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TITLE:

Overexpressing Long Noncoding RNAs Using Gene-activating CRISPR

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KEYWORDS:

lncRNA, CRISPR, IFNG-AS1, overexpression, lentiviral, real-time PCR

SUMMARY:

Traditional cDNA-based overexpression techniques have a limited applicability for the overexpression of long noncoding RNAs due to their multiple splice forms with potential functionality. This review reports a protocol using CRISPR technology to overexpress multiple splice variants of a long noncoding RNA.

ABSTRACT:

Long noncoding RNA (lncRNA) biology is a new and exciting field of research, with the number of publications from this field growing exponentially since 2007. These studies have confirmed that lncRNAs are altered in almost all diseases. However, studying the functional roles for lncRNAs in the context of disease remains difficult due to the lack of protein products, tissue-specific expression, low expression levels, complexities in splice forms, and lack of conservation among species. Given the species-specific expression, lncRNA studies are often restricted to human research contexts when studying disease processes. Since lncRNAs function at the molecular level, one way to dissect lncRNA biology is to either remove the lncRNA or overexpress the lncRNA and measure cellular effects. In this article, a written and visualized protocol to

overexpress lncRNAs in vitro is presented. As a representative experiment, an lncRNA associated with inflammatory bowel disease, Interferon Gamma Antisense 1 (IFNG-AS1), is shown to be overexpressed in a Jurkat T-cell model. To accomplish this, the activating clustered regularly interspaced short palindromic repeats (CRISPR) technique is used to enable overexpression at the endogenous genomic loci. The activating CRISPR technique targets a set of transcription factors to the transcriptional start site of a gene, enabling a robust overexpression of multiple lncRNA splice forms. This procedure will be broken down into three steps, namely (i) guide RNA (gRNA) design and vector construction, (ii) virus generation and transduction, and (iii) colony screening for overexpression. For this representative experiment, a greater than 20-fold enhancement in IFNG-AS1 in Jurkat T cells was observed.

INTRODUCTION:

While most biomedical research has focused on protein-coding transcripts, the majority of transcribed genes actually consists of noncoding RNAs (Ensembl release 93). Current research is beginning to explore this field, with the number of publications on lncRNAs in disease processes rising exponentially between 2007 and 2017[1]. These publications demonstrate that many lncRNAs are associated with disease. However, the molecular mechanisms of these lncRNAs are difficult to study due to their diverse functions as compared to mRNAs. Compounding the problem of understanding the role of lncRNAs in disease, lncRNAs are often expressed at lower levels than coding RNAs[2]. Additionally, lncRNAs are poorly conserved, which limits the functional studies in human-cell-line-based techniques[3]. One method to study the mechanism of these novel genes is to endogenously overexpress them in cultured cells. Overexpression studies can provide key information as to the function of specific genes and enable researchers to dissect key molecular pathways.

A new method to activate the transcription of lncRNA genes is based on CRISPR technologies developed initially by the Gersbach laboratory[4]. This protocol has been adapted for use in lncRNA biology and for the expression of these genes in other model systems. In the CRISPR overexpressing technique, a protein called CRISPR-associated protein 9 (Cas9) can be directed toward a DNA sequence via an antisense gRNA that is recognized by Cas9. Normally, Cas9 will induce DNA cleavage; however, mutations in Cas9, previously developed for the overexpression technique, deactivate this step[5]. When a transcriptional activator is fused to a “dead” Cas9 (dCas9) and transduced into cell lines, the endogenous overexpression of lncRNAs can be achieved[4,6]. The addition of additional modifications to the gRNA, enabling additional transcriptional factors to bind to the gRNA, increased the efficacy of the dCas9 gene activation system 10-fold[7]. Importantly, it was demonstrated that transcriptional activation requires close proximity (<200 bp) to the transcriptional start site genes, enabling a specific upregulation in gene-rich areas[7]. Unlike CRISPR knockout technologies, dCas9 and gRNA cassettes need to be integrated into the genome to allow for cells to retain an overexpression over multiple generations. One method to achieve this is to use lentiviruses to integrate the dCas9- and gRNA-containing cassettes. After integration, the lncRNA gene expression can be determined.

In this article, a protocol for lncRNA overexpression in a Jurkat T-cell model will be demonstrated. A step-by-step procedure is shown and can also be adapted to adherent cells.

PROTOCOL:

NOTE: It is important to note that this protocol uses replication-deficient lentiviruses. Perform viral handling only after appropriate lab safety training. Bleach all items and surfaces that come in contact with live viruses for a minimum of 10 min after handling. Use a disposable lab coat and face/eye protection, as well as double gloves, at all times. Perform virus work in biosafety level 2+ labs with viral certification. Dedicate tissue culture hoods and incubators to viral work.

1. Vector design and generation

NOTE: The best way to identify gRNA sequences is to use online design tools (e.g., http://crispr-era.stanford.edu) (**Supplemental Figure 1: gRNA design**). For the accompanying representative experiment, a company designed and created the gRNA vectors used in the representative experiment. The dCas9 plasmid was also purchased online.

1.1. The gRNA sequence is located upstream of the nucleotide sequence "NGG", where "N" is any nucleotide, which also is within 100 base-pairs of the transcriptional start site. Search genetic databases such as BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start) for the gRNA sequence, to make sure there are no other sites in the genome with similar sequences (**Supplemental Figure 2: BLAT**). This will ensure that the gRNA sequence is unique to the gene of interest (GOI).

1.2. Store the gRNA and dCas9 plasmids, which come as bacterial stubs, at 4 °C. Streak out *Escherichia coli* on a Luria broth (LB) agar plate with ampicillin (**Supplemental Table 1**) and grow the bacteria overnight at 37 °C.

1.3. Pick a colony and grow the bacteria in 5 mL of LB with ampicillin (**Supplemental Table 1**) overnight, vigorously shaking the plate in a 37 °C incubator. The next day, add 2 mL of bacteria to 200 mL of LB with ampicillin grow in a 2 L flask, vigorously shaking the flask overnight in a 37 °C incubator.

1.4. Spin down the bacteria at 3,724 x *g* for 20 mins. Remove the media and place the tube containing the bacteria on ice.

1.5. Purify bacterial DNA using a plasmid purification kit per the manufacturer's protocol.

NOTE: The plasmid purification kits contain proprietary resuspension buffer, lysis buffer, wash buffer, equilibration buffer, and elution buffer.

1.5.1. Add 10 mL of resuspension buffer from the kit to the bacteria and pipet the bacteria into the solution. Then, add 10 mL of lysis buffer from the kit to the resuspended bacteria and mix them by inverting the tube. Wait 5 min to lyse the bacteria; then, add 10 mL of ice-cold neutralization buffer from the kit to the lysed bacteria and mix them by inverting the tube.

1.5.2. Equilibrate the DNA column from the kit with 10 mL of equilibration buffer for 5 min. Pour the samples into the DNA column filter and let the solution pass through the filter.

1.5.3. Wash the samples 2x with 30 mL of wash buffer and, then, elute the DNA with 30 mL of elution buffer in a 15 mL conical tube. Slowly layer 10.5 mL of isopropanol at room temperature onto the eluted DNA, invert the tube, and immediately spin it at 16,200 x *g* for 30 min at 4 °C.

