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Genome-Wide CRISPRi/a Screening in an In Vitro Coculture Assay of Human Immune Cells with Tumor Cells

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Abstract

Cell-based immunotherapy has achieved preclinical success in certain types of cancer patients, with a few approved cell-based products for clinical use. These achievements revitalized the field of cell engineering/immunotherapy and brought attention to the opportunities that cell-based immunotherapeutics can offer to patients. On the other hand, obvious indications emphasize the need for a better understanding of the biological mechanisms involved in the immune response. This knowledge may not only ameliorate safety and efficacy, but also determine the possibilities and limitations in use of immune cell engineering for cancer treatment, and facilitate developing novel immunotherapeutic strategies. Recently developed technology based on CRISPR-dCas9 has an immense potential to systematically uncover genetic mechanisms by identifying subsets of essential genes involved in interactions of cancer cells with the immune system. This chapter will present a reliable and reproducible general protocol for the application of genome-wide sgRNA gene-editing tools in the recently established two-cell type co-culture, consisting of immune cells as effectors and cancer cells as targets, utilizing CRISPRi/a-dCas9-based technology.

Keywords

CRISPRi/a; Genome-wide screening; Two-cell type coculture assay; Cell-based immunotherapy

1 Introduction

The ongoing development of precise molecular, cell-manipulation tools is opening up a wide prospect for specific cell-based therapeutic approaches. In particular, recent applications of

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¹⁴.Prior to the actual experiment, it is also advised to test a wide range of lentiviral particle dilutions (1:10–1:100), in order to determine the optimal dilution for the desired downstream applications. Please note that transducing too many cells, relative to the number of lentiviral particles, significantly reduces transduction efficiency, resulting in large amounts of cell death upon drug selection.

³⁰.To minimize associated errors in sgRNA amplification, it is important to use a high-fidelity polymerase such as Pfu Ultra II (e.g., Agilent) or Kapa HiFi (Kapa Biosystems).

³¹.The corresponding sequencing primers are listed in Table 2.

the CRISPR/Cas9 system have rapidly advanced the landscape of genome engineering in the fields of cancer biology and therapeutic gene-editing applications in human disease treatment [1]. Multiple ongoing clinical trials are utilizing CRISPR/Cas9 to knockout (KO) PD-1 from T cells of patients with lung cancer (NCT02793856) [2], prostate cancer (NCT02867345), bladder cancer (NCT02863913), esophageal cancer (NCT03081715), leukemia and lymphoma (NCT03166878, NCT03398967), renal cell cancer (NCT02867332), and others (NCT03044743, NCT03399448). These studies are the first proof of concept of the application of CRISPR/Cas9 technology in human cancer treatment. CRISPR screens have also been widely adopted to conduct comprehensive genome-wide scale gene disruption to facilitate the discovery of essential and/or drug resistance genes in various cancer types [1, 3]. Subsequent modifications of the CRISPR/Cas9 system, through the establishment of CRISPR interference (CRISPRi) and activation (CRISPRa) tools (Fig. 1), facilitate controlling transcript levels of endogenous genes [4, 5].

CRISPR screens allow the identification of genes, which influence a specific phenotype in an unbiased fashion. Hence, the chosen approach requires a number of technical and rational considerations.

1. The Cas9 enzyme is natively an RNA-guided DNA endonuclease. The most typical outcome after a double-strand DNA break is repair via the nonhomologous end-joining (NHEJ) pathway, which often introduces small insertions or deletions [6]. Several modified genetic screening platforms for mammalian cells based on this pathway, also known as CRISPR nuclease (CRISPRn), have been established [1]. A mutant form of Cas9 (dCas9) was engineered to inactivate the nuclease activity of Cas9, without affecting its ability to bind DNA through sgRNA targeting [7]. This in turn sterically prevents transcription factors and RNA polymerase from accessing DNA promoter regions. This tool can be used to either inhibit or activate transcriptional activity as CRISPRi and CRISPRa approaches, respectively [8] (Fig. 1). CRISPRi is based on the fusion of dCas9 to a transcriptional repressor domain (e.g., Krüppel-associated box (KRAB) domain), which promotes heterochromatin-mediated gene silencing in mammalian-derived cells [7] (Fig. 1a). CRISPRa constructs recruit multiple activator domains that are directly fused to dCas9. There are currently several CRISPRa approaches, including the VPR approach, via a direct fusion of multiple activator domains (e.g., VP64, p65, and RTA (VPR)) to dCas9 [9] (Fig. 1b); the SunTag approach, via a protein scaffold consisting of VP64, which is fused to superfolder GFP (sfGFP) and an antibody single-chain variable fragment (scFv), which is able to target the transcriptional activator GCN4 epitope [7]; and the SAM approach, via an RNA scaffold that consists of MS2 RNA hairpins, which bind to dimers of MS2 coat protein (MCP) fused to p65 and HSF1 transcriptional activation domains [10]. Genome scale screens, based on CRISPRn, CRISPRi, and CRISPRa technologies, have already been successfully tested in pooled screens [1].
2. The sgRNA expression construct library pools are stably integrated into the genomes of mammalian cells, via lentiviral transduction and designed to target

on specific pathway components and/or genomes on a wide scale, utilizing 5–10 sgRNAs to target each gene. Although the system is very reliable, it is recommended to optimize and validate optimal infection conditions. Although the number of sgRNAs per gene can be varied (at least 3–5 sgRNA/per gene), it is recommended to design 5–10 sgRNAs/per gene in a primary screen, in order to achieve more reliable results for specific gene targeting. There are numerous resources available that can assist in sgRNA design, such as:

