APPLICATION NOTE

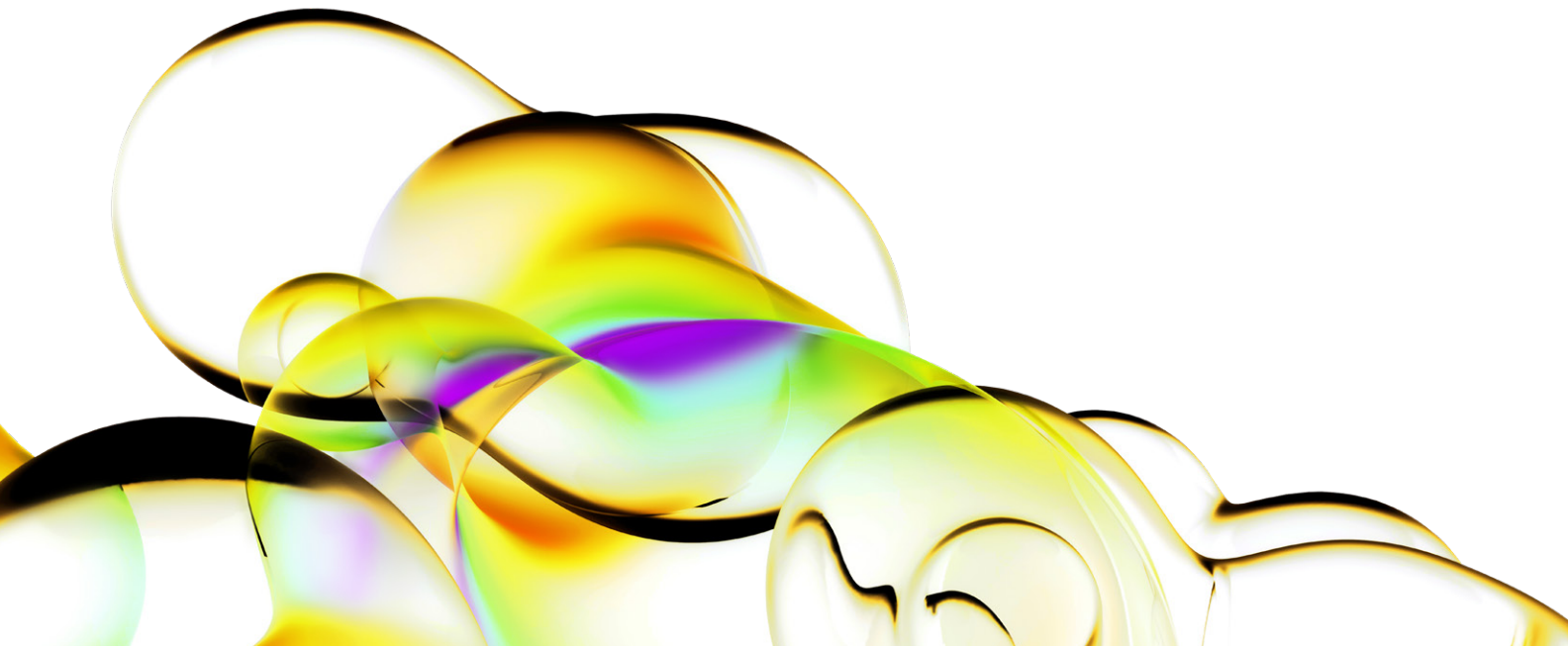
Duration of CRISPRa-guided target overexpression using dCas9-VPR-EGFP mRNA.

Introduction

Overxpression experiments are a perfect complement to loss-of-function studies, either as rescue or stand-alone experiments. CRISPRa enables upregulation of specific gene target expression within the gene's native context and can be used for single gene experiments or scaled-up to a large-scale screen.

The Dharmacon™ CRISPRa system employs a specialized catalytically-dead Cas9 (dCas9) fused to a triad of transcriptional activator proteins (VP64, p65 and Rta) (VPR) and a guide RNA (gRNA), predesigned to specifically guide dCas9-VPR to the region immediately upstream of a gene's transcriptional start site (TSS). Following dCas9-VPR gRNA-guided targeting, transcription of the adjacent gene is upregulated in a specific and robust manner, and within the context of the native expression cassette, ensuring that expected downstream post-transcriptional modifications occur. When choosing an overexpression system, a major experimental consideration is the overexpression timeline, or duration of target activation.

Several options exist for introducing CRISPRa reagents for targeted overexpression, each enabling variations in the duration of target overexpression. To this end, we have developed the Dharmacon™ CRISPRmod CRISPRa dCas9-VPR stable cell lines, dCas9-VPR-lentiviral particles, and dCas9-VPR mRNA to provide a selection of overexpression options. While dCas9-VPR stable cell lines (either pre-made or generated via lentiviral particles) can enable stable gene target overexpression for extended timepoint assays,



their use is not optimal in cases where a more temporary overexpression phenotype is required, or cells that are sensitive to introduction or integration of exogenous DNA. In this case, introduction of dCas9-VPR via mRNA template is an option, and a simple co-transfection with synthetic CRISPRa crRNA:tracrRNA includes all components necessary for transient, but targeted overexpression of a specific gene target[1]. Further, expression of a heterogenous protein from an mRNA template avoids the requisite steps of nuclear entry and transcription when utilizing a plasmid template, thus facilitating accelerated overexpression[2].

mRNA is less stable than DNA, and protein yield per molecule of transfected mRNA can be less that that per molecule of plasmid DNA or in stable cell lines[3]. As such, the temporal kinetics of mRNA-templated, CRISPRa-induced overexpression can be useful in experiments where a more transient overexpression phenotype is desired. Here we present the persistence of CRISPRa components in U2OS cells, and duration of downstream target protein overexpression, when dCas9-VPR is introduced via mRNA template, along with chemically modified synthetic crRNA:tracrRNA guides.

We demonstrate here that dCas9-VPR-EGFP mRNA enables robust and rapid dCas9-VPR expression for 72 hours following mRNA template introduction. This system leads to potent and transient target gene overexpression, facilitating study of cells before, during and after introduction of CRISPRa components.

Results

dCas9-VPR mRNA translation is rapid and robust 72 hours following transfection

A cancer cell line from an epithelial sarcoma (U2OS) was transfected with dCas9-VPR-EGFP mRNA and CRISPRa crRNA:tracrRNA, using DharmaFECT Duo. At 24-hours post-transfection, cells were imaged using brightfield and fluorescent (FITC) microscopy (Figure 1) and in subsequent 24-hour increments (Figure 2). EGFP and dCas9-VPR are in a single expression cassette, separated by a linker that results in translation of separate, isolated proteins.

Thus, EGFP fluorescence can be utilized as a proxy for dCas9-VPR expression as both constructs will be expressed simultaneously. As depicted in Figure 1, EGFP fluorescence can be detected throughout the population of transfected cells. EGFP expression peaks in the 24 hours after transfection and becomes undetectable after 4 days, indicating the transient nature of dCas9-VPR-EGFP mRNA.

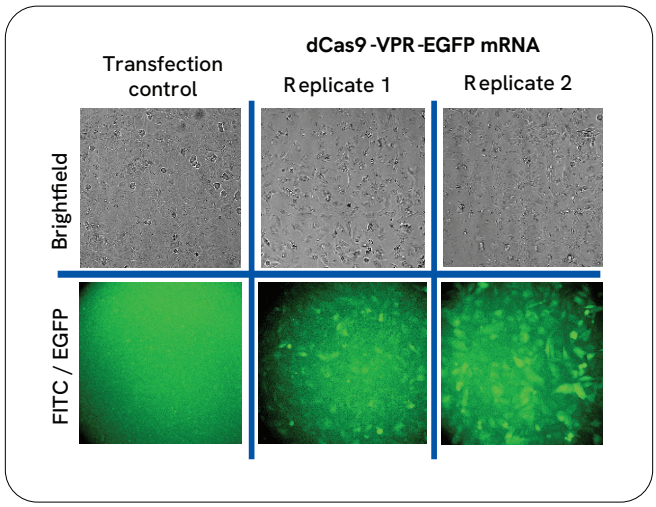


Figure 1: Brightfield and fluorescent images of U2OS cells 24 hours post-transfection with dCas9-VPR-EGFP mRNA.

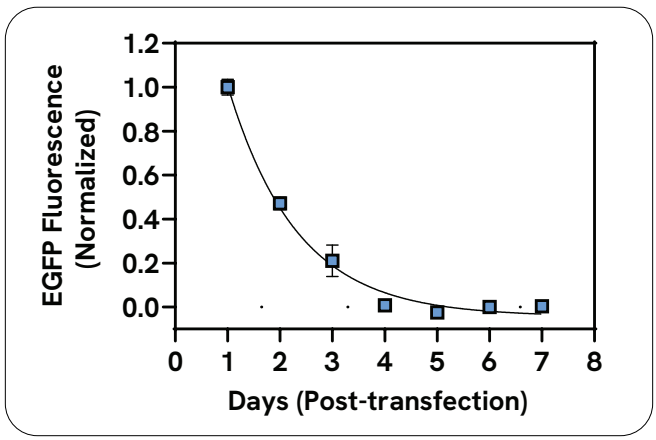


Figure 2: EGFP fluorescence in U2OS cells transfected with dCas9-VPR-EGFP mRNA up to seven days after transfection

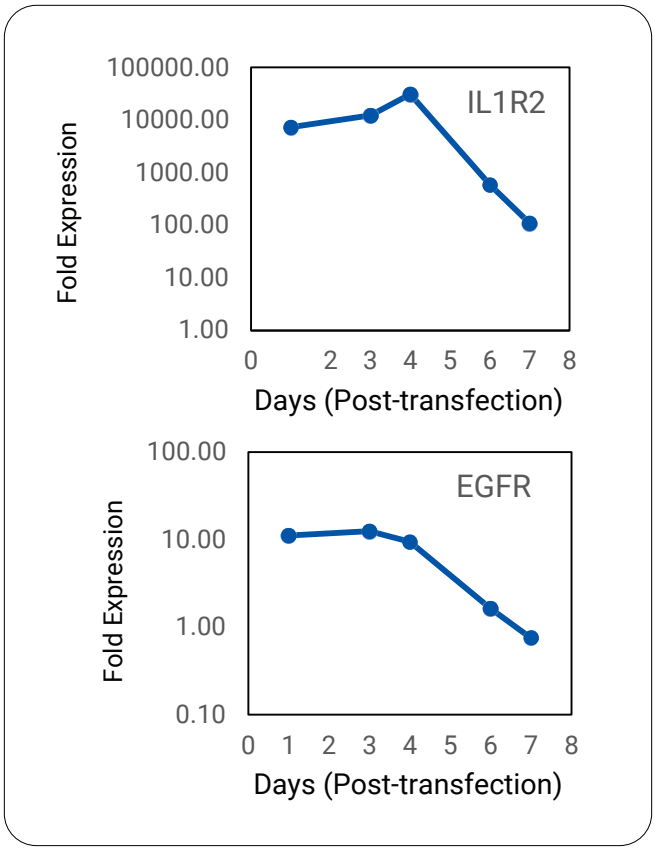


Figure 3: Relative target gene RNA transcriptional fold-activation after dCas9-VPR-EGFP mRNA and crRNA:tracrRNA co-transfection in U2OS cells measured by qPCR. Trace indicates qPCR signal relative to non-targeting control.

Duration of target overexpression following CRISPRa mRNA and guide transfection

CRISPRa experiments following these same conditions included CRISPRa crRNA:tracrRNA targeting extracellular growth factor receptor (EGFR) and interleukin 1 receptor type 2 (IL1R2) genes. To enhance activation, pooled mixtures of crRNA guides were used, each targeting an independent locus upstream of the TSS. Degree and duration of EGFR and IL1R2 expression was assessed with quantitative PCR (qPCR). Elevations in target mRNA were immediate and sustained for up to 4 days, returning to near-basal levels around days 6–7 post-transfection (Figure 3). U2OS cells from parallel transfections were processed for immunocytochemical microscopy and imaged. In-line with observations of target gene RNA levels, acute overexpression of target proteins was measurable within 24 hours of transfection (Figure 4) and persisted until a return to basal levels within 4–6 days post-transfection.

Discussion

Choice of targeted gene overexpression platform is informed by desired duration of gene overexpression, sensitivity of cell type to exogenous DNA, ease of use, and experimental scale. Here we demonstrate the use of CRISPRa dCas9-VPR-EGFP mRNA for overexpression experiments where a rapid, reliable, and reversible rise in endogenous target protein levels can be initiated by co-transfecting with synthetic gRNA.

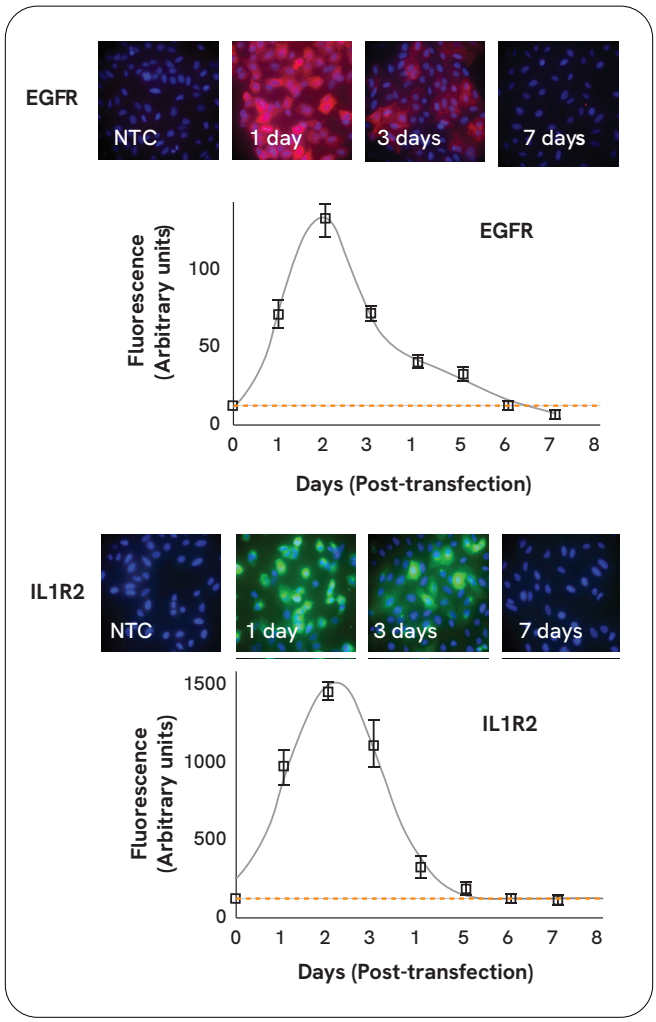


Figure 4: Relative fold target gene protein expression measured by immunofluorescent microscopy after dCas9-VPR-EGFP mRNA and CRISPRa crRNA:tracrRNA co-transfection in U2OS cells. Traces indicate fluorescent signal relative to non-targeting control. Background blue coloration is Hoeschst 33342 dye bound to nuclear DNA.

By measuring target RNA and protein levels, we show targeted overexpression quickly peaks 2–3 days post-transfection of CRISPRa reagents, followed by a gradual return to basal levels in U20S cells. These conditions are ideal where experimental conditions require rapid target overexpression followed by a return to basal conditions, where cellular phenotype or response can be assessed before, during, and after target oscillation. In addition, due to the ease with which overexpression can be initiated with co-transfection of mRNA and synthetic gRNA, the CRISPRmod CRISPRa platform can be scaled up for arrayed high-content screening experiments.

For applications that require an extended duration of target gene overexpression, we offer Dharmacon CRISPRmod CRISPRa lentiviral reagents (dCas9-VPR and sgRNA) and stable cell lines for long-term activation.

Materials and methods

Tissue culture

U2OS cells (ATCC, HTB-96) were maintained in established media (high glucose DMEM with 10% (v/v) FBS and 2 mM L-glutamine) at 37 °C and 5% CO_2. Cells were passaged by rinsing with calcium-free PBS, incubating with 0.25% trypsin for 5 minutes at 37 °C, then diluting with full media and reseeding on 96-well plates.

Transfection

Transient dCas9-VPR experiments: U2OS cells were seeded in 96-well, glass bottom plates at 10,000 cells/well one day prior to transfection. Cells were transfected with crRNA:tracrRNA (Revvity, Dharmacon CRISPRmod CRISPRa crRNA SMARTpools for EGFR (P-003114-01) and IL1R2 (P-007690-01), (Revvity, Dharmacon Edit-R tracrRNA (U-002005)), and dCas9-VPR-EGFP mRNA (Revvity, Dharmacon CRISPRmod CRISPRa dCas9-VPR-EGFP mRNA (CAS12212)) at final concentrations of 25 mM, 25 mM, and 200 ng/well, respectively. Synthetic crRNA:tracrRNA and dCas9-VPR-EGFP mRNA were complexed with 0.5 µL/well DharmaFECT Duo Transfection Reagent (Revvity, DharmaFECT Duo Transfection Reagent (T-2010)) in serum free media for 20 minutes at room temperature. Cell growth media was removed and replaced with complexed transfection mixtures, after being diluted 5-fold into complete growth media. Each condition was replicated eight-fold to ensure reproducibility through the experimental timeline.

RT-qPCR

Relative target and control gene expression was determined at 24-hour timepoints from 1–7 days post-transfection. Total RNA was isolated from cell lysates using an SV 96 Total RNA Isolation System (Promega, Z3500). From RNA isolates, cDNA was synthesized using a Maxima First Strand cDNA Synthesis Kit (Thermo, K1672). 7.5 µL TaqMan Master Mix (Thermo, 4304437) was mixed with 2.0 µL undiluted cDNA and 0.5 uL of appropriate TaqMan probe (Thermo, GAPDH, 02786624_g1; EGFR, 01076090_m1; IL1R2, 00174759_m1). Samples were mixed and 10 uL of each reaction was transferred to individual wells on a 384-well plate and loaded onto a Roche LightCycler 480 II for thermal cycling and analysis. Each sample was performed in technical duplicate. The relative expression of each target gene was calculated with the ΔΔCq method using GAPDH as the housekeeping gene and normalized to a non-targeting control

Fluorescence microscopy and analysis

For immunocytochemical analysis, cells were processed on the 96-well glass culture support. After removing media, 4% paraformaldehyde (PFA) fixative was added for 30 minutes at room temperature, then washed twice with PBS. Cells were permeablized by incubating with 0.5% (v/v) Triton X-100 in PBS for 15 minutes at room temperature and washed three times with PBS. After blocking cells with 3% (w/v) BSA in PBS for 30 minutes at room temperature, 5 µg/mL mouse-derived anti-EGFR (Abcam ab30) and 5 µg/mL goat-derived anti-IL1R2 (Novus, AF-263) were added to blocking solution for 1 hour incubation at room temperature with gentle rotation. Cells were then washed three times with PBS with 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100, then incubated with fluorescent secondary detection antibodies (1:1000 dilutions of each goat anti-mouse conjugated to AF555 [Thermo, A32727] and donkey anti-goat conjugated to AF488 [Thermo, A32814]) in PBS. After three washes with PBS with 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100, 1 µg/mL Hoeschst 33342 in PBS was added for 15 minutes at room temperature followed by three washes in PBS. Wells were left in 100 µL PBS and sealed for imaging. Fluorescent images were captured at 20X magnification using a Nikon Eclipse Ti and processed with CellProfiler 4 image analysis software[4] and Prism (GraphPad).

References

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