1.5.4. Remove the supernatant and add 1 mL of 70% ethanol to the DNA pellet. Resuspend the pellet by flicking the tube. Spin it at 16,200 x *g* for 10 min at 4 °C and use a 200 µL pipet tip to remove most of the ethanol. Air-dry the pellet for 5 min and resuspend it in 200 µL of water, and store the DNA at -20 °C. The expected concentration will be >1 µg/µL.

2. Virus generation and particle count

2.1. When ready to create the dCAS9-containing lentivirus, coat 100 mm tissue culture dishes with 5 mL of 0.01% poly-L-lysine (**Supplemental Table 1**). Remove the excess liquid thoroughly from the plates and let them air-dry for a few minutes in a biosafety cabinet.

2.2. Plate 5 x 10^6 HEK 293T cells per dish in 10 mL of complete Dulbecco's modified Eagle's medium (DMEM) (**Supplemental Table 1**) and incubate them overnight at 37 °C with 5% CO_2. The next day, remove the medium from the HEK 293T dishes and add 10 mL of complete DMEM.

2.3. Mix 6.5 µg of plasmid pMDLg/pRRE containing the gag/pol (components of the virus), 3.5 µg of the plasmid pMDG2.G containing the VSV-G (a component of the virus), 2.5 µg of the plasmid pRSV-Rev containing Rev (a component of the virus), 10 µg of an long terminal repeat (LTR)-containing gRNA vector or an LTR-containing dCas9 vector and add water to a total volume of 450 µL. Filter the mixture through a 0.2 µm filter tip attached to a syringe.

2.4. Add 50 µL of 2.5 M $CaCl_2$ (**Supplemental Table 1**) to each transfection sample of DNA and mix gently. Filter the calcium/DNA mixture through a 0.2 µm filter tip attached to a syringe. Pipet 500 µL of 2x HBS (**Supplemental Table 1**) into a 5 mL polystyrene tube. Add the 500 µL DNA/$CaCl_2$ mix dropwise and gently vortex. Incubate at room temperature for 3 min.

2.5. Slowly add 1 mL of the DNA/$CaCl_2$/HBS suspension to each dish. Immediately swirl the dishes to distribute the contents evenly. Incubate each dish overnight in a 5% CO_2 incubator at 37 °C.

2.6. On day 3, slowly remove and discard the media from the dishes. Carefully wash the cells 1x with phosphate-buffered saline (PBS). Add 6 mL of complete DMEM supplemented with 20 mM HEPES and 10 mM sodium butyrate. Incubate the cells for 5–6 h in a 5% CO_2 incubator at 37 °C.

2.7. Wash the cells 1x with PBS and add 5 mL of complete DMEM with 20 mM HEPES to the HEK 293T cells. Incubate for 12 h overnight in a 5% CO_2 incubator at 37 °C.

2.8. On day 4, collect the HEK 293T cell supernatants and filter the supernatant. Freeze 1 mL aliquots at -80 °C.

2.9. When ready to use the virus, thaw an aliquot of the viral particle containing conditioned media (stored as described in step 2.8) on ice. Bring all reagents to room temperature.

2.10. Use a p24 Enzyme-Linked Immunosorbent Assay (ELISA) kit to quantify the number of viral particles per milliliter.

2.10.1. Dilute the wash concentrate from the kit by adding 19 parts of distilled deionized water. Dilute the positive control from the kit to 200 ng/mL, using RMPI 1640 as the diluent, and make the dilutions for standard curve in 1.5 mL tubes according to the p24 ELISA dilution table (**Supplemental Table 2**).

2.10.2. Add 20 µL of 5% Triton X-100 to all wells except the substrate blank. Add 200 µL of RPMI 1640 to the negative control wells. Add 200 µL of each standard (in duplicate) to the designated wells.

2.10.3. Using a spectrophotometer, measure the concentration of the virus within the samples, using a standard curve. Start the sample dilution at 1:1,000 and modify the volume as necessary in order to be within the range of the standard curve. Dilute the samples with Triton X-100 to a final concentration of 0.5% and add 200 µL of each sample in RPMI 1640 to designated wells. Seal the plate and incubate it for 2 h at 37 °C.

2.10.4. Wash the plate 6x with 300 µL of 1x wash buffer per well. Remove any excess fluid by inverting the plate and tapping it on a paper towel. Add 100 µL of detector antibody from the kit to all wells except the substrate blank. Seal the plate and incubate it for 1 h at 37 °C. Wash the plate and, then, remove any excess fluid by inverting the plate and tapping it on a paper towel.

2.10.5. In order to measure the detector antibody, prepare streptavidin-horseradish peroxidase (SA-HRP) within 15 min of use. Dilute the SA-HRP at 1:100 with SA-HRP diluent. Mix the diluted SA-HRP thoroughly and add 100 µL to all wells except the blank. Seal and incubate the plate for 30 min at room temperature.

2.10.6. Wash the plate with 1x wash buffer and tap away any excess liquid as in step 2.10.4. Use ortho-phenylenediamine (OPD) substrate solution, which provides the peroxidase the necessary substrates to produce chemiluminescence, within 15 min of preparation. Use one OPD tablet to 11 mL of substrate diluent for each plate.

2.10.7. Vortex the OPD solution vigorously to dissolve it completely and protect it from light. Add 100 µL of OPD substrate solution to all wells, including the blank. Using a spectrophotometer, read absorbance at 450 nm immediately, repeat this 10x at 1 s intervals, and take the average measurement.

3. dCas9-VP64 transduction

3.1. Culture Jurkat T cells in complete DMEM in a T75 flask with a density of 2 x 10^6 cells in 15 mL of media. Culture the cells in a 37 °C incubator with 5% CO_2.

3.2. Spin down the cells for 5 min at 233 x *g* and, then, resuspend them in 10 mL of reduced serum media. Count the cells with a hemocytometer or an automated cell counter.

3.3. Resuspend and plate 1 x 10^6 Jurkat cells in 5 mL of reduced serum media with polybrene (**Supplemental Table 1**) in a T75 flask. Add HEK 293T-conditioned media containing 1 x 10^6 dCas9-containing viral particles. The number of viral particles can be calculated roughly from the p24 ELISA because 1 µg/mL p24 equals 1 x 10^7 viral particles. Put the flasks in 37 °C incubator with 5% CO_2.

4. Selection and clone creation

4.1. Three days post-infection, spin down the cells at 233 x *g* for 5 min and resuspend them in 10 mL of complete DMEM with puromycin (**Supplemental Table 1**). Plate the cells in T75 flasks and culture them at 37 °C with 5% CO_2. Every third day, for a period of 9 days, spin down the cells at 233 x *g* for 5 min and replace the media with complete DMEM with puromycin.

4.2. Count the cells using a hemocytometer or an automated cell counter. On a 96-well plate treated with tissue culture, plate 10,000 cells in 100 µL of complete DMEM with puromycin in the first well and serially dilute 1:1 the contents of the following wells with complete DMEM with puromycin. Perform 24 dilutions and repeat this 4x per plate in order to ensure that there is an adequate number of cells per well. Incubate the cells at 37 °C with 5% CO_2.

