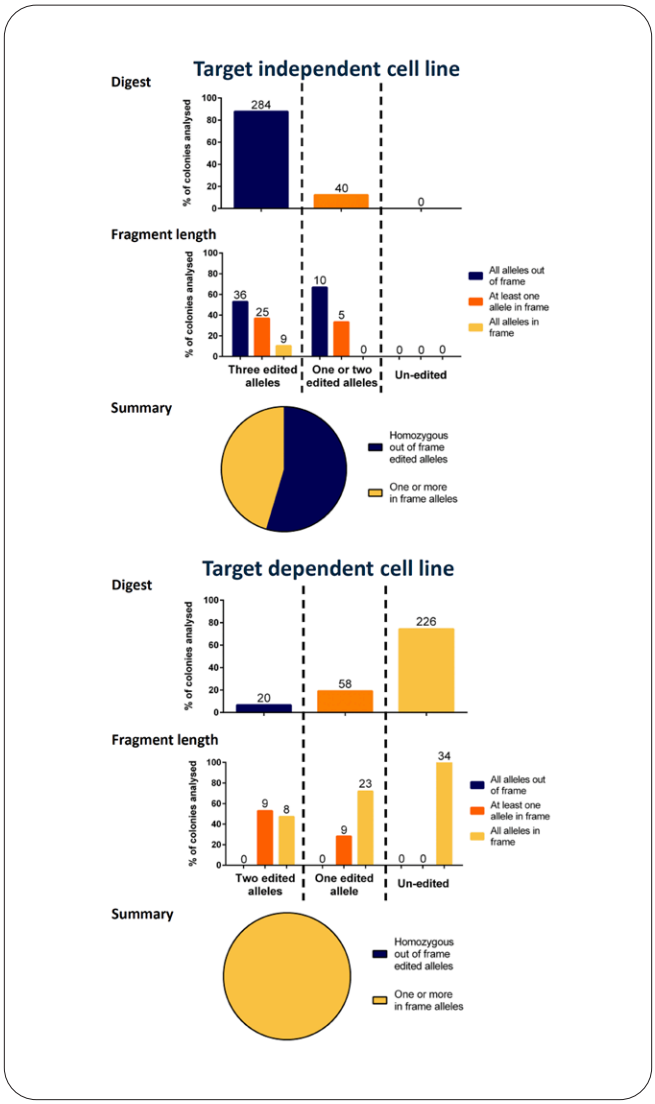


Figure 3: Combined data from restriction digest and fragment length assays. In the target independent cell line the majority of clones have all alleles edited and a significant proportion of surviving clones show out of frame editing of all alleles. This is in direct contrast to the target dependant line which shows a very small proportion of clones in which all alleles are edited, and of these none of the surviving clones show out of frame editing of all alleles.

Conclusions

We have developed a novel medium-throughput assay to analyse gene essentiality in cell lines that are amenable to single cell dilution cloning. This method should aid both the understanding of whether the protein from a target gene of interest needs to be completely absent for a phenotypic effect or whether reduced expression is good enough to induce the phenotype of interest. Such data will support the development of more robust cancer therapeutics.

Revvity support

Revvity offer the target essentiality assay described here as an off-the-shelf Target Essentiality Assay which includes:

- Disruption of the gene of interest in four cell lines from our list of suitable cell lines using pre-validated guides (additional cell lines can be tested for suitability at extra cost)
- Single cell dilution and culture of colonies from single cells
- Analysis of gene disruption status by restriction digest assay and fragment length analysis
- Evaluation of the frequency of in-frame mutations

Alternatively the target essentiality assay can be tailored to your needs in a bespoke fashion.

Revvity offer a range of services and products utilising CRISPR-Cas9 technology, such as sgRNA pooled library screens and X-MAN® isogenic cell lines.

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