HORIZON DISCOVERY

Application Note

RNAi in Target Identification and Validation

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INTRODUCTION

Genetic screening based on loss-of-function phenotype analysis is a powerful tool in both Target ID and Validation. RNA interference (RNAi) remains an established and efficient technology in this arena. Horizon has developed a functional genomic screening and validation platform based on the use of siRNA. Here we present an overview of one of our synthetic lethality studies, from the primary screen to target deconvolution and secondary evaluation.

We used our XMAN® isogenic cell lines for screening, where the mutant cell line differs only in the alteration of a single gene found frequently mutated in cancer. This system provides an exceptionally clean baseline for primary screening and is a valuable model system for target discovery. For primary screening we use a druggable genome library, which targets ~2200 genes of high interest in oncology (Figure 1).

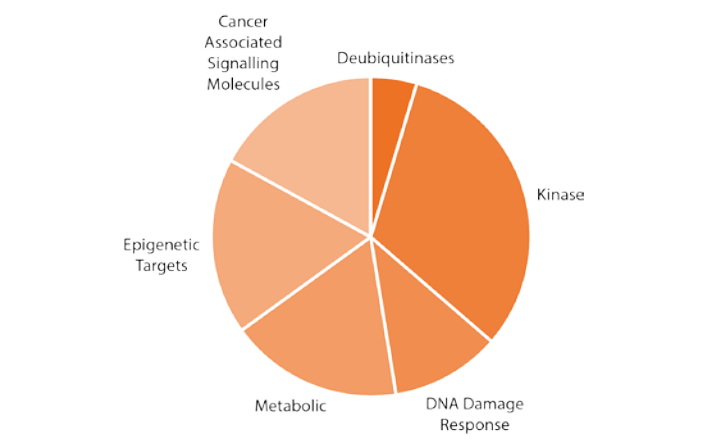

Figure 1. Composition of Horizon Discovery's druggable genome siRNA library. This arrayed library uses SMARTpools targeting 2175 unique genes in seven broad gene classifications.

RESULTS & DISCUSSION

Primary screening was conducted under extensively optimised conditions using a pair of Horizon's isogenic cell lines. Following plate normalisation, synthetic lethal hits were identified by examining the relative viability of cells following siRNA treatment in the mutant genotype compared with the paired wild-type cell line.

This process yields a synthetic lethality score, which we used to rank and stratify hits. Selected hits were then triaged through a confirmation, deconvolution and validation cascade (see Figure 2).

In this screen, we identified a number of promising synthetic lethal candidates (Figure 3). Hits were nominated through a combination of unbiased and subjective query of available data and literature evaluation, including the druggable potential of the targets.

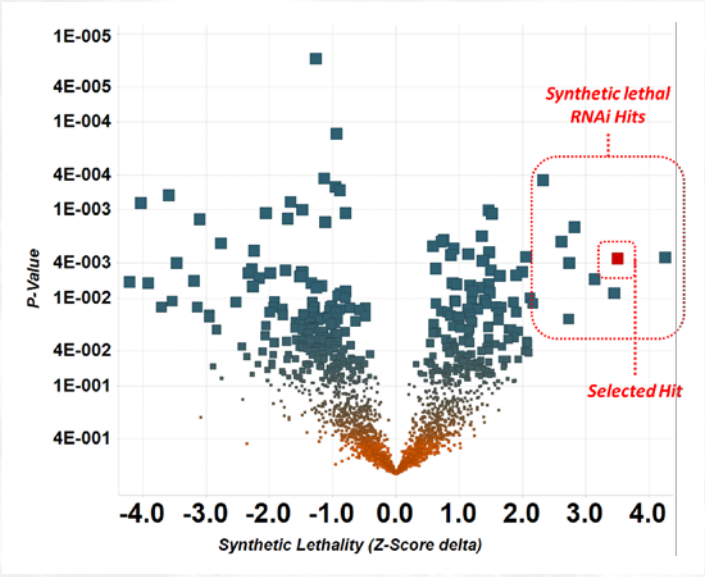

Figure 3. Primary screen data and hit identification. P-values for each hit are plotted against the magnitude of synthetic lethality.

Hit confirmation was conducted in parallel with a medium-throughput analysis that allowed simultaneous determination of hit deconvolution and viability analysis in 2D growth; RT-qPCR analysis of mRNA knockdown; western blot analysis of protein knockdown; and viability analysis in 3D growth.

This partially-automated, medium-throughput programme provided a rapid workflow for the collection of multiple datasets for multiple primary screen hits. In this app note, we discuss the data derived for only one of these hits as an example.

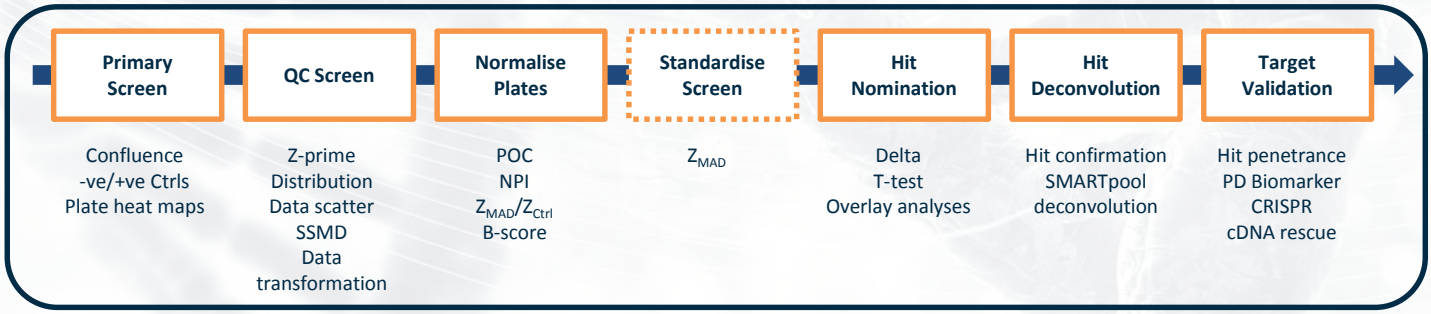

Figure 2. Hit identification from primary screen to target validation

Deconvolution of the SMARTpool revealed that each of the individual reagents targeting this hit contributed to the overall phenotype observed for the pooled reagent, but that knockdown with reagent D2 showed the greatest effect.

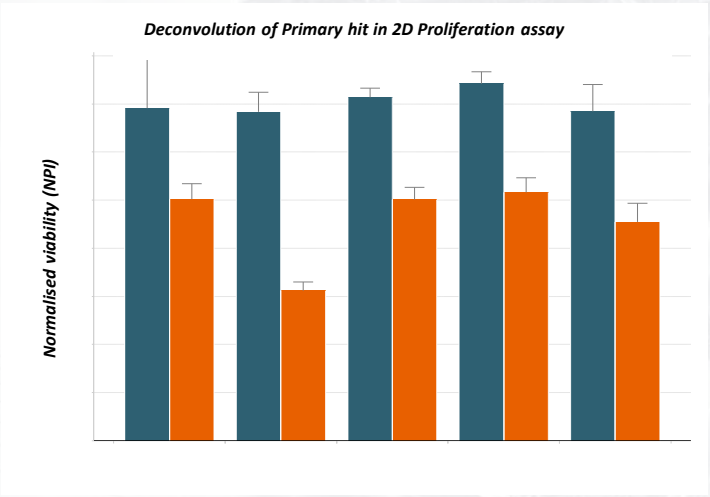

Figure 4. 2D phenotypic assay for deconvolution of SMARTpool regents. Parental wild-type cells are shown in blue, with the mutant isogenic clone in orange.

As part of the combined confirmation and validation assay, an expanded panel of reagents targeting this hit was prepared, using siRNAs from additional vendors and design algorithms to confirm the effect. The phenotype of the knockdown in the isogenic cell pair was then evaluated in 3D culture. This assay allows us to examine the phenotypic effect of knockdown in a condition which more closely mimics *in vivo* cell growth. This assay can provide insights into therapeutic problems associated with metabolic and oxygen gradients commonly found in tumours and also to directly address cell–cell and cell–matrix interactions that may influence chemoresistance mechanisms.

For this hit, we found that the synthetic lethal phenotype observed in 2D growth culture was exacerbated when the cells were grown in a soft agar 3D assay (Figure 5).

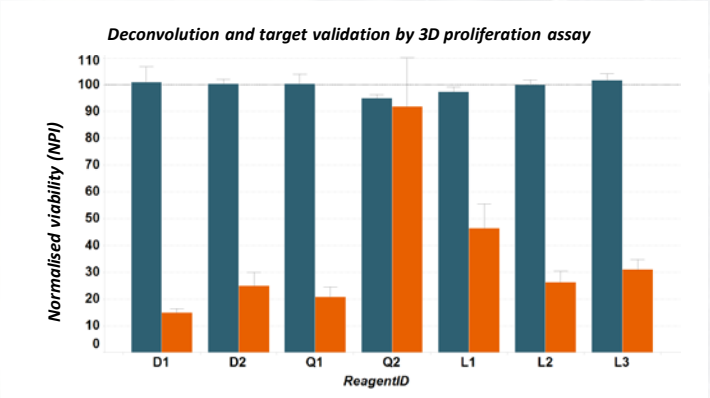

Figure 5. 3D phenotypic assay in soft agar. Parental wild-type cells are shown in blue, with the mutant isogenic clone in orange.

For one siRNA reagent (Q2), no significant phenotype was observed in the 3D growth assay. When the knockdown of this target was analysed by RT-qPCR we found that this reagent was the least efficacious at depleting the mRNA (only ~30% knockdown, Figure 6), corresponding directly with the observed phenotype.

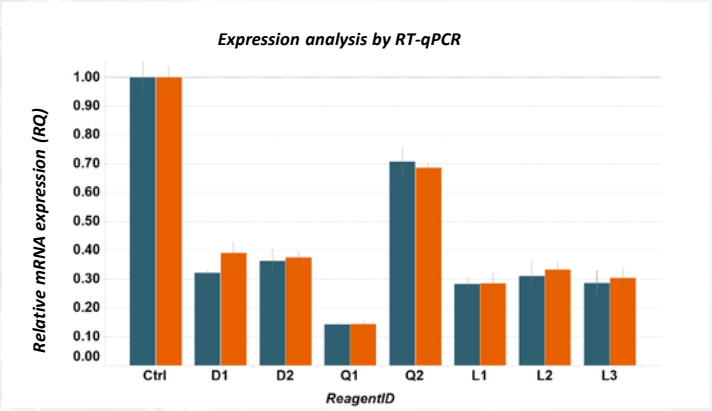

Figure 6. Analysis of knockdown using RT-qPCR to evaluate mRNA expression levels. Parental wild-type cells are shown in blue, with the mutant isogenic clone in orange.

The knockdown levels of the protein (Figure 7) correlate well with the mRNA and the observed phenotypic effect. An established biomarker for the target was also evaluated by western blotting. Knockdown of the synthetic lethal target resulted in a striking concordant loss of this biomarker in both parental and mutant cells (Figure 7). We are now evaluating this promising target in greater detail.

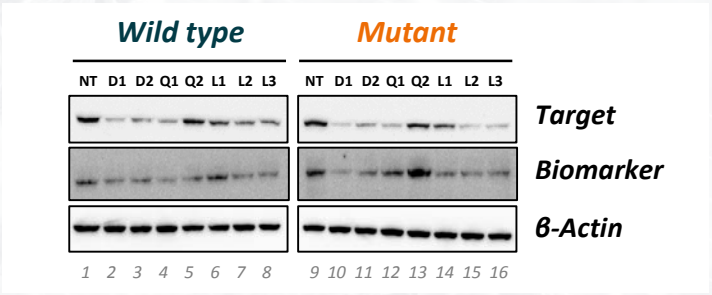

Figure 7. Western blot analysis of target knockdown using and effect on biomarker.

METHODS

All isogenic lines were created using rAAV-mediated homologous recombination. RNAi screening was conducted using Horizon's druggable genome library of Dhamacon siGENOME SMARTpool reagents. Secondary screening used Dhamacon deconvoluted siGENOME reagents, Qiagen Flexitube siRNA reagents and Ambion (LifeTech) Silencer Select siRNA reagents. Proliferation was monitored using either Hoechst staining or AlamarBlue reagent (LifeTech). RT-qPCR analysis of mRNA was with LifeTech TaqMan assays and 3D growth was measured using soft agar assays in medium throughput experiments.

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