4.3. Expand clonal cells into two 24-well plates, then into a 6-well plate, and eventually into a T75 flask. In order to focus on three of the clones, plate 2 x 10^6 cells per well of a 6-well plate for three of the clones. Plate the other three wells with nontransduced Jurkat cells as controls. Put the cells in a cell culture incubator overnight.

4.4. For RNA extraction, use a commercially available RNA isolation kit. Spin down the cells at 233 x *g* for 5 min at room temperature and remove the media. Use a 1 mL pipet tip to resuspend the cells in 350 µL of lysis buffer. Add 350 µL of 70% ethanol to the lysed cells, mix thoroughly with a 1 mL pipet, and transfer the sample to the RNA column.

4.5. Spin the lysates at 10,000 x *g* for 1 min, remove the flow-through, and add 700 µL of low-stringency wash buffer from the kit to the column. Spin the lysates at 10,000 x *g* for 1 min, remove the flow-through, and add 80 µL of DNase I solution from the kit to the column; let the samples incubate for 15 min at room temperature.

4.6. Spin the lysates at 10,000 x *g* for 1 min, remove the flow-through, and add 700 µL of high-stringency wash buffer from the kit to the column. Spin the lysates at 10,000 x *g* for 1 min, remove the flow-through, and add 700 µL of low-stringency wash buffer to the column.

4.7. Remove the column from the collection tube and transfer it to a new collection tube. Spin the lysates at 10,000 x *g* for 1 min and, then, transfer the RNA column to a 1.5 mL tube. Add 50 µL of water to the column and spin at 10,000 x *g* for 1 min.

4.8. Use a spectrophotometer to quantify the concentration of RNA. Expect the concentration to be between 100–500 ng/µL, with a 260/280 absorbance between 1.9–2.1. Store the RNA at -80 °C.

4.9. In 250 µL tubes, add 1 µg of RNA, 4 µL of complementary DNA (cDNA) synthesis buffer, 1 µL of reverse transcriptase, and water to a final volume of 20 µL. Include no reverse transcriptase controls. In a thermal cycler, synthesize cDNA at 42 °C for 30 min and at 95 °C for 5 min to inactivate the reverse transcriptase. Dilute the cDNA with 60 µL of water after the synthesis.

4.10. Prepare polymerase chain reactions (PCRs) containing 5 µL of SYBR green, 4 µL of cDNA, 0.5 µL of forward primer (10 µM), and 0.5 µL of reverse primer (10 µM). Run the PCR as follows: step 1 = 95 °C for 3 min, step 2 = 95 °C for 10 s, step 3 = 60 °C for 10 s, and then, repeat steps 2 and 3 for 30x.

NOTE: The sequence for the dCas9 forward primer is 5'-TCGCCACAGCATAAAGAAGA and the sequence for the dCas9 reverse primer is 5'-CTTTTCATGGTACGCCACCT. The forward primer for Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) is 5'-GACCAGTCAACAGGGGACAT and the reverse primer is 5'-GCTTGCGACCTTGACCATCT.

5. gRNA transduction, selection, clone creation, and screening

5.1. Retransduce the validated Jurkat-dCas9 cells with gRNA-containing viruses as outlined in section 3. Select cells in DMEM with hygromycin (**Supplemental Table 1**) for 10 days. Spin down the cells and change the media every 3 days.

5.2. Perform a serial dilution of cells (as described in step 4.2) and expand individual colonies (as described in step 4.3). Purify RNA from the colonies exactly as described in step 4.5 and perform cDNA synthesis as described in step 4.9. Perform real-time PCR against the GOI and HPRT1 or an appropriate housekeeping gene, similarly to the PCR described in step 4.10, except in this case, image the wells each cycle after step 3.

5.3. Use the comparative cycle threshold (Ct) method to determine fold change in the GOI[8]. Briefly, use the following equation to calculate relative transcript levels (RTLs): $2^{-(\text{average Ct of GOI} - \text{average Ct of housekeeping gene})}$. Then, calculate the average RTL of the controls and divide all RTL values by the average control RTL to generate a fold change compared to the control samples.

REPRESENTATIVE RESULTS:

A dual vector system to overexpress the lncRNA IFNG-AS1

The example experiment in this manuscript is the overexpression of a Jurkat T-cell model system expressing the lncRNA IFNG-AS1[9]. IFNG-AS1 is an lncRNA associated with inflammatory bowel disease, that has been seen to regulate Interferon Gamma[10]. The IFNG-AS1 gene contains three splice variants that all use the same transcriptional start site (**Figure 1A**). Therefore, gRNA sequences that were 10 and 100 bp away from the transcriptional start site and had an "NGG" sequence upstream were used for directing the Cas9-activating complex to the transcriptional locus of IFNG-AS1 (**Figure 1A**). A two-plasmid system was used to transduce either dCas9 or gRNAs/enhancers into cells as a single plasmid alone makes viral particle generation difficult due to plasmid size (**Figure 1B**). To enable the selection of double-transduced cells, the dCas9 vector contained a puromycin (aminonucleoside) resistance gene, and the gRNA-containing plasmid contained a hygromycin (aminoglycoside) resistance gene. gRNAs were fused to an MS2 scaffold sequence that enabled the MS2 scaffold protein to bind to the gRNAs. Fused to the MS2 protein are additional transcriptional activators to enhance the overexpression of IFNG-AS1[7]. Using these vectors, viral particles can be created to transduce this overexpression system into most human cell lines.

Viral titering and colony screening

After generating the dCas9- and gRNA-containing plasmids, plasmid purifications were performed and lentiviruses were created. As lentiviruses randomly integrate their cassettes into the genome, a quantification of the number of viral particles enables the least number of integrations possible. To accomplish this, conditioned-media-containing viruses were measured with a p24 ELISA kit, allowing for the calculation of the number of virions per milliliter. After measuring, both viral supernatants had nearly 1 µg/mL of p24, which allowed the use of 100 µL of virus to transduce the Jurkat cells. After transduction, antibiotic-selected cells were serially diluted and clones were expanded. Cas9 expression was then analyzed by real-time PCR and agarose gel electrophoresis (**Figure 2B**). Both clones selected were positive for Cas9 expression. To confirm mRNA expression, reverse transcriptase was omitted from the cDNA reaction (**Figure 2B**). Primers against HPRT1 were used to confirm the presence of RNA in the nontransduced cells.

Measuring IFNG-AS1 gene expression

Using the dCas9 clones as a parental cell line, cells were transduced with either a nontargeting gRNA-containing virus or a virus containing gRNAs against IFNG-AS1. After gRNA transduction, selection, and clone creation analogous to dCas9 cell line creation, the IFNG-AS1 expression was measured to verify overexpression. IFNG-AS1 produces three splice variants, each of which can be detected individually with transcript-specific primers (red) or against all known IFNG-AS1 transcripts (blue) (**Figure 3A**). All fluorescence curves were exponential with HPRT1 reaching the exponential phase (or, Ct) within a half cycle between control and IFNG-AS1-gRNA-expressing cells (**Figure 3B,C**). Primers against all known IFNG-AS1 transcripts were the most detectable between experiments with measurements of 20-fold increases in IFNG-AS1 (**Figure 3B**). While primers against transcripts against 1 and 2 successfully amplified in peripheral blood mononuclear cells (PBMCs), these transcripts were not detectable in Jurkat cells (data not

shown). However, when the third transcript of IFNG-AS1 was detectable in concentrated RNA, a five- to tenfold significant increase in IFNG-AS1 levels was seen. These data suggest either alternative splicing of IFNG-AS1 in Jurkat cells exist compared to primary cells. Primers against IFNG-AS1 detected large increases in this gene, thereby validating the activating CRISPR overexpression system.

FIGURE LEGENDS:

Figure 1: A dual vector system to overexpress the lncRNA IFNG-AS1. (**A**) A schematic of the IFNG-AS1 gene structure and the relationship between guide RNA (gRNA) binding sites and the transcriptional start site (TSS). (**B**) The features of the gRNA and dCAS9 vectors.

Figure 2: Viral titering and colony screening. (**A**) An example p24 ELISA standard curve for lentivirus-containing, conditioned media. The black dots represent standard samples and the red dots unknown samples. (**B**) After transducing, selecting, and generating colonies, real-time PCR and gel electrophoresis against dCas9 were performed on the RNA from either nontransduced Jurkat cells (parental) or dCas9-transduced clones. No reverse transcriptase (No RT) controls were performed.

Figure 3: Measuring IFNG-AS1 gene expression. (**A**) A diagram of the relationship between primer sets and transcript variants. The red arrows represent transcript-specific primers, while the blue arrows indicate primers against all known transcripts. (**B** and **C**) Representative average PCR curves and fold-change quantifications for control and IFNG-AS1-overexpressing cells. RFU = relative fluorescence units. $N = 3$ samples per group. Mean ± SD. $*p < 0.05$, $***p < 0.001$. A Student's *t*-test was used to calculate the *p*-values.

Supplemental Figure 1: gRNA design

Supplemental Figure 2: BLAT

Supplemental Table 1: Solutions

Supplemental Table 2: ELISA dilutions

DISCUSSION:

This manuscript presents a protocol for using activating CRISPR to overexpress lncRNAs in vitro. This is an especially important technique when studying long noncoding RNAs as the transcriptomic product is the functional unit. After overexpression, the researcher can, then, use these cells to increase the signal-to-noise ratio when studying binding partners and even measure the cellular consequences of increased levels of lncRNAs. Additionally, as lncRNAs frequently act on cis-genes, this endogenous overexpression technique enables these events to be studied[11,12].

This manuscript highlights a generalized protocol for gRNA creation and lentivirus generation, transduction, and selection, and a representative example of lncRNA overexpression in a

peripheral blood T cell line was outlined. Additionally, this technique has already been shown to be successful in bone-marrow-derived immune cells[13], neurons[7], mouse embryonic stem cells[14], and kidney epithelial cells[4]. While this protocol is generally applicable for most cell lines, cells that are hard to transduce might require individual titers as to enable higher infection rates. Additionally, it is important to perform antibiotic kill curves when using new cell lines, as this protocol utilizes a positive selection strategy.

Of note, this protocol has several technical aspects that require special attention. Reproducible and consistent pipetting for real-time PCR is critical to establish reproducible results. Even small differences in volumes will cause highly variable values, thereby making interpretation difficult. In addition to a proper quantitative PCR primer design, controls, including the no-reverse-transcriptase control for the real-time PCR primers, are critical as they confirm that the RNA being quantified is not genomic DNA. The selection of housekeeping genes for real-time PCR is also important in order to adequately perform these experiments. While HPRT1 was chosen in this protocol, other genes of interests targeted by this technique might alter HPRT1[15]. A careful selection of housekeeping genes based on these factors should be taken into consideration.

There are a few drawbacks to activating CRISPR and to particular aspects of this protocol. One potential limitation is the expression of transcripts in highly gene-rich regions as other neighboring genes could be turned on. It is possible that neighboring genes in close proximity may be activated and controls should be performed to assess this. In addition, other limitations include the lack of binding for particular gRNAs to a given DNA sequence. The selection of multiple gRNAs may be required to identify the appropriate gRNA to drive expression. Another caveat to the system is that the cell of interest has to have the capacity to drive the promoters in the cassettes in order to drive the expression of the gRNAs and dCas9. For the studies presented here, the Jurkat T-cell model was able to drive the expression of these components for a successful overexpression system.

Ideally, the protocol described here should be paired with other corroborating information, such as single transcript overexpression data and functional effects from knockdown or knockout studies, to bolster the case for any of the subsequent findings. This technique offers a robust way of overexpressing any gene in a particular cell type. Given the limitations of lncRNA biology, such as poor species conservation, techniques such as the one described here are critical to exploring their function in relevant cell types. This study focused on one lncRNA that has been implicated in inflammatory bowel disease pathophysiology but serves as a model of studying the function of other lncRNAs in human disease biology.

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DISCLOSURES:

The authors have nothing to disclose.

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15. Matouskova, P. et al. Reference genes for real-time PCR quantification of messenger RNAs and microRNAs in mouse model of obesity. *PLoS One.* **9** (1), e86033 (2014).

Figure 1

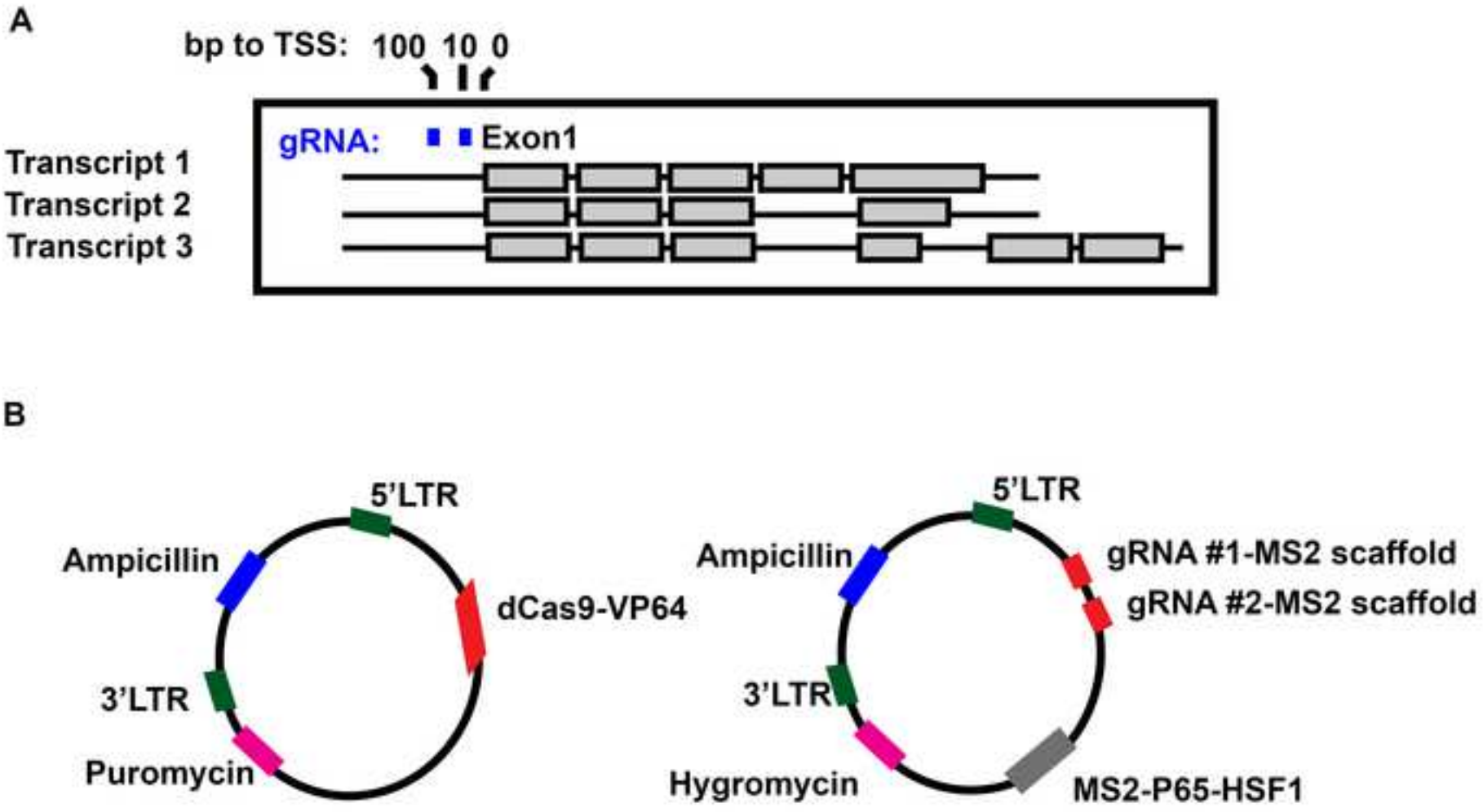
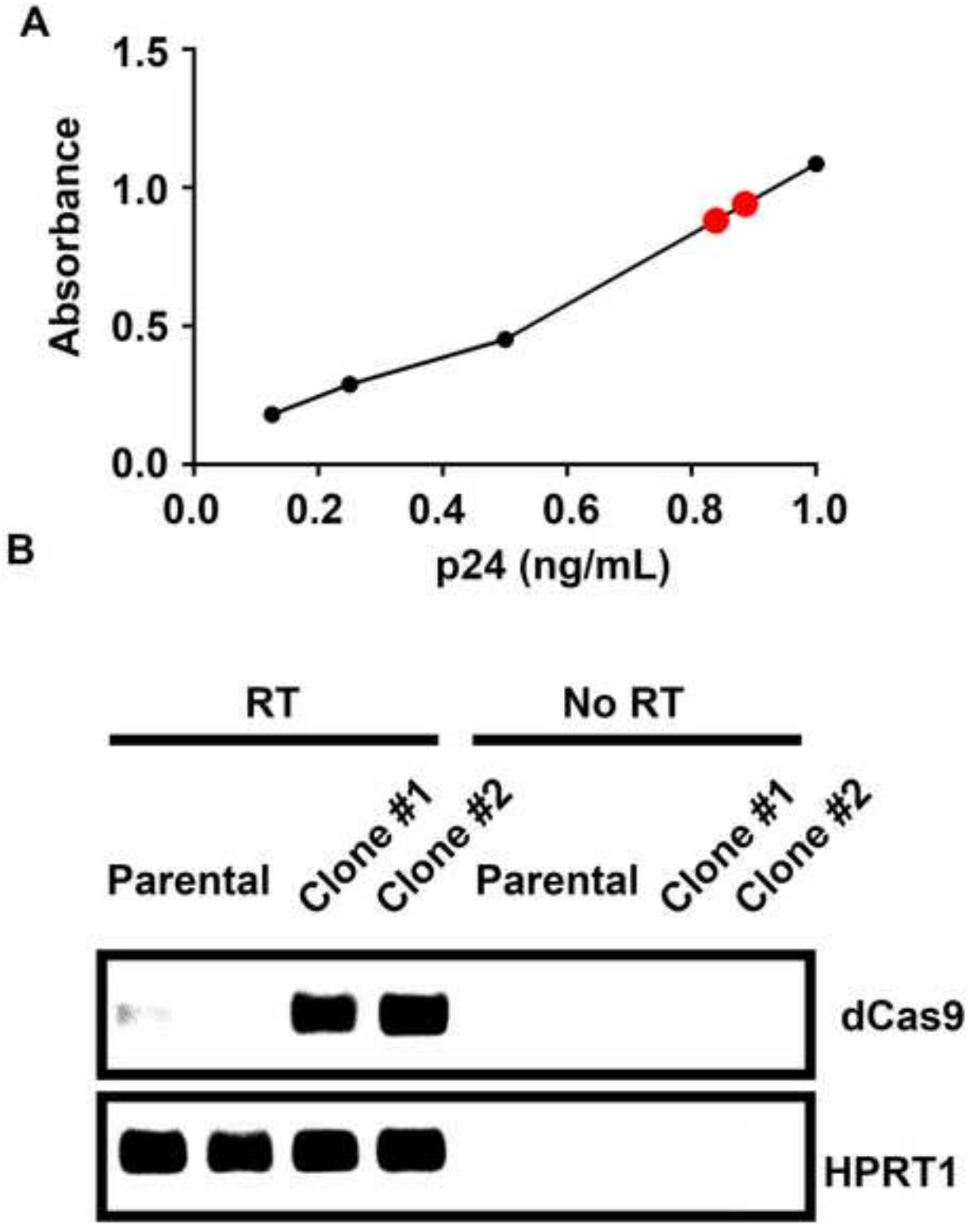
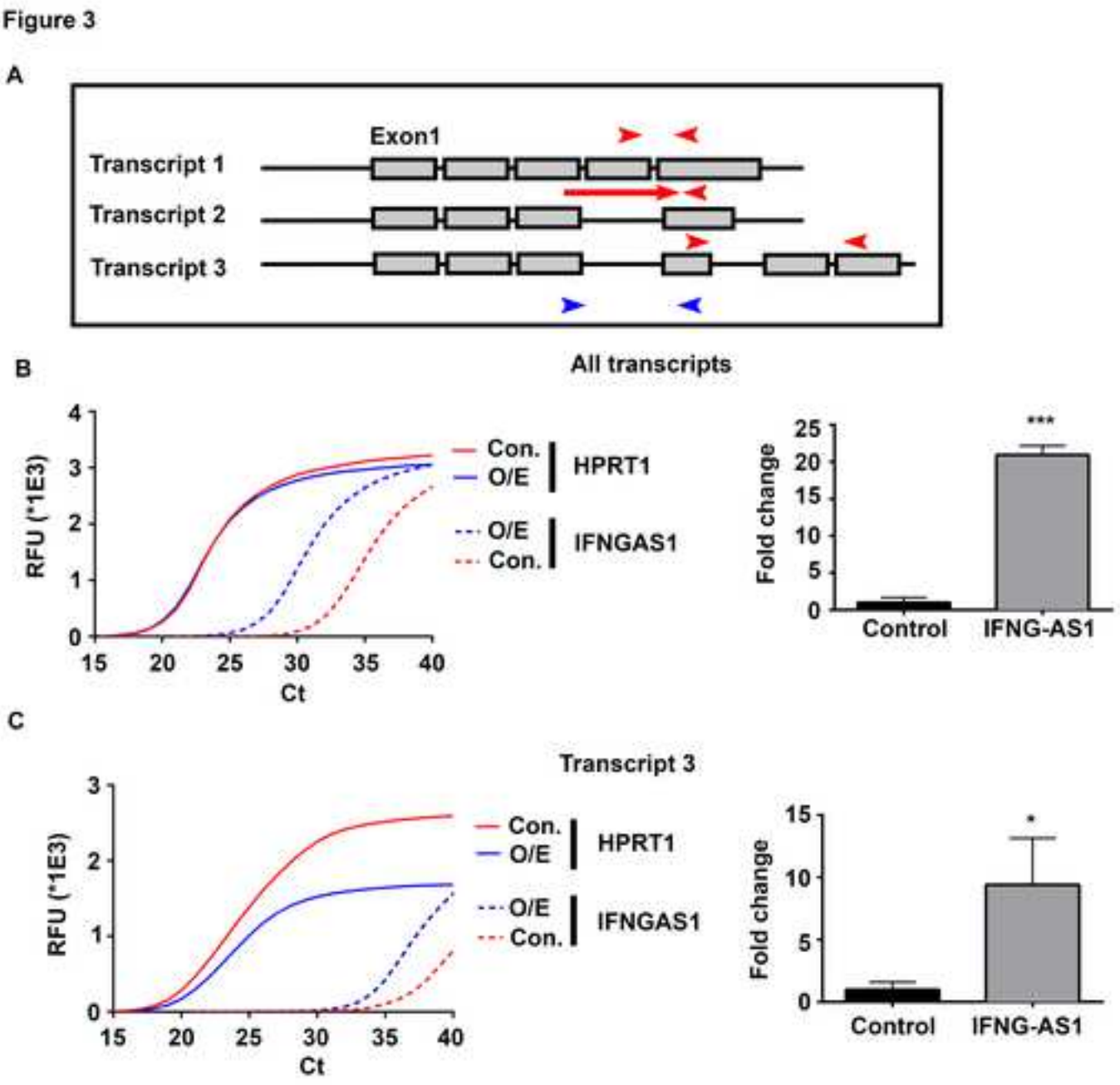


Figure 2





Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Ampicillin	Fisher Scientific	BP1760-5	
$CaCl_2$	Sigma Aldrich	C1016	
cDNA synthesis kit (iScript)	BioRad	1708891	
dCas9 forward primer	Integrated DNA Technologies	n/a	5'-TCGCCACAGCATAAAGAAGA
dCas9 reverse primer	Integrated DNA Technologies	n/a	5'-CTTTTCATGGTACGCCACCT
dCas9 vector	Addgene	50918	
DMEM	Corning	10013CM	
Ethanol	Acros Organics	61509-0010	
FBS	Sigma Aldrich	F2442	
gRNA plasmid	VectorBuilder	VB180119-1195qxv	
HEK293T cells	ATCC	CRL-1573	
HEPES	Sigma Aldrich	H3375	
HPRT1 forward primer	Integrated DNA Technologies	n/a	GACCAGTCAACAGGGGACAT
HPRT1 reverse primer	Integrated DNA Technologies	n/a	GCTTGCGACCTTGACCATCT
Hygromycin B	Corning	MT3024CR	
Isopropanol	Fisher Scientific	BP2618-500	
Jurkat cells	ATCC	TIB-152	clone E6-1
L-glutamine	Corning	25005Cl	
LB agar	Fisher Scientific	BP1425-500	
LB broth	Fisher Scientific	BP1426-2	
Maxi-prep kit (Plasmid Purification Kit)	Qiagen	12362	
Na_2HPO_4	Sigma Aldrich	NIST2186II	
Optimem I reduced serum media	Gibco	31985070	
p24 elisa	Perkin Elmer	NEK050B	
PBS	Corning	21-040-CMR	
Penicillin and Streptomycin	Corning	30-002-Cl	
pMDG2.G	Addgene	#12259	
pMDLg/pRRE	Addgene	#60488	
Poly-L-Lysine	Sigma Aldrich	P-4832	
Polybrene	EMD Millipore	TR-1003	

pRSV-REV	Addgene	#12253
Puromycin dihydrochloride	Sigma Aldrich	P8833
RNA purification kit (Aurum RNA mini)	BioRad	7326820
Sodium Butyrate	Sigma Aldrich	B5887
SYBR green (iTaq universal)	BioRad	1725122
Triton X-100	Sigma Aldrich	X100

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Author(s): Carl Parker, Janet Treger, Emmanuelle Kuwar, Lilian Barbarowicz, Charlotte Ruth Leng, David Padua

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CORRESPONDING AUTHOR

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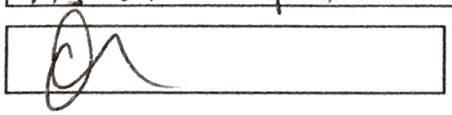
Signature:	
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12/3/18

Dear Dr. DSouza

Below you will find the responses to the comments from the editor. Please let me know if you need any additional information
Thank you,
David Padua

Comment 1: Our format does not allow for this, please define all abbreviations as they appear in the text instead.

We have removed this section and interspersed the abbreviations throughout the text as they appear.

Comment 2: The highlighting (4 pages) currently exceeds 2.75 pages, please edit the highlighting to meet the limits. Please ensure continuity between highlighted steps.

We have highlighted a section to be within the limits.

Comment 3: Nothing to film so I made this step into a note.

Ok

Comment 4: Please move the commercial names to the table of materials and avoid referencing them in the manuscript.

We have removed the commercial name references

Comment 5: I am not sure what we can film here. Can you provide an example gRNA sequence and screenshots of how you perform this search? The screenshot(s) can be provided as supplementary files.

Supplemental Figures have been provided and the text edited

Comment 6: Which strain? Please add to the table of materials.

E. coli

Comment 7: define

We have defined LB to Luria Broth

Comment 8: tube containing bacteria, correct?

yes

Comment 9: I've made these into substeps, is this appropriate?

Yes

Comment 10: What are the specs? Is this part of the kit?

These columns are provided by the kit, the text has been edited to reflect this

Comment 11: The day after the DNA purification? How is the pellet stored in the meantime? Please mention storage temperate and conditions.

We have edited the text to state that "when ready to create the dCAS9 containing lentivirus," We have added storage of the DNA in section 1.5.4

Comment 12: Stored in 2.8?

Yes, we have edited this section

Comment 13: I've made this into substeps, please check that this is appropriate.

Yes

Comment 14, 15: from the kit?

Yes

Comment 16: On a spectrophotometer?

Yes, we have edited the text

Comment 17: define

We have edited the text

Comment 18: unclear, please revise:

We have edited the text to state "Using a spectrophotomer read 450 nm immediately, repeat 10 times at 1 second intervals"

Comment 19: From the kit?

Yes

Comment 20: Please re-write using imperative voice as the previous steps.

The text has been edited

Comment 21: Made this into a note

Ok

Comment 22: We cannot film calculations so I have unlighted this.

Ok

Comment 23: Fig 1C was not discussed.

We removed the "C" label, combining B and C together

Comment 24: Titer?

We have edited the text

Comment 25: Panel C needs to be defined

We removed the "C" label, combining B and C together and re-defined panel B

Comment 26: Titer?

The text was edited

Comment 27: Define RFU.

The text was edited

Dear Dr. DSouza,

Thank you for the opportunity to revise our manuscript. We have attempted to address the editor's comments as well as the reviewers' comments. Please let me know if there are any additional questions or concerns. We would be happy to address any issues that arise. Below is a point-by-point response.

Thank you,

David Padua, M.D. Ph.D.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Thank you for the opportunity to review the manuscript. We have thoroughly reviewed the revised manuscript for spelling or grammatical errors.

2. Keywords: Please provide at least 6 keywords or phrases.
We now include 6 keywords for the manuscript.

3. Please define all abbreviations before use.
All abbreviations are now listed after the keyword section.

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
SI abbreviations are now used for all units.

5. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.
All numerical values have a space between their corresponding units.

6. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
We now list centrifuge speeds in centrifugal force.

7. Please remove commercial language: Qiagen, Triton X-100, Optimem, etc.
We have removed the wording for Optimem and replaced it with "reduced serum media". Triton X-100 is the name of a chemical compound so we believe removing it and replacing it with something else would confuse the readers.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
The manuscript has been edited to avoid the use of personal nouns.

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc.

However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
We have changed all of the actions to the imperative tense.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
We have now gone into more detail throughout the protocol. Additionally, we now list the step for RNA extraction, cDNA synthesis and real time PCR. We also have addressed the examples below.

11. 1.2.6: Please describe how to maxi-prep bacteria.
We have now listed a detailed protocol for maxi-prepping bacteria in section 1.

12. 2.2: Please provide the composition of fibroblast culture medium.
While the fibroblast culture medium composition was in the previous solutions table, we have now altered its name to "DMEM complete" as these media are interchangeable

13. 2.5: What is DPBS? Please split it into two steps.
As there is no significant difference between DPBS (Dulbecco's phosphate-buffered saline) and PBS, we now just use PBS to make it easier to understand.

14. 2.7: Please provide the composition of Wash Concentrate.
The Wash Concentrate is part of the p24 ELISA kit. We do not know its composition. We now state that a p24 ELISA kit is used at the beginning of the section to clarify this.

15. 3.1: Please specify the growth conditions.
We now state the growth conditions for the cells in section 3.

16. 3.2: Please specify centrifugation conditions (force and time).
We now state centrifuge conditions in section 3.

17. 4.6: Please specify the PCR primers used.
The sequences for the dCas9/HPRT1 PCR primers are now listed in section 4.11.

18. Lines 180-181: Please remove the embedded table. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file. Reference the table in the protocol.
We have moved the p24 ELISA dilution table to an excel sheet and referenced the table in the protocol.

19. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Please include single-line spaces between all paragraphs, headings, steps, etc.
We have combined the shorter protocol steps so that each individual step generally contains 2-3 actions with a maximum of 4 actions.

20. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the

essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

21. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

22. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

The sections have been highlighted.

23. Please remove the embedded figure(s) from the manuscript. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
We have removed the embedded figures and uploaded .tiff images of each figure.

24. Figure 3: Please define the error bars in the figure legend.
We have defined the error bars for figure 3.

25. Lines 393-423: Please include solution composition in a table and reference the table in the protocol.
We now include the solution composition as a table and reference the table in the protocol.

26. Discussion: Please discuss critical steps within the protocol.
We now discuss the critical steps within the protocol in the discussion, lines 442-449.

27. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.
We now use the JoVE endnote style for the references.

28. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
29. References: Please do not abbreviate journal titles. If there are six or more authors, list the first author and then “et al.”.
We now use the JoVE endnote style for the references.

30. Lines 425-459: Please move such information to the Table of Materials.
Response: We now only list all materials in an excel sheet labeled “Table of Materials”.

31. Table of Materials: Please sort the items in alphabetical order according to the Name of Material/Equipment.
The table of materials is listed in alphabetical order now.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors describe point-by-point method for CRISPR-based activation of lncRNAs (or genes in general). While the methodology is not really novel, however it may be a useful resource for researcher seeking to understand the basics of the CRISPR-based activation strategy.

Below are a few comments that need to be addressed:

Major Concerns:

1) The authors demonstrate successful activation of IFNG-AS1. They describe their gRNA design strategy. They mention to "ensure that the gRNA should be the reverse complement to the DNA sequence."

What is the evidence that targeting the reverse complement strand is important?

The two studies below do not support this:

In Gilbert et. al. 2014, they also found that "…the DNA strand that was targeted [did not] strongly correlated with sgRNA activity…"

Similarly, Konermann, et. al. 2015 reported: "high efficiency of guides within 200bp of the TSS regardless of strand orientation"

Can the authors comment on this?

The gRNAs in our representative experiment were designed by the company, Vector Builder. The protocol we listed was a reverse engineering of their design. We did not mean to suggest that the strand orientation mattered. We now clarify this in section 1 and suggest bioinformatic software for choosing gRNA sites.

2) In the diagram below, the gRNAs appear to target downstream of the TSS. I can see from the "bp to TSS" that the actual TSS is downstream of the thin line that is drawn. What does the thin line represent? It's most intuitive that it should represent the entire transcript sequence and hence the beginning of the line should be the TSS. Can the authors fix this?

The thin line represents the location of the TSS on the genome. On our screen the thin line at “0” bp matches up with the TSS. The gRNAs are upstream of the TSS as denoted by the negative sign before bp, which is the common notation.

3) In figure 2B: Were high Cas9 levels the basis for clonal selection? Did IFNG-AS1 levels always correlated with higher Cas9 levels?

We observed high Cas9 levels in all clones selected. We clonally selected cells to ensure a stable population before transduction with the gRNA containing virus.

a. It would be great to plot Cas9 vs. IFNG-AS1 transcript levels to demonstrate this relationship and/or to determine whether there's a threshold effect.
b. Can the authors comment?

We did not test Cas9 levels versus IFNG-AS1 levels.

The paper could benefit with additional discussions. Other techniques such as the SAM system could be discussed especially when talking about difficult to induce transcripts. In our experience the promoters in the vectors can play a role in whether a construct works in your cell line of choice so some discussion on this topic could also be beneficial.

The system we used was the SAM system, we reference this in Maeder et al. We did not test different promoters in our system. We now mention in the discussion (lines 455-456) that depending on the cell line/tissue of origin different promoters might not work.

Minor Concerns:
In the abstract there is a word missing in the sentence "In order to
study the molecular functions, lncRNA's can either removed or over-expressed so as to measure their cellular effects." I assume it should be "can be either…"

We have corrected the missed word.

Step 1.1.4 guides can be obtained from commercial vendors, I think it is important to state where the ones are from that you are using. Addgene ID numbers could be useful here

We now state the source of the vectors in section 1 and refer to the table of materials for the catalog numbers.

Reviewer #2:

Manuscript Summary:
The manuscript described a method to over-express long non-coding RNAs using gene-activating CRISPR.

Major Concerns:

"1.1.1 Identify a gRNA sequence upstream of the nucleotide sequence "NGG" that also is within 100 base-pairs of transcriptional start site." What the "NGG" sequence and how to identify it? The "NGG" sequence as well as the gRNA specific for the target gene should be shown.

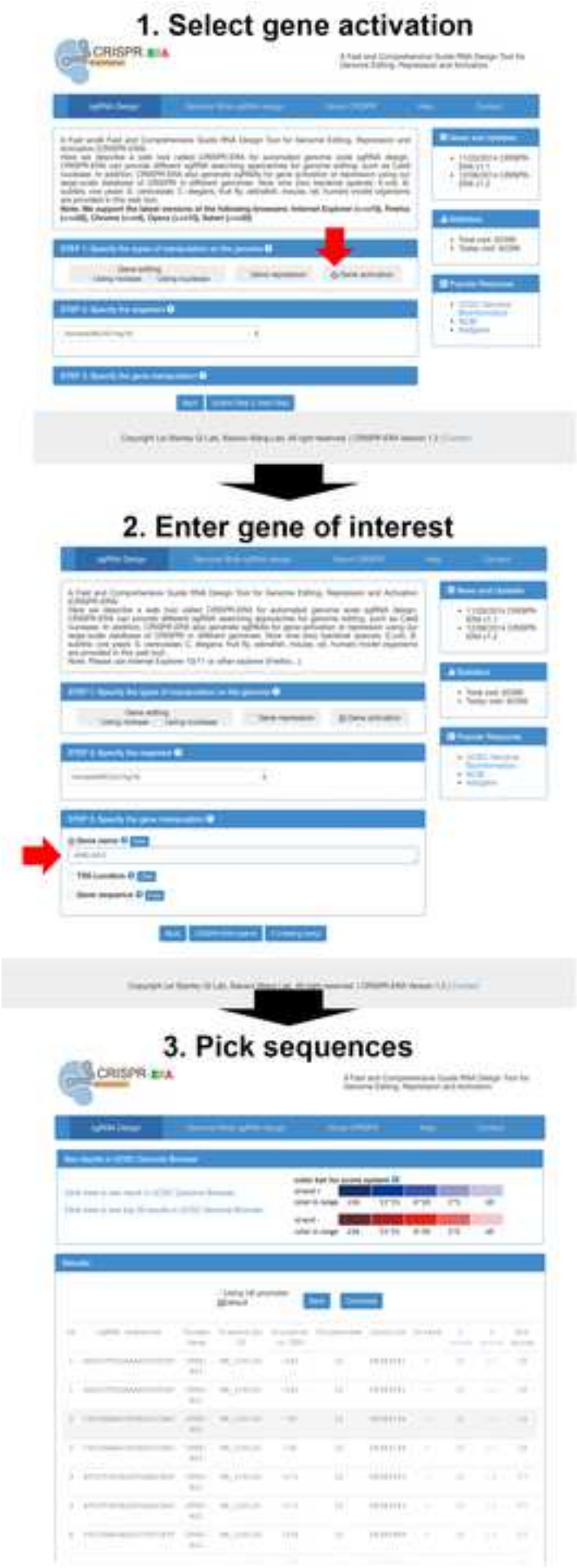
We now explain what "N" means and refer to bioinformatics software to choose gRNA sequences in the first section of the protocol.

What is the difference between the manuscript and the references 4-6(Nat Methods, 2013, 10:973-6; Science,367 2012,337:816-21; Nat Methods 2013,10(10):977-9.)?

While the main difference between this manuscript and references 4-6 is the addition of the visualized protocol, there are also a few other big differences. First this is a complete protocol going with highly detail instruction about how to procure the reagents, preform all of the techniques required and analyze the results. We also discuss the pitfalls and limitations of this technique, many which were not stated in those references. We also created a different gRNA vector than stated in the above references.

Supplemental Fig 1- gRNA design

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental figure - gRNA design.tif



Supplemental Fig 2- BLAT

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental figure - BLAT.tif

1. Enter gRNA sequence

2. Analyze genomic hits

Solutions Table

Reduced serum media with polybrene

10 mL optimem I +10 µL 10 mg/mL polybrene (1 µg/mL).

Some adherent cell lines can tolerate up to 10 µg/mL of polybrene.

DMEM complete

DMEM + 10% FBS + 1% penicillin and streptomycin

DMEM complete with puromycin

DMEM complete + 1 µg/mL puromycin. Some adherent cell lines require puromycin concentrations up to 10 µg/mL. 10 mg/mL aliquots of Puromycin can be stored in aliquots at -20 ^{o}C.

DMEM complete with hygromycin

DMEM complete + 100 µg/mL hygromycin. Some adherent cell lines require puromycin concentrations up to 500 µg/mL.

Ampicillin

100 mg ampicilin in 100 mL water, store 1 mL aliquots at -20 ^{o}C.

LB agar with ampicillin

Mix 20 g LB agar in 500 mL water, autoclave and add 500 µL ampicillin once samples are below 55 ^{o}C

LB broth with ampicillin

Mix 12.5 g LB broth in 500 mL water, autoclave and add 500 µL ampicillin once samples are at room temperature

2X HBS

280 mM NaCl, 1.5 mM Na_2HPO_4, 100 mM HEPES. For 500 mL: Mix 28 mL 5 M NaCl, 1.5 mL 0.5 M Na_2HPO_4, 50 mL 1 M HEPES, pH to 7.12, filter sterilize through 0.2 µm filter, dispense in 10 mL aliquots, freeze solution at -20 ^{o}C (stable for several months)

2.5 M $CaCl_2$

100 g CaCl2 in 250 mL water. Filter sterilize through 0.2 µm filter, dispense in 10 mL aliquots, freeze solution at -20 ^{o}C .

DNase I solution

This is part of the RNA isolation kit. Add 5 µL DNaseI to 75 µL DNaseI buffer per sample. Store aliqouts of DNaseI at 20 ^{o}C and make this solution fresh each time.

Supplemental Table 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);p24 ELISA Dilutions.xlsx

Standard Conc. (ng/mL)	Tube Label	Addition (µL)	Diluent (µL)
4	1	20 of Pos. Control	980
2	2	500 of tube 1	500
1	3	500 of tube 2	500
0.5	4	500 of tube 3	500
0.25	5	500 of tube 4	500
0.125	6	500 of tube 5	500
0.063	7	500 of tube 6	500
0.032	8	500 of tube 7	500
0.016	9	500 of tube 8	500
0.008	10	500 of tube 9	500