- a. <https://omictools.com/crispr-grna-design-tool-tool>
- b. <https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design-crisprai>

CRISPRa and CRISPRi pooled libraries generally select a representative transcription start site (TSS) for a gene (Fig. 1a, b). In addition to genome-wide sgRNA pools, sgRNA subsets that target genes according to their biofunctional theme are also commercially available (e.g., Kinases Screen, Cancer and Apoptosis Screen, Membrane Proteins Screen, and Mitochondrial Proteins Screen). An alternative approach is to customize the CRISPRi/a library pools, which can be done in-house or through commercial vendors. The relative abundance of each sgRNA in a given experimental cell pool can be quantified by comparing the frequencies of cells expressing sgRNA at an initial time point, compared to those at a later time point [1]. It is important to consider certain limitations regarding the end point of the utilized cell viability screens, and many mechanisms of genetic interactions between perturbation/depletion and cell state specific gene expression changes over time have been noted [11–13]. The isolation of cells at multiple time points may provide clearer distinctions, but it is also associated with a higher cost.

3. In a pooled genetic CRISPR-based screen, lentiviral delivery of dCas9 and the sgRNA often utilize a drug resistance gene (e.g., puromycin, hygromycin, neomycin, blasticidin) or fluorescence-reporter gene tag (e.g., GFP, BFP, mCherry) to select for pure target cell populations. In general, puromycin treatment results in quicker elimination of nonresistant cells, while neomycin or hygromycin treatments may take weeks, with the appropriate treatment doses varying across selected cell types [14].
4. The most crucial step in the general screening strategy is to deliver the dCas9 gene prior to sgRNA pools. In most cases, the delivery dCas9 and/or sgRNA pools (being inserted in expression plasmids, carrying either drug resistance and/or fluorescent protein-coding genes) can be accomplished by several well-established approaches, with lentiviral delivery often achieving the best transfection efficiencies [1, 8, 15]. During the next step, it is absolutely crucial to confirm dCas9 presence and stability by evaluating its expression levels, both at genetic (e.g., RNA by RT-qPCR) and protein levels (e.g., Western blot). Utilization of a pre-established dCas9 expressing cell line, prior to the introduction of sgRNA pools, can significantly improve the quality of library

screening and further provides a universal background during the screening procedure.

5. Ultimately, the critical step in a pooled CRISPR-based screen is the isolation of genomic DNA. Many commercial kits and homemade protocols are readily available, although there is no one-size-fits-all solution, due to variations in yield and quality across different cell types. The quantification of library abundance, as a read-out of library screens, requires a two-step PCR amplification step on the isolated genomic DNA, prior to subjecting the PCR product to next-generation sequencing.

Presented below is a reliable and reproducible general protocol for genome-wide sgRNA screening in a two-cell type in vitro co-culture assay, consisting of immune cells as effectors and cancer cells as targets, utilizing the recently established CRISPRi/a-dCas9-based technology. This protocol is also compatible with Tet-inducible CRISPRi/a-dCas9 system for gene-editing applications in either all types of immune or cancer cells.

2 Materials

2.1 Equipment

1. Thermocycler (Bio-Rad T100™, temperature range: 0–100 °C).
2. Bench centrifuge or microcentrifuge.
3. Spectrophotometer.
4. CO₂ incubator (temperature range 37 °C).
5. Microbiological culture hood.
6. Temperature-regulated shaker (temperature range covering 37 °C).
7. Electroporator and electroporating cuvettes.
8. Large gel system.
9. QuBit fluorometer.
10. Bioanalyzer 2100.
11. Access to an Illumina sequencing facility (preferably HiSeq 4000 sequencer, Illumina).

2.2 Plasmid Vectors

1. pHR-TRE3G-KRAB-dCas9-mCherry and/or pHR-UCOE-TRE3G-dCas9-VPR-mCherry.
2. pHR-Tet3G.
3. pU6-sgRNA-Ef1 α -Puro-T2A-BFP.
4. hCRISPRi sgRNA library pool and/or hCRISPRa sgRNA library pool.
5. Lentiviral packaging plasmids: pCMV-dR8.91 and pMD2-G.

2.3 Cells and Culture Media

1. HEK293T cell line.
2. Effector cells (e.g., NK cells, T cells, TILs).
3. Target cells (e.g., cancer cells, primary or cell line).
4. Complete HEK 293 T cell culture medium (DMEM, 10% FBS, 1× Pen-Strep).
5. Cell culture medium for effector and target cells.

2.4 Supplies and Reagents

1. Pipette tips (RNase and DNase free).
2. Sterile culture dish (15 cm²).
3. Sterile 6-well plates.
4. Sterile 1.7-ml tubes.
5. Sterile 15-ml falcon tubes.
6. Sterile T25 flasks.
7. Centrifuge tubes (Max volume: 1.5 ml; RNase and DNase free).
8. PCR tubes or 96-well PCR plate (Max volume: 50 µl; RNase and DNase free).
9. X-tremeGENE HP DNA transfection reagent.
10. Polybrene (final working concentration: 8 µg/ml).
11. DEAE dextran sulfate (final working concentration: 6 µg/ml).
12. Puromycin (final working concentration: 1 or 2 µg/ml).
13. Tetracycline (stock in DMSO: 10 mg/ml, final working concentration: 500 ng to 1 µg/ml).
14. 2× annealing buffer (200-mM potassium acetate; 60-mM HEPES-KOH, pH 7.4).
15. 1× PBS.
16. DMSO.
17. Designed oligonucleotides (Table 1).
18. Restriction enzymes: BstXI and BlnI.
19. 10× CutSmart buffer.
20. Ultrapure agarose.
21. Electrophoresis buffer (10× TAE or 10× TBE).
22. DNA loading dye (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol FF).
23. SYBR Green I nucleic acid gel stain-10,000×.

24. Premade LB-Agar plates supplemented with ampicillin (100 µg/ml).
25. *DH5a* chemically competent cells and/or MegaX DH10B electrocompetent cells.
26. Lenti-X concentrator.
27. Genomic DNA extraction kit (e.g. Macherey Nagel Blood Kit).
28. NucleoSpin Blood Kits.
29. Quick-gRNA MidiPrep Kit.
30. SbfI-HF restriction enzyme.
31. Gel extraction kit.
32. Phusion high-fidelity DNA polymerase.
33. dNTPs mix.
34. Custom PCR and sequencing primers (Table 2).
35. SPRI select beads.
36. Magnetic stand.
37. 1-Kb plus DNA ladder.
38. 50-bp DNA ladder.
39. Agilent DNA 1000 reagents.
40. p24 ELISA kit.

3 Methods

3.1 *Annealing of Single-Stranded Complementary Oligonucleotides (See Note 1)*

1. Dissolve each oligonucleotide in a volume of annealing buffer to create a 100 µM stock solution.
2. Mix equal volumes of the dissolved complementary oligonucleotides in a PCR tube.
3. Heat to 95 °C and maintain the temperature for 2–5 min, cool to 25 °C over 45 min, and then cool to 4 °C for temporary storage.
4. Use a 1:20 dilution of annealed Oligos in ddH₂O for the subsequent steps.

¹The oligonucleotides do not require phosphorylation. When annealing, slow cooling to room temperature by putting the tubes on the lab bench rather than the default cooling ramp rate on a conventional PCR machine is critical. Annealed oligos can be stored at –20 °C and are stable through at least 2–3 freeze-thaw cycles. If many sgRNAs are to be cloned at once, it is recommended to buy the corresponding oligos in presuspended form at 100 µM each, in a plate or an arrayed format, which allows easy combining of complimentary oligos by multichannel handling. For higher throughput, it is advised to keep everything in PCR plates throughout the protocol until bacteria cultures have been plated.

3.2 Cloning of Annealed Oligonucleotides (sgRNAs)

1. Add 2–10 µg of pU6-sgRNA-Ef1 α -puro-T2A-BFP plasmid to 1 µl of BstXI, 1 µl of BlnI, 1 µl of 10× CutSmart buffer and water to 10 µl total volume, incubate the digestion mixture at 37 °C for at least 1 h.
2. Confirm successful plasmid digestion by agarose gel electrophoresis, and proceed to plasmid purification procedures, by using a commercially available plasmid purification kit (e.g., ZymoPure plasmid purification kit), according to the manufacturer's instructions.
3. Verify the concentration of the plasmid by spectrophotometric analysis.

3.3 Ligation of Annealed Oligonucleotides with Digested Plasmid Backbone

1. Add 100 ng of digested pU6-sgRNA-Ef1 α -puro-T2A-BFP plasmid (*see* Subheading 3.2) to 2 µl of annealed oligonucleotides (1:20 dilution) (*see* Subheading 3.1), 2 µl of 10× T4 DNA ligase buffer, 1 µl of T4 DNA ligase and water to 20 µl total reaction volume.
2. Incubate at room temperature for 1–4 h or at 16 °C over night.

3.4 Transformation into DH5 α Bacteria

1. Add 1 µl of individual ligation reaction (*see* Subheading 3.3) to 20 µl of thawed *DH5 α* cells and incubate on ice for 30 min, followed by 42 °C for 45 s, and then immediately on ice for at least 2 min.
2. Add 1 ml of prewarmed sterile LB medium to the transformed *DH5 α* cells, incubate them at 37 °C for at least 1 h for recuperation in a temperature-regulated shaker, set at 200 rpm.
3. Spread 100 µl of recuperated transformed *DH5 α* cells on prewarmed LB-agar plates, supplemented with ampicillin (100 µg/ml) to achieve single colonies.
4. Incubate the plated LB-agar plates in the incubator at 37 °C overnight.
5. Pick several single colonies per plasmid-oligonucleotide construct and amplify colonies in LB medium, supplemented with ampicillin (100 µg/ml) at 37 °C for at least 10–16 h in a temperature-regulated shaker, set at 200 rpm.
6. Subject the amplified bacterial culture to plasmid extraction procedures, by using an appropriate plasmid purification kit (e.g., ZymoPure Plasmid purification kit or QIAprep Spin Miniprep kit), according to the manufacturer's instructions.
7. For individually extracted plasmid-oligonucleotide constructs, determine the concentration by spectrophotometric analysis (e.g., Nanodrop) and verify the integrity and purity by agarose gel electrophoresis.
8. Verify the incorporation and sequence of individually ligated oligonucleotides inside the pU6-sgRNA-Ef1 α -puro-T2A-BFP plasmid by sequencing, with the following sequencing primer: MP177–5'-gagatccagtttggttagtaccggg-3'.

9. To avoid unnecessary time- and cost-consuming vector preparation and sequencing for a larger sgRNA pool (more than 100 sgRNAs), the use of a robust Gibson Assembly PCR strategy [16] or purchase from a commercially available resource (e.g., Addgene) is recommended.

3.5 Amplification of Individual sgRNA or a Mixed sgRNA Pool

1. Mix a predefined number of sgRNA expression vectors, based on the set of genes to target, in equimolar amounts.
2. Dilute the mixed sgRNA sample to 100 ng/μl in 1× TE buffer.
3. Prewarm the recovery medium and plates to room temperature.
4. For each tube, add 1 μl of the mixed sgRNAs pool (100 ng/ μl) to 50 μl of MegaX DH10B electrocompetent cells in an electroporation cuvette and electroporate at 1.8 kV and 180 Ω (*see Note 2*).
5. Add 1 ml of recovery medium into the cuvette, transfer the electroporated cells to a sterile cell culture falcon tube, and add an additional 3 ml of recovery medium.
6. Place the cell culture tubes in the shaking incubator for 1 h at 37 °C, set at 250 rpm.
7. For each of the transformed sgRNA pools, individually transfer 5 μl of the culture to an individual sterile 1.8-ml eppendorf tube, containing 995 μl of recovery medium and mix well by pipetting (**do not vortex!**).
8. From each tube (**step 7**), transfer 40 μl to an individual sterile 1.8-ml eppendorf tube, containing 200 μl of recovery medium, mix well by pipetting (**do not vortex!**), and replate on individual prewarmed LB-agar plates, supplemented with ampicillin (100 μg/ml).
9. Incubate the LB-agar plates at 37 °C for 14–16 h and count the number of colonies on each plate. To obtain the real colony number for each of the electroporations, multiply each counted colony number by the dilution factor of 4,000,000 (*see Note 3*).
10. For each electroporation, divide the remaining culture (3.995 ml) into two sterile 500 ml Erlenmeyer flasks, containing 250-ml prewarmed LB medium and supplemented with ampicillin (100 μg/ml). Incubate the eight Erlenmeyer flasks in the shaking incubator overnight at 37 °C, set to 250 rpm.
11. Collect and centrifuge each of the bacterial cultures in sterile falcon tubes (50 ml) by centrifugation at 4000 × *g* for 30 min.

²Enough DNA (up to 400 ng) and 50–100 μl of competent bacteria have to be used, to ensure the representation of all sgRNAs of the corresponding hCRISPRi/a libraries.

³The ideal efficiency should be equivalent to or greater than 200 colonies/per sgRNA, to ensure equal representation of each sgRNA in the library. Therefore, proceed only if the total number of colonies is at least 1.4×10^7 .

12. Proceed with isolation and purification of the amplified sgRNA-containing plasmids by utilizing the EndoFree plasmid maxi kit, according to the manufacturer's instructions (*see* Note 4).
13. Determine the concentration by Nano-drop ND1000, and verify the integrity and purity of amplified pools of plasmids with sgRNA by electrophoresis (*see* Note 5).

3.6 Production of Lentiviral Particles (Fig. 2)

1. The day prior to transfection, plate 7.5×10^6 HEK293T cells in a total of 25 ml of complete medium in a 15-cm sterile culture dish.
2. Proceed only when the cell confluence has reached between 30% and 40%.
3. Prewarm $1 \times$ PBS, Opti-MEM, and corresponding culture dishes to 37 °C.
4. Remove complete medium and wash cells with sterile prewarmed $1 \times$ PBS to remove any residual FBS.
5. Mix 1500 μ l of prewarmed Opti-MEM with 45- μ l X-treme- GENE HP DNA transfection reagent in 1.8-ml tubes and incubate the mixtures at room temperature for 5 min.
6. To the 1.8-ml tube, add the desired lentiviral plasmid (9 μ g), with the corresponding packaging vectors (8 μ g of pCMV dR8.91 and 1 μ g of pMD2-G) (*see* Note 6).
7. Carefully close the cap of 1.8-ml microcentrifuge tube, mix well (**do not vortex!**), and incubate at room temperature for 15–30 min.
8. Pipette the mixtures slowly by drop-wise addition onto the cultured HEK293T cells in 10 ml of complete medium with 10% FBS.
9. The next day, add 8 ml of complete medium with 10% FBS and then allow viral particle production to continue for 48–72 h before harvesting (*see* Note 7).
10. Carefully collect all the culture supernatant (expected volume: ~18 ml), avoiding any detached cells.
11. Filter collected supernatant through a 0.45- μ m filter and/or apply the collected supernatant to a Lenti-X concentrator and use it according to the manufacturer's instructions (*see* Note 8).

⁴ Depending on the pellet size and weight, the use of multiple Endofree-Maxiprep columns is required, in order to avoid any column binding oversaturation during extraction.

⁵ Expected yield should be around 2 mg. It is recommended to aliquot the amplified libraries at 1000 ng/ μ l with 20 μ l/tube. The amplified hCRISPRi/a libraries may be stored at –80 °C, and multiple freeze-thaw cycles should be avoided. Deep sequencing of the amplified and purified hCRISPRi/a libraries is strongly recommended, prior to be used in subsequent experimental steps or procedures.

⁶ The plasmids used in this protocol (e.g., pHR-TRE3G-dCas9) contain LTR repeat sequences, LTR-mediated recombination can happen in *E. coli* after transformation, resulting in a plasmid of reduced size. Hence, it is recommended to use Stbl3/MegaX competent cells for plasmid amplification, which will minimize any recombination events.

⁷ The cells start releasing viral particles into the culture supernatant 4–48 h post transfection, and usually after 24 h post transfection the supernatant containing the lentiviruses can be collected.

12. Incubate obtained mixture overnight at 4 °C, and centrifuge at $1500 \times g$ for 45 min at 4 °C, resulting in a visible off-white pellet. Carefully remove the supernatant and gently resuspend the pellet in 1/10 or 1/100 of the original volume of complete DMEM, $1 \times$ PBS, or other appropriate media (*see* Note 9).
13. The lentiviral titer estimation can be optimally achieved through the commercially available p24 ELISA kit, according to the manufacturer's instructions. The expected yield of lentiviral particles should be at least 1×10^6 TU/ml (*see* Note 10).
14. Lentiviral particles should be immediately aliquoted at desired concentrations and frozen at -80 °C. Lentiviral particles can be stored at 4 °C for about 3–7 days and at -80 °C for up to 1 year. Multiple freeze-thaw cycles are not recommended and result in low transfection efficiencies (*see* Note 11).

3.7 Generation of a Stable Cell Line with the Tet-Inducible dCas9 and sgRNA Pools

1. Prepare a batch of prewarmed complete medium (*see* Note 12), supplemented with polybrene (final concentration: 8 μ g/ml) or DEAE (final concentration: 6 μ g/ml).
2. Rapidly thaw the lentiviral particles containing TRE3G- KRAB- dCas9-mCherry or TRE3G-dCas9-VPR-mCherry at room temperature.
3. Seed $1-5 \times 10^4$ /ml of either adherent or suspension target cells with complete medium, supplemented with polybrene (8 μ g/ml) and the corresponding lentiviral particle dilution, into one well of a 6-well culture plate, with the total volume not exceeding 1 ml/well (*see* Notes 13–15).

⁸The use of the Lenti-X concentrator significantly increases the lentiviral titer. It is recommended to only use cellulose acetate or polyethersulfone (PES) (low protein binding) filters. Do not use nitrocellulose filters, as nitrocellulose binds to lentiviral envelope surface proteins, subsequently resulting in virus destruction.

⁹All experimental waste has to be disposed, according to local hazardous waste disposal guidelines. According to Biohazard level 2+ waste disposal regulations, all equipment and reagents, which have come into contact with lentiviral particles, are required to undergo decontamination procedures with 10% bleach solution for at least 30 min, before waste disposal.

¹⁰Viral titers are represented by functional infectious titer, measured in transduction units (TU/ml), or physical titer, measured in viral particles (VP/ml). The functional titer is a measurement of how much virus can infect the cell and is typically 100- to 1000-fold lower than the physical titer. Direct functional titer is a more accurate measurement for calculating MOI (Multiplicity of Infection), but it is more time-consuming and sometimes not feasible. The physical titer is a measurement of how much virus is present and is usually calculated based on the level of protein, such as p24 or viral nucleic acid. The physical titer is sufficient for most lentiviral experiments, and the functional titer can be calculated from physical titer.

The yield of lentiviral particles and final lentiviral titer highly depends on the size of the lentiviral construct. Please note that large lentiviral constructs (e.g., dCas9 fusions) yield five- to tenfold lower titers compared to smaller lentiviral constructs (e.g., sgRNAs). The final lentiviral titer is also highly dependent on HEK293T cell health and transfection efficiency. Virus producing HEK293T cells have a distinct round shape and phenotype. Do not allow the cultured HEK293T cells to become overconfluent and avoid cell clumps by ensuring that the cells are well trypsinized before plating, as clumps of cells can significantly reduce the final lentiviral titers. As viral production can vary between batches, it is recommended to purify and titer each lentiviral batch accordingly.

¹¹For experimental consistency, it is highly recommended to purify and titer the samples or store as single-use aliquots at -80 °C. Avoid multiple freeze-thaw cycles for the lentiviral supernatant.

¹²Medium should be warmed to room temperature to minimize cell stress.

¹³The health of the target cell line is critical for obtaining accurate results. Regularly check the cell culture for the absence of mycoplasma. Maintain the cells at ideal growing conditions for the specific cell line that you are working with. Thaw a new cell viral after 20–30 passages and restart the culture process. Do not add penicillin/streptomycin to the media during the lentiviral transduction.

¹⁵The lentiviral transduction of a final spin-infection step is optional for most protocols, as it generally results in an increased infection rate, but it is not absolutely required. In case of introducing this step during experimental procedures, the recommended spin should be carried out at $180-450 \times g$ for 5 min and/or up to 2 h. It is advised to test several protocols, prior to the final experiment, as the setup will highly depend on the cell type to be transduced.

4. Three days after adding the virus, carefully collect the cells from the well into a 15-ml falcon tube and pellet them by centrifugation at $450 \times g$ for 5 min.
5. Carefully remove the culture medium, without disrupting the cell pellet and resuspend the cell pellet in a falcon tube with 3 ml of complete medium, transfer the cell suspension into a T25 flask, and add an additional 7 ml of complete medium.
6. Culture the cells for 3 days in 1 $\mu\text{g/ml}$ tetracycline (*see* Note 16) and then FACS purify mCherry positive cells.
7. Expand the whole population of FACS purified mCherry positive cells for a frozen stock and further introduction of the sgRNA library.
8. Rapidly thaw the lentiviral particles containing a pool of individual pU6-sgRNA-Ef1 α -puro-T2A-BFP plasmid aliquots, harboring the corresponding sgRNAs.
9. Seed $1-5 \times 10^4/\text{ml}$ of cells from **step 7** with complete medium, supplemented with polybrene (8 $\mu\text{g/ml}$) and the lentiviral particle dilution (*see* Notes 13–15). For 50,000 gRNAs, divide lentivirus evenly into the wells of one 6-well plate.
10. Three days after adding the virus, carefully collect the cells from each well into 50-ml falcon tubes and pellet them by centrifugation at $450 \times g$ for 5 min.
11. Carefully remove the culture medium, without disrupting the cell pellet, and resuspend the cell pellet in complete medium, transfer the cell suspension into T75 flasks, and add additional 30 ml complete medium, supplemented with puromycin (1–2 $\mu\text{g/ml}$) (*see* Note 17).
12. Culture the T75 flasks for an additional 3 days, monitoring each flask for the presence of dead cells and/or medium color change, on a daily basis (*see* Note 18).
13. Once transduced and polyclonal cell populations are growing well and have been sufficiently expanded (typically between days 10 and 14), cell stocks can be prepared, according to standard freezing protocols, or be used for downstream applications (*see* Note 19).

¹⁶In our hands, this concentration of tetracycline works well, but different cell lines might require optimization.

¹⁷The concentration of puromycin needs to be determined for each target cell as the minimal concentration that yields 100% death in 1 week of incubation.

¹⁸Depending on the transduction efficiency, you will see different degrees of cell death upon antibiotic selection. In general, the infection efficiency and cell viability will vary between different cells. It is advised to test several transduced conditions, prior to the final experiment. With a transduction efficiency of 50%, expect approximately half of the cells to die within one week of incubation. Media color should not over acidify and is typically changed every 3 days.

¹⁹To achieve a stable cell pool, the antibiotic selection should last at least as long, as it takes the control (untransduced) cells to completely die. After that, the cells may undergo additional growth in the presence of the antibiotic. At this time, some researchers reduce the concentration of the antibiotic in culture or entirely remove the antibiotic entirely. If the antibiotic is reduced or removed from the culture, check the cells regularly to confirm transgene expression (either through mCherry and/or BFP expression by FACS or other gene and/or protein expression validation).

3.8 General Pooled sgRNA Library Screening Plan for a Two-Cell Type Coculture Assay (Fig. 3)

1. Three days before co-culture, set up three parallel cultures of the target cells, each with enough cells for complete library representation (500 cells/sgRNA) (*see* Subheading 3.7), in effector cell media (*see* Notes 20 and 21).
2. Two days before co-culture, add 1 µg/ml of tetracycline to activate dCas9 expression in two of the three cultures (*see* Note 22).
3. Co-culture the effector cells with the target cells at a predetermined effector (E): target (T) ratio (e.g., E:T, 0.25 to 50:1) and time span (e.g., 2–48 h) for one of the two cultures that received tetracycline. The remaining two groups of target cells (one that received tetracycline, and one that did not) are cultured without effector cells at the same conditions as the third group and serve as controls (*see* Note 23).
4. Mix cells in 50-ml falcon tubes and pellet cells, using centrifugation at $180 \times g$ for 5 min.
5. Maintain cell pellets in 50-ml falcon tubes with a loosened lid and culture them in the CO₂ incubator for the predetermined period of time.
6. To improve the efficiency of gRNA library amplification from the target cells, the majority of the effector cells should be removed from the culture. This can be accomplished either by allowing the effector cells to die without supplying an essential growth factor for several days (e.g., T cells without IL-2) or based on any unique antigens on either cell line. Target cells are maintained for additional 48 h to 2 weeks in the cell culture incubator for recovery and stored by standard cryo-freezing procedures and/or processed for later downstream steps (e.g., sgRNA enrichment analysis) (*see* Note 24).

3.9 Genomic DNA Isolation, PCR Amplification, and Next-Generation Sequencing for sgRNA Enrichment Analysis

1. Isolate genomic DNA (gDNA) from fresh or frozen cell pellets by using the NucleoSpin Blood Kits or Zymo research quickgDNA MidiPrep Kit, according to the manufacturer's protocol (*see* Notes 25 and 26).

²⁰Target cells typically survive in effector cell medium over the course of 3 days. However, if the target cells lose viability, the media will need to be optimized such that the target cells survive and the effector cells still kill.

²¹Most sgRNA-pooled libraries contain 3–6 sgRNAs per target gene, and maintaining the distribution of each sgRNA within the cell population is crucial. Hence, at the beginning of the screen, the working cell number of treated stably transformed cells should be kept at 300–500 cells for representing each individual sgRNA. The cell number is dependent on the screening library pool size.

²²Target cells should be validated for incorporation of the library by BFP and mCherry expression. Take approximately 100 µl of the corresponding cell suspension and fix it with 200–300 µl of 1% PFA for 20 min at room temperature, protecting the cell suspensions from light (e.g., wrapping the corresponding tubes with aluminum foil). Measure BFP and mCherry fluorescence of the cells by standard flow cytometry, looking for the percentage of cells expressing both fluorescence markers.

²³This screen approach can also be used with additional variable drug treatments, although the corresponding drug treatments and their individual concentrations have to be thoroughly tested (e.g., IC₅₀) and optimized, in order to achieve the best drug effect with the least cell toxicity.

²⁴Cryo-freeze target cells in cryo-freezing tubes in a total volume of 1 ml freezing solution (90% FCS and 10% DMSO) and store them immediately at –80 °C or liquid nitrogen. Freezing can occur at multiple points of the screen to capture, as the cell population may change over time.

2. Digest 10 µg of the extracted gDNA with 5 µl SbfI-HF (400 U/mg), 5.0 µl of 10× CutSmart buffer, and water to a total volume of 50 µl at 37 °C overnight (*see* Note 26).
3. Prior to large agarose gel preparation, clean the corresponding gel tray and large well combs, by rinsing them well with ultrapure (RNAse and DNAse free) water.
4. Dry the gel tray and place previously cleaned combs.
5. Pour hand warm dissolved agarose (final percentage 0.8%) in 1× TBE, supplemented with SYBR safe into the preset gel tray.
6. Allow the agarose gel to solidify in the cold room for around 30 min.
7. Fill the gel tank with 1× TBE, aspirate any TBE that may have entered the wells.
8. Load digested gDNA sample per well with 1× loading dye and DNA ladder (50–2500 bp) (*see* Note 27).
9. Run samples at 150 V, for 1–1.5 h or until the purple dye front has travelled half way down the gel.
10. Excise gel pieces from each individual lane using a razor blade under long wave UV light, with the band size range corresponding between 350 and 700 bp. Make sure to change blades between different sample lanes.
11. Weigh and place each excised gel piece in individual 50-ml falcon tubes (*see* Note 28).
12. Extract digested gDNA fragments from individually excised gel pieces, by appropriate gel purification kit (e.g., Macherey Nagel gel extraction kit or Zymoclean gel DNA Recovery kit), according to the manufacturer's instructions.
13. Measure the concentration of extracted digested gDNA fragments.
14. Store size selected (350–700 bp) and purified gDNA fragments at –20 °C or continue directly with the next step.
15. Set up a PCR reaction with 10 µl of 10× buffer, 0.4 µl of 3' common primer (stock concentration 100 µM), 0.4 µl of 5' primer with index (stock concentration 100 µM), 2.0-µl dNTP (stock concentration 10 mM), 3.0-µl DMSO

²⁵Frozen cell pellets or isolated gDNA can be stored at –20 °C for several months. Make sure to tighten the connection between the reservoir and the column during the extraction procedure. Centrifuge for sufficient time and speed to remove any residual buffer in the column. An additional final dry spin is recommended for residual washing buffer removal. For maximum recovery of gDNA, if more than 150 million cells are harvested, two elution columns should be used with an expected yield of 1.0–1.5 mg of gDNA per column.

²⁶The sgRNA content of the sample up to the PCR step is at extremely low abundance, compared to plasmid or post-g DNA PCR.

This makes the samples very susceptible to contamination, which can be falsely amplified during subsequent PCR steps. It is highly recommended to do all of the genomic extractions, digests, PCR setup on a separate bench, to avoid sample cross-contamination.

²⁷The SbfI digest and subsequent gel isolation can theoretically enrich for sgRNAs > 600-fold, but some material may be lost.

Therefore, this step is optional, but highly recommended for samples, where a large number of cells (generally greater than 15 million) have been processed. For sgRNA pooled samples, where the amount of input DNA (assuming 6.6 pg of gDNA per cell), under these circumstances, the 500-fold sgRNA representation is 3.3 ng/per sgRNA.

²⁸The recommended input of 500 ng gDNA/per 100 µl reaction can be adjusted, although higher inputs (>1 µg) can lead to inhibition of PCR reactions or higher observed background. If very high number of PCRs, based on the 500 ng input, is set up, it is recommended that an unimportant stock sample, containing sgRNA pooled library-containing cells, is initially used, in order to optimize this step.

(stock concentration 100%), 1.0- μ l Pfu Ultra II, 500-ng gRNA DNA, and water to a total reaction volume of 100 μ l (*see* Notes 29–32).

16. Use the following PCR conditions: (step 1) 98 °C for 30 s, (step 2) 98 °C for 30 s, 56 °C for 15 s, 72 °C for 15 s, for 23 total cycles, (step 3) 72 °C for 10 min, (step 4) hold at 12 °C.
17. After the PCR completion, pool all PCR reactions for a given sample in a 5-ml or 15-ml tube and mix well. All of the sgRNAs (gDNA fragments) in the pool should be represented at much greater abundance, so you may proceed with only a fraction of the sample for PCR sample for subsequent purification. You can directly test the PCR product by running a small aliquot on a 20% TBE agarose gel with 50-bp DNA ladder, with expected enriched PCR product being at ~274 bp and primer dimers at ~150 bp (*see* Note 33).
18. Purify the PCR product, derived from individually excised gel pieces by an appropriate gel purification kit, according to the manufacturer's instructions.
19. Measure PCR cleanup yield by Nanodrop NT-100 and/or Qubit, and dilute a small portion of your sample to 400 pg/ μ l, based on the measurement.
20. Run purified samples on a Bioanalyzer (Agilent) with a High Sensitivity DNA chip. The enriched PCR product is expected to be at 274 bp, but the Bioanalyzer peak should appear around 276–280 bp.
21. Submit samples for sequencing using one pair of the twelve suggested primer pairs (*see* Note 33 and Table 2).

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²⁹The PCR is designed to amplify the extracted gDNA fragments (containing sgRNAs), and for adding Illumina sequencing adapters for subsequent steps. For detailed primer information (Table 2), please refer to: <https://weissmanlab.edu/CRISPR/illuminaSequencingSamplePrep>.

³²Multiple samples can be pooled, prior to sequencing analysis. Alternatively, each sample can be analyzed in a separate channel, in order to obtain a more accurate concentration of the sequencing sample, prior to being pooled. Before proceeding, plan out how you want to pool/run your samples on the flow cell. Each sample should be PCR amplified, in order for them to have a unique index.

³³This protocol is optimized for the Illumina HiSeq4000 sequencer. If you are using other sequencers, consult your core facility or test the requirements for sequence diversity. Consult with your sequencing facility to find out how many reads to expect per lane, and plan for 250–500 reads per sgRNA per sample. For example, a typical genome-scale 200,000 gRNA library screen should have at least 50 million reads per sample (e.g., control and experimental group).

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A Tet-Inducible CRISPRi-dCas9

B Tet-Inducible CRISPRa-dCas9

Fig. 1.

Basic principles of the CRISPRi/a-dCas9 system. The necessary components and basic mechanism of CRISPRi-dCas9 (gene repression) (a) and CRISPRa-dCas9 (gene activation) (b) are shown. Important functional components are color coded for easy identification. The KRAB domain is the main component resulting in gene repression, while VP64-ReIA-RtaAD (VRP)-linked domains result in gene activation. This system is based on the Tet-inducible system, utilizing the Tet-On-3G domain and the addition of Tetracycline, for proper functioning

Fig. 2.

General outline for genome-wide sgRNA gene-editing screen in two-cell type co-culture system. **(a)** On the left side: An immune cell kills a target tumor cell using granzymes and perforin, in addition to producing various cytokines. On the right side: CRISPR/dCas9 targets a gene in the tumor cell that makes it resistant to immune cell killing. **(b)** Screen steps are depicted starting with target cells with different sgRNAs, which are then added to immune cells. After a period of time for cell recovery, genomic DNA is isolated, sequenced, analyzed for enrichment, and validated as a target

Fig. 3.

General outline for the transduction of target cells lines with CRISPRi/a-dCas9 and pooled sgRNAs. The sgRNA library is transfected into 293 cells for lentiviral packaging and viral particle production. The viral particles are then used to transduce the target cells

Table 1

Designed oligonucleotides for insertion of gRNA into pU6-sgRNA plasmid

Primer	Sequence (5'-3')
5' Forward	TTGNNNNNNNNNGTTAAGAGC
3' Reverse	CTTGTGNNNNNNNNNGTTAAGAGCTAA

Note: NNNNNNNNNN refers sgRNA sequence

Table 2

PCR primers for Illumina Hiseq 4000 sequencing

Primers	Sequence
Truseq 1- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTt AAGTAGAG gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT AAGTAGAG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtcgactcggtgccatttttc
Truseq 2- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTat ACACGATC gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT ACACGATC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTatcgactcggtgccatttttc
Truseq 3- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTgat CGCGCGGT gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT CGCGCGGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgatcgactcggtgccatttttc
Truseq 4- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTcgat CATGATCG gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT CATGATCG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcgatcgactcggtgccatttttc
Truseq 5- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTtcgat CGTTACCA gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT CGTTACCA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtcgatcgactcggtgccatttttc
Truseq 6- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTatcgat TCCTTGGT gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT TCCTTGGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTatcgatcgactcggtgccatttttc
Truseq 7- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTgatcgat AACGCATT gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT AACGCATT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgatcgatcgactcggtgccatttttc
Truseq 8- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTcgatcgat ACAGGTAT gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT ACAGGTAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcgatcgatcgactcggtgccatttttc
Truseq 9- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTacgatcgat AGGTAAGG gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT AGGTAAGG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTacgatcgatcgactcggtgccatttttc
Truseq 10- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTt AACAATGG gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT AACAATGG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtcgactcggtgccatttttc
Truseq 11- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTat ACTGTATC gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT ACTGTATC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTatcgactcggtgccatttttc
Truseq 12- Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTgat AGGTCGCA gcacaaaaggaaactcacct
Reverse	CAAGCAGAAGACGGCATACGAGAT AGGTCGCA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgatcgactcggtgccatttttc

Illumina Hiseq4000 compatible Primers (for PCR and sequencing)

Set A

Sample	TruSeq ID	Set A 5 Primer (orange indicates sample index for demultiplexing after sequencing)
1	12	aatgatacggcgaccaccgagatctacacgatcgaagagcacacgtctgaactccagtcacCTTGTAgcacaaaaggaaact cacct
3	14	aatgatacggcgaccaccgagatctacacgatcgaagagcacacgtctgaactccagtcacAGTTCCgcacaaaaggaaact cacct
5	3	aatgatacggcgaccaccgagatctacacgatcgaagagcacacgtctgaactccagtcacTTAGGCgcacaaaaggaaact cacct

Set B

Sample	Truseq ID	Set B 3 Primer
2	6	aatgatacggcgaccaccgagatctacacgatcgaagagcacacgtctgaactccagtcacGCCAATcgactcgggtgccacttt ttc
4	10	aatgatacggcgaccaccgagatctacacgatcgaagagcacacgtctgaactccagtcacTAGCTTcgactcgggtgