APPLICATION NOTE

All-in-one pooled CRISPR screening to accelerate drug discovery, development, and pathway analysis.

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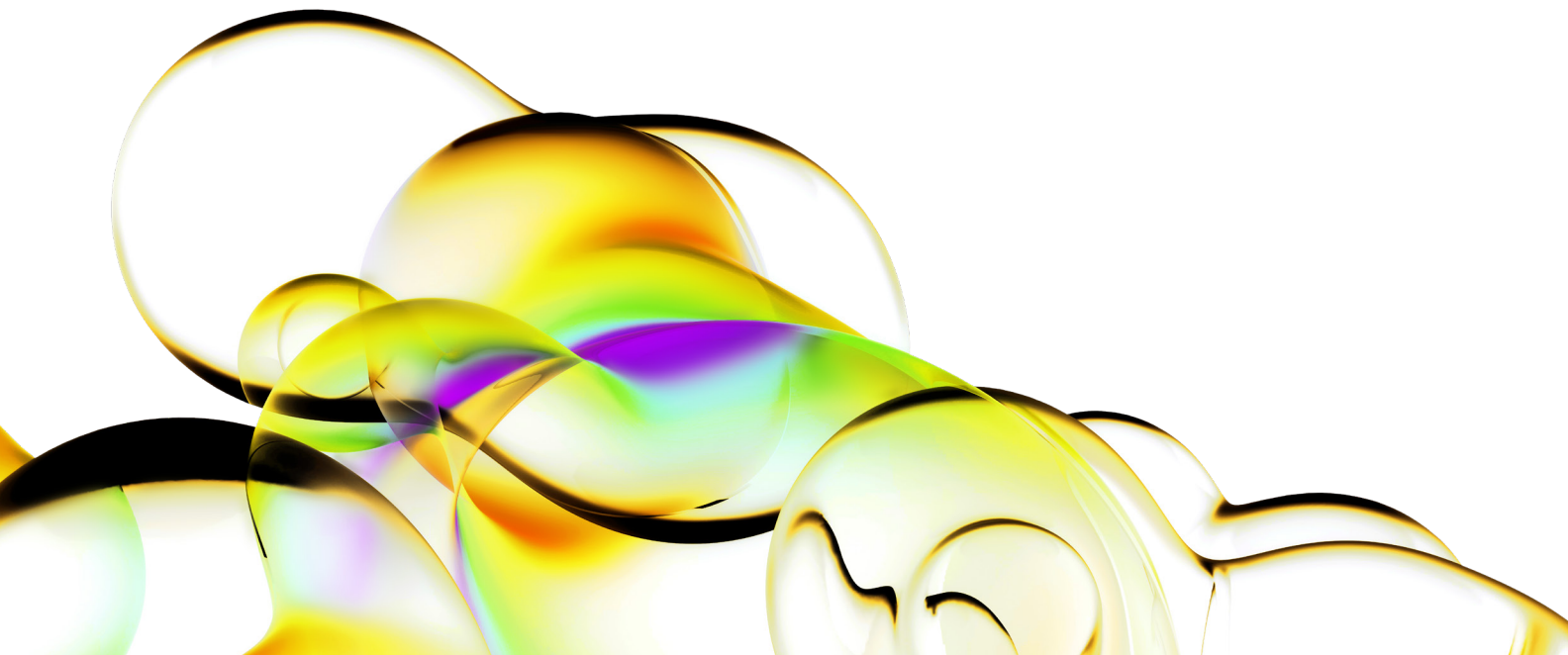
Introduction

The discovery of the bacterial CRISPR-Cas9 system, and the subsequent adaptation for use in eukaryotic cells has driven substantial expansion in gene editing applications across academic, biopharmaceutical, and biotechnological fields[1]. To modulate the level of target gene expression, groups have pioneered the use of the CRISPR-Cas9 system to include inactivated Cas9 fused to activators or inhibitors for the purpose of positively or negatively regulating transcription at the single gene level [2,3]. These CRISPR-Cas9 based applications – gene knockout, gene activation (CRISPRa) or gene inhibition (CRISPRi) – have also been incorporated into screening platforms to identify targets for drug development and validate the gene function through phenotypic observations.

Functional genomic screens are typically arranged in arrayed or pooled formats, with distinct benefits and limitations. In arrayed CRISPR screens, synthetic RNA guides (sgRNAs) are transfected into cells that constitutively express the platform-relevant Cas9 construct. This approach assesses one gene target per well, usually with a set of multiple guide RNA designs per gene target, which enables complex readouts (e.g. multi-parameter cell morphology assessments). Since the arrayed format features several individually-synthesized sgRNAs distributed amongst separate wells, large-scale screens necessitate automated sample handling systems and involve higher material consumption.



Similar to the arrayed setup, pooled lentiviral CRISPR screens typically begin with cells that stably express Cas9 or a dCas9-effector protein relevant to the experimental platform. The key difference is that pooled screens utilize a mixture of sgRNAs representing hundreds to thousands of gene targets that are introduced to a large cell population, where ultimately each cell hosts a single sgRNA integrant. In a pooled screen, each sgRNA is associated with a unique spacer sequence that serves to identify the modulated gene in each cell using NGS. This setup creates a powerful experimental system where modulation of target genes that lead to increases in viability or proliferation is detected via higher sgRNA abundance. Conversely, disruption of genes essential for cell viability or health results in decreased viability and lower sgRNA abundance. When applied to cell line disease models, this experimental framework can identify specific gene targets that are candidates for therapeutic intervention.

Additionally, by comparing data from pooled, whole-genome screens performed with and without a drug of interest, researchers can assess the enrichment or depletion of cells associated with perturbed target genes to determine which genes are associated with drug resistance or sensitivity. Parallel usage of multiple CRISPR screening platforms in both the arrayed and pooled setup (orthogonal validation) can increase the rigor and utility of downstream findings. For example, assessing drug sensitivity targets using both pooled CRISPRi and CRISPR knockout can identify shared target hits, providing confirmation and confidence in results.

Alternatively, combining pooled CRISPRa and CRISPRi screening experiments in tandem can help confirm target pathways in drug sensitivity screens. Identifying gene targets in shared pathways that antagonize drug mechanisms in CRISPRa screens while enhancing the drug efficacy in CRISPRi screens can accelerate the elucidation of drug mechanisms and identify pathways involved in compound resistance or sensitivity.

CRISPR screening efficiency hinges on minimally disruptive delivery of guide RNAs and CRISPR effectors into cells. Traditional sequential delivery methods can compromise cellular homeostasis and viability, particularly in sensitive cell types, while extending preparation time through multiple selection steps. Therefore, a single-step delivery that integrates both components simultaneously can offer substantial advantages in preserving cell health and accelerating experimental timelines.

Here we present a pooled all-in-one lentiviral system capable of introducing a whole genome library of guide RNAs and associated CRISPR effector for target gene knockout, activation or interference in one step. We will discuss the All-in-one platform, including the vector design, experimental workflow, and biological quality validation. Finally, we will explore a proof-of-concept study focusing on combining multiple all-in-one platforms to enhance drug discovery and cellular pathway analysis.

Results

All-in-one platform design

Dharmacon™ All-in-one lentiviral vectors (Figure 1) feature either *S. pyogenes* Cas9 for gene knockout, dCas9-VPR for gene activation or dCas9-SALL1-SDS3 for gene interference. The Cas9 effector of choice is expressed as a single transcript along with the puromycin resistance marker (PuroR) and linked by a 2A peptide sequence to ensure protein separation. The sgRNA, directed against a specific gene target, is expressed under the control of a separate promoter and integrity of sgRNA libraries was confirmed with NGS sequencing. To ensure optimal expression and activity across cell lines, two promoter options are available for system optimization.

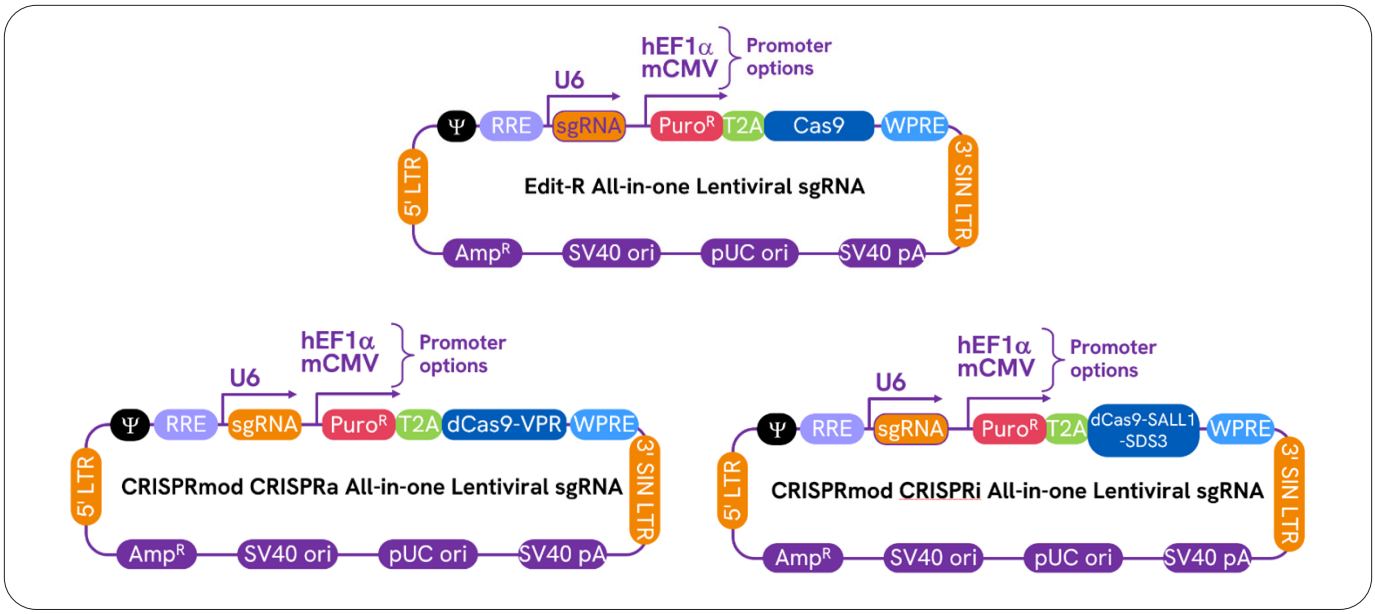


Figure 1: **Schematic maps of the All-in-one lentiviral vectors for gene knockout (Edit-R), gene activation (CRISPRa) and gene repression (CRISPRi)**. sgRNA is expressed under the type III RNA polymerase U6 promoter. Selection marker (Puro^R) and Cas9 construct (either Cas9, dCas9-VPR or dCas9-SALL9-SDS3) are expressed under control of either hEF1α or mCMV promoters.T2A ribosomal skipping sequence enables generation of separate protein products from a single transcript. Long terminal repeats (LTR), Psi packaging element (ψRev response element (RRE) and Woodchuck Posttranscriptional Regulatory Element (WPRE) are lentivirus-specific elements. Amp^R, SV40 ori, pUC ori and SV40 pA elements are for plasmid maintenance and transcription.

Pooled all-in-one screening methods and workflow

Dharmacon All-in-one whole genome pooled libraries for CRISPRko, CRISPRi, and CRISPRa containing 4 sgRNAs per gene were constructed and packaged by Revvity. A design-matched single vector dCas9-KRAB CRISPRi library was also generated to benchmark the Dharmacon CRISPRmod CRISPRi system.

Functional titration was used to identify the transduction conditions that enabled the use of a low MOI (~0.3) where transduced cells are infected with a single viral particle. This is particularly important for screening where the generation of single knockout events is the main goal. Once each lentiviral library had been functionally titrated in A375 cells, the cells were trypsinized, seeded in complete medium and transduced with each library according to standard lentiviral protocols. At 48 hours post-transduction, cells were treated with puromycin for 4 days, then split into duplicate control and experimental populations. Experimental cell populations were passaged in complete media with 2 µM Vemurafenib for approximately 14 doublings, while control populations were passaged in complete media with DMSO vehicle. At least 300 cells per sgRNA in each library (fold-representation) were maintained at all times throughout the screening procedure and in all treatment conditions.

Following harvest and gDNA extraction, gDNA was amplified and prepared for high-throughput sequencing on an Illumina platform. The integrated sgRNA sequences in both reference and experimental samples were identified and relative abundance compared. Gene essentiality was determined between an initial timepoint and the screening endpoint using the MAGeCK algorithm[9]. The open-source drugZ screening algorithm[6] was used to compare Vemurafenib-treated populations to control populations with Log2 fold change (LogFC) also calculated to determine the change in abundance of each guide per sample.

Non-targeting All-in-one controls (NTCs) are also incorporated in the screening library. Optimized transduction conditions, including optimal cell densities and promoter activity should be determined in advance and applied during the screening workflow (Figure 2) such that cells are transduced at a multiplicity of infection (MOI) around 0.3 to minimize multiple integration events of All-in-one cassettes. Following transduction, cells with lentiviral integrations are enriched through Puromycin selection. In the screening phase, experimental selective pressure, such as a drug or other compound, is applied to the population of transduced cells. This applied pressure serves to shift the abundance of specific populations of cells expressing sgRNAs that provide a contextual advantage or disadvantage in cell growth or survival.

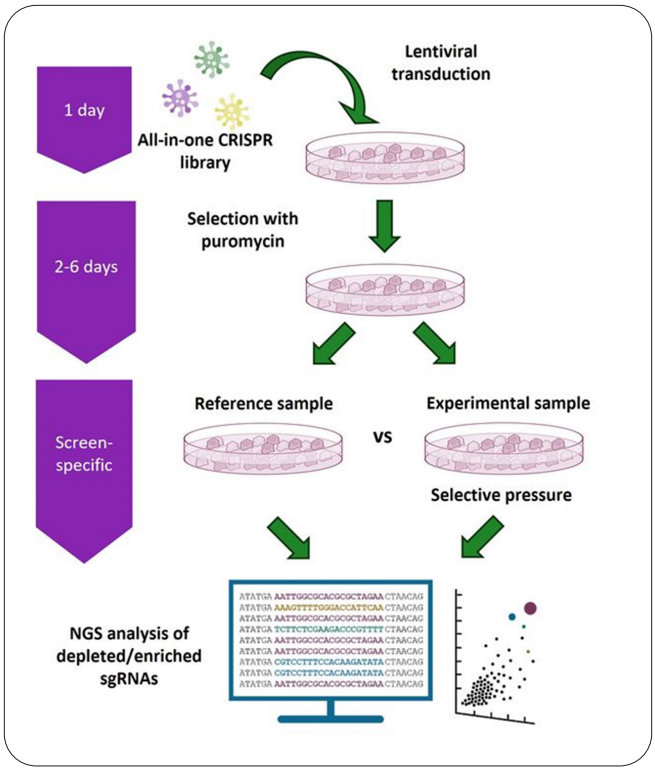


Figure 2: **Workflow illustrating gene expression screening workflow using CRISPRko, CRISPRmod CRISPRa or CRISPRmod CRISPRi All-in-one lentiviral pooled library platforms.** On day 1, cells are transduced at a low MOI with an All-in-one lentiviral pooled library and selected with puromycin for 2-6 days. Transduced and selected cells are split into reference and experimental populations where screen-specific compounds or other selective pressure can be applied. Genomic DNA is isolated from both cell populations and integrated sgRNA sequences are PCR-amplified using the Dharmacon Hit-identification primers and NGS Library Prep Kit. High-throughput NGS sequencing is used to quantitate and compare the abundance of integrated sgRNA sequences in reference and experimental samples. sgRNA constructs that are enriched or depleted in the experimental population are identified as hits and corresponding gene candidates can be further studied with individual All-in-one Lentiviral sgRNAs for target gene confirmation and additional downstream analysis.

Biological quality control for gene knockout and suppression platforms in pooled whole genome experiments

To assess the efficacy of the whole genome All-in-one screens, we analyzed a subset of guide RNAs that target a group of 684 core essential (CEG) and 1000 non-essential genes (NEG)[5] for gene knockout (Cas9) or repression (either dCas9-KRAB or dCas9-SALL-SDS3) in the reference population (DMSO-treated only) of A375 melanoma cells. By assessing the degree of essential "gene dropout" for sgRNA targets that become depleted relative to an initial timepoint, one can establish an unbiased measure of platform performance. Violin plots in Figure 3 illustrate the degree of CEG and NEG dropout in the three loss-of-function platforms. Each screening platform exhibits significant essential gene dropout, and neutral non-essential gene behavior, serving to validate the incorporated whole-genome screen in each case.

Dropout of core essential genes in the CRISPRko experiment (mean Log2 FC –2.70) occurs at a higher frequency than each of the two CRISPR interference platforms, an expected result when functions of essential genes are ablated in a knockout experiment. In contrast, gene repression experiments with dCas9-KRAB or dCas9-SALL-SDS3 exhibit comparably less dropout of essential gene targets, as partial function of essential genes can be retained. Comparison of the two gene repression screens indicate a higher degree of essential gene dropout when using dCas9-SDS3-SALL1 (mean Log2 FC –2.15), demonstrating more efficient repression of gene expression than when using dCas9-KRAB (mean Log2 FC –1.87). This differential efficiency between CRISPRko and CRISPRi platforms provides researchers a strategic option for experimental design, allowing selection of the optimal system based on whether complete knockout or gene silencing is required for the biological question under investigation.

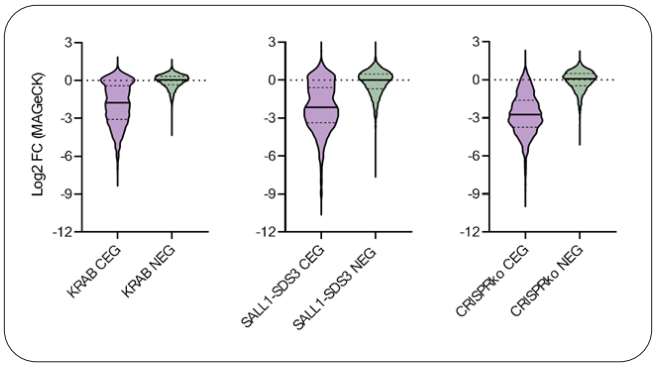


Figure 3: **Assessment of essential and non-essential gene dropout with the All-in-one dCas9-SALL1-SDS3 CRISPRi system, CRISPRi KRAB system and CRISPRko system.** Evaluation of core essential gene (CEG) and non-essential gene (NEG) dropout provides a metric by which to compare performance of different loss-of-function technologies at a genome-wide scale.

Parallel gene knockout and interference experiments in pooled all-in-one screening

To highlight the use of the All-in-one lentiviral screening platform, and present a case for orthogonal validation, A375 cells were transduced with either dCas9-SALL1-SDS3 or Cas9 All-in-one lentiviral sgRNA whole genome pooled libraries for gene inhibition or knockout, respectively. After the one-step transduction, selection and drug treatment, harvested gDNA was processed as described in methods in order to determine gene targets that perturb drug sensitivity when modulated in cells. This analysis is presented in Figure 4 and illustrates gene targets that lead to vemurafenib resistance upon being knocked out (CRISPRko) or knocked down (CRISPRi). Simultaneous analysis of orthogonal knockout and knockdown experiments can instill confidence in gene target hit identification and help to validate results. Focusing on gene target hits that generate a shared phenotypic presentation in parallel gene knockdown/knockout experiments (e.g. highlighted Shared hits in Figure 4) is a time-efficient approach for narrowing down a list of prospective genes to a select group of high-confidence targets for further study or therapeutic development.

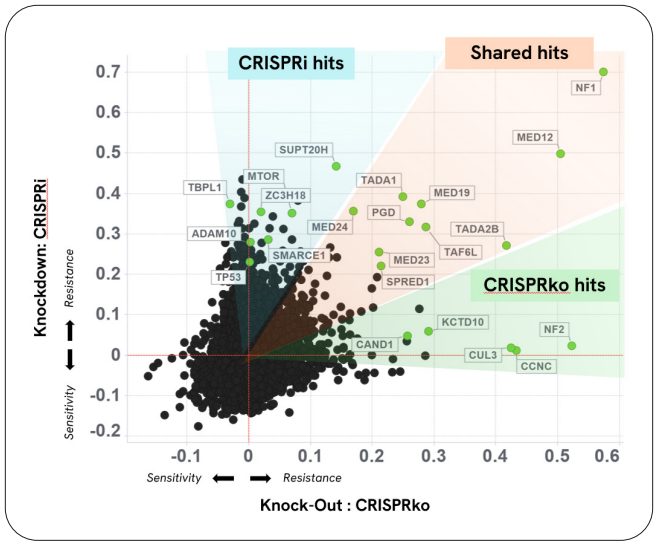


Figure 4: **Identification of shared gene targets in orthologous CRISPRi and CRISPRko experiments.** Combined dual loss of function screening using parallel loss-of-function CRISPRi and CRISPRko screening platforms can identify shared hits (orange shaded region), increasing confidence of hits identified in single platforms.

Parallel dual-direction modulation experiments with all-in-one pooled screening

Combining platforms which diametrically regulate gene target expression is another approach to establish a drug's mechanism of action. In contrast to the experimental design where orthogonal gene reduction platforms are run in parallel to validate target hits, a dual-regulation paradigm involves parallel experiments where sgRNAs at the whole genome-scale are combined with separate platforms that differentially regulate gene targets. To demonstrate this concept, A375 cells were transduced with pooled whole genome All-in-one CRISPRa (dCas9-VPR) or CRISPRi (dCas9-SALL1-SDS3) lentiviral particles, followed by selection. The two pools of cells were then treated with the chemotherapeutic agent vemurafenib and processed to determine relative gene target enrichment. Figure 5 presents the combined results of the dual-direction drug sensitivity screen, which was analyzed by drugZ. By focusing on gene targets that are enriched after the CRISPRa pooled screen, genes are highlighted that act as drug antagonists and thus drive resistance to the mechanism of action.

In contrast, CRISPRi loss-of-function screening can elucidate genes whose expression enhance drug activity and thereby drive resistance when depleted. By combining these two platforms in parallel pooled screening experiments, complementary target associations can be made, enhancing drug screening, drug discovery and pathway reconstruction. For example, NF1[7] and EGFR[8], two of the top hits on the dual-direction screen, have been confirmed as drivers of vemurafenib resistance.

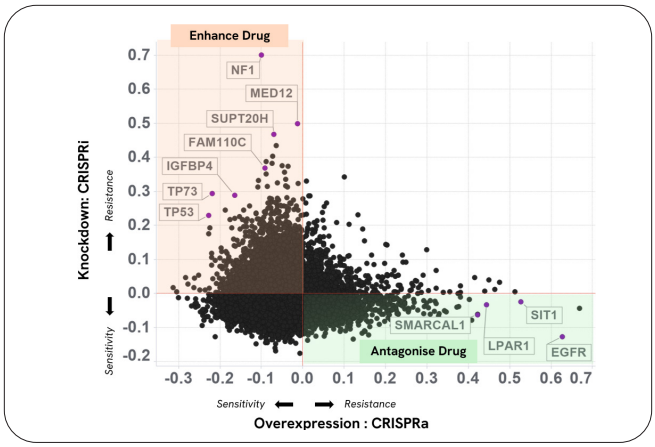


Figure 5: **Dual-direction screening identifies targets that can enhance and antagonize drug action.** Target enrichment or depletion following drug treatment of cells in CRISPRa and CRISPRi whole-genome screens are overlaid, indicating targets that either enhance or antagonize drug action. Dual-direction hit identification can enable drug pathway reconstruction (network analysis) and development of parallel therapeutics.

Discussion

In this study, we demonstrated the utility and effectiveness of pooled whole genome All-in-one CRISPR platforms for functional genomic screening. The All-in-one approach represents a significant advancement in CRISPR screening technology by integrating both the sgRNA and CRISPR effector (Cas9, dCas9-SALL1-SDS3, or dCas9-VPR) into a single lentiviral vector, enabling a one-step delivery into target cells.

This consolidated approach offers several substantial advantages over traditional two-component systems. First, it dramatically reduces the experimental timeline by eliminating sequential transduction and selection steps, potentially shortening preparation time by weeks. Second, it minimizes cellular stress by requiring only a single transduction event, which is particularly beneficial for sensitive, difficult-to-transduce, or unstable cell lines that may not tolerate multiple rounds of lentiviral infection or antibiotic selection. Third, the streamlined workflow reduces material consumption and technical complexity, making CRISPR screening at the whole genome scale more accessible to a broader range of researchers.

The versatility of our All-in-one platform is demonstrated through its adaptability to various experimental contexts. The availability of different CRISPR effectors—Cas9 for gene knockout, dCas9-SALL1-SDS3 for gene interference, and dCas9-VPR for gene activation—enables researchers to select the most appropriate approach for their specific research questions. Additionally, the option to choose between different promoters allows optimization of expression levels across diverse cell types, further enhancing the platform's flexibility.

Our biological quality control experiments clearly demonstrated the functionality of each All-in-one system, with expected patterns of essential gene dropout across the different platforms. Notably, the dCas9-SALL1-SDS3 interference system showed improved repression efficiency compared to the conventional dCas9-KRAB system, highlighting the continued innovation in CRISPR effector design.

Perhaps most significantly, our proof-of-concept studies illustrate how parallel implementation of complementary All-in-one platforms can generate robust, high-confidence results through orthogonal validation. The parallel CRISPRko and CRISPRi screens for vemurafenib resistance identified shared gene targets, providing stronger evidence for their biological relevance than either screen alone. Similarly, the dual-direction approach combining CRISPRa and CRISPRi screens revealed complementary insights into drug resistance mechanisms, identifying established resistance factors such as NF1 and EGFR.

These orthogonal screening strategies not only enhance confidence in hit identification but also provide deeper mechanistic insights into cellular pathways and drug responses. By simultaneously observing how both loss and gain of function for thousands of genes affect a phenotype of interest, researchers can more rapidly elucidate complex biological networks and identify potential therapeutic targets.

The All-in-one CRISPR screening platform we have developed addresses key limitations of conventional screening approaches while maintaining experimental rigor and data quality. Its simplified workflow, reduced cell manipulation requirements, and versatile applications make it an invaluable tool for functional genomics research across diverse biological questions—from basic mechanistic studies to therapeutic target discovery and drug resistance mechanisms.

Whether you are a newcomer or screening expert, our dedicated technical support team is available to provide personalized guidance, troubleshooting assistance and expert recommendations to get your science off to a successful start. Contact us at technical.horizon@revvity.com, or through our convenient chat and phone support channels to maximize your experimental success.

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