APPLICATION NOTE

Dual-guide CRISPR Cas9-knockout screening for gene-gene interaction analysis and beyond.

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Introduction

Genetic redundancy and compensatory pathways pose significant hurdles in the drug discovery and development process. When a single gene is targeted, these pathways often step in to maintain cellular function, which can lead to treatment resistance. However, the dual guide CRISPR knockout screening method addresses this challenge by enabling the simultaneous knockout of two genes, thereby revealing synthetic lethal interactions and compensatory mechanisms that can remain hidden in traditional single-gene approaches.

This application note describes an optimized dual-guide CRISPR knockout screening platform, its applications, and the advantages it offers over conventional single-guide screening methods.

Applications

1. Identifying synthetic lethal interactions

Dual guide CRISPRko screening enables the identification of gene pairs that, when simultaneously knocked out, result in cell death while individual knockouts remain viable. This approach is particularly valuable for discovering novel combination therapy strategies and identifying vulnerabilities in specific genetic backgrounds.

2. Uncovering drug resistance mechanisms

By systematically knocking out pairs of genes and measuring drug sensitivity, researchers can identify compensatory pathways that contribute to treatment resistance, discover novel drug targets to overcome resistance, and develop rational combination therapies.

3. Pathway analysis and target validation

Dual guide screening provides deeper insights into complex biological pathways by revealing functional redundancies between genes, identifying key nodes in signalling networks, and validating potential drug targets in specific genetic contexts.

Here, the capabilities to perform dual CRISPRko screens to assess the impact of gene interactions and synthetic lethality are demonstrated. This proof-of-concept confirms that dual CRISPRko can be effectively used to probe dual guide interactions or the influence of these interactions in sensitizing or creating resistance in cells to a drug.

Methods

Generation of stable cell lines and assessment of Cas9 activity by FACS reporter assay

Cas9 stable cell lines are routinely used to generate knockout cells and to perform pooled and arrayed CRISPR screens. We generated a COR-L23-Cas9 expression cell line.

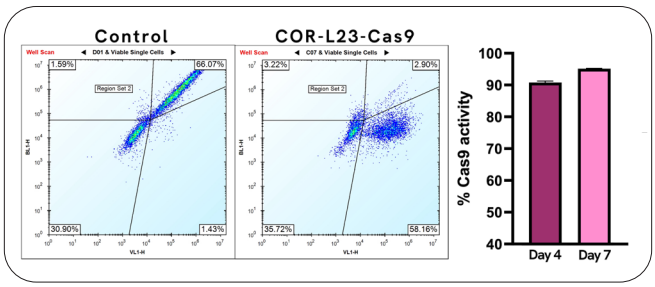

Figure 1: Reporter assay for Cas9 activity | COR-L23-Cas9 cell line assessment

To assess Cas9 activity levels, we employed a fluorescent-based reporter assay similar to that described by Tzelepis et al.[2] The COR-L23-Cas9 cell line demonstrated a high percentage of Cas9 activity (>90%) (Figure 1).

Dual guide construct design

Revvity's dual guide CRISPRko system utilizes a specialized construct design that enables efficient expression of two guide RNAs from a single vector (Figure. 2). Our team produced and tested various dual guide CRISPRko constructs, including those with human U6 promoters in the first position paired with mouse U6 in the second position, as described by Shen et al.[1]. The first position contains a guide RNA with a CR2 Tracr sequence, while the second position utilizes a guide RNA with a modified HZD Tracr sequence to minimize recombination. The arrangement was cloned in Revvity's CRISPR lentiguide puro vector backbone and used to transduce Cas9 stable cell lines.

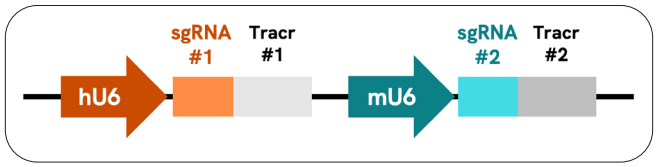

Figure 2: Construct Design. The chosen construct design utilizes a human U6 (hU6) promoter driving expression of the first sgRNA followed by a mouse U6 (mU6) promoter driving the second sgRNA. The construct incorporates distinct Tracr sequences: CR2 for the first position and HZD for the second position.

Dual guide CRISPRko test guides

To test our dual guide CRISPRko system, sgRNAs targeting EGFR, ERBB2 (HER2), or non-targeting controls were cloned into each position to assess knockout efficiency and potential positional effects. These constructs were tested in COR-L23 Cas9 stable-expressing cells.

Following selection, EGFR and ERBB2 expression were detected in COR-L23 Cas9 stable-expressing cells by flow cytometry using BioLegend antibodies Alexa Fluor® 488 Anti-human EGFR (Cat. No. 352908), detected with the BL-1 channel, and Brilliant Violet 421™ Anti-human CD340 ERBB2 (Cat. no. 324420) detected with the VL-1 channel using an iQUE3® instrument (Sartorius).

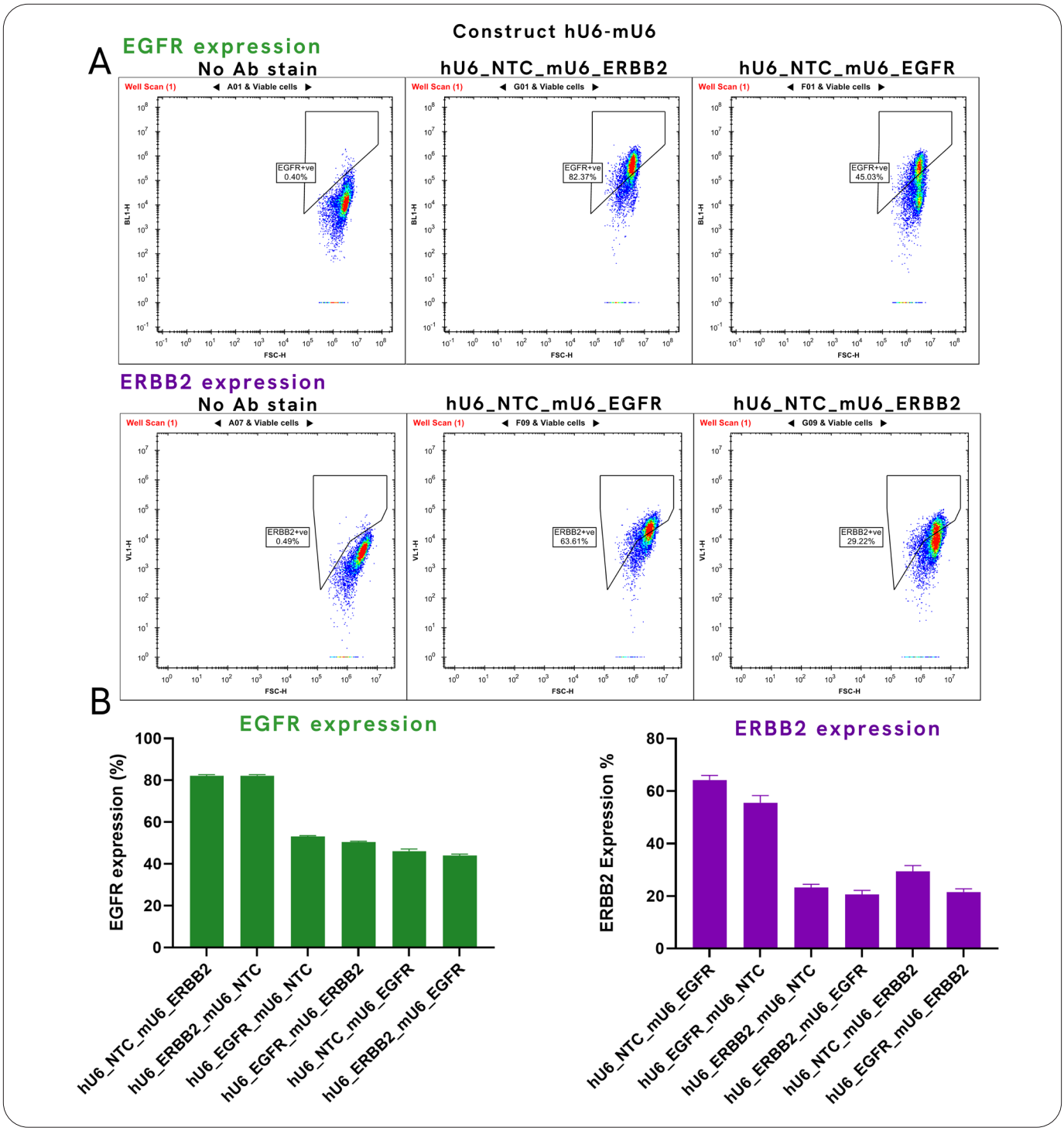

Figure 3: Strong performance with the mouse U6 promoter driving guide expression in the second position **A.** EGFR and ERBB2 expression in unstained control (No Ab stain), hU6_NTC_mU6_ERBB2 and hU6_NTC_mU6_EGFR, single knockouts of ERBB2 and EGFR, respectively, with the targeting guide in the second position for the construct hU6_mU6. **B.** Analysis of all tested guide combinations revealed equivalent reduction in EGFR and ERBB2 expression, regardless of whether the targeting guides were positioned first or second in the construct.

Results

Performance of the construct

The human U6-mouse U6 promoter combination showed the best and most consistent performance across guide positions. EGFR expression levels significantly decreased in constructs with EGFR-targeting guides, with slightly more effective knockdown when the EGFR guide was in the second position (driven by the mU6 promoter). EGFR expression remained unaffected in constructs that contained only ERBB2-targeting guides. ERBB2 levels consistently decreased in constructs with ERBB2-targeting guides, with comparable knockdown efficiency regardless of whether the ERBB2 guide was located at the first (hU6 promoter) or second (mU6 promoter) position. ERBB2 expression was not impacted in constructs with only EGFR-targeting guides. This balanced performance across guide positions makes the hU6-mU6 construct the preferred choice for dual guide CRISPRko applications, as it demonstrated overall superior performance with similar knockdown efficiency regardless of guide location (Figure 3A and 3B).

Conclusions

This application demonstrates Revvity's capabilities in preclinical services to perform dual CRISPRko screens for examining gene interactions and synthetic lethality. It shows that dual CRISPRko can be effectively used in custom library pooled screens to study gene-gene interactions and their impact on drug sensitivity or resistance. Revvity's dual guide CRISPRko screening platform enables researchers to analyze these interactions with precision, making it especially useful for cancer research, drug resistance investigations, and developing combination therapies. By systematically exploring genetic interactions that remain hidden in traditional single-gene approaches, researchers can identify new therapeutic targets and strategies that could transform treatment methods across various disease areas.

Recommendations for dual CRISPRko screen implementation

When designing dual guide CRISPRko screens, we recommend focusing on custom libraries targeting specific pathways or gene sets, typically including 100-300 genes in all pairwise combinations along with appropriate control constructs containing non-targeting guides. Cell line selection is critical. Choose disease-relevant models with stable Cas9 expression and consider the expression levels of your target genes to ensure optimal knockout impact. For analysis, employ next-generation sequencing for accurate guide quantification, implement robust statistical methods specifically designed for interaction scoring, and validate top hits with individual constructs to confirm the observed phenotypes. This targeted approach maximizes the value of dual guide screening while keeping library complexity manageable and ensuring reliable results.

At Revvity, our experienced preclinical services team handles all these complex considerations for you, from custom library design to sophisticated data analysis, allowing you to focus on the biological insights rather than the technical challenges of implementing dual guide screens.

References

1. Shen et al., 2017. Combinatorial CRISPR-Cas9 screens for de novo mapping of genetic interactions. Nat Methods. DOI: 10.1038/nmeth.4225

2. Tzelepis K, et al. 2016. A CRISPR dropout screen identifies genetic vulnerabilities and therapeutic targets in acute myeloid leukemia. Cell Rep. DOI: 10.1016/j.celrep.2016.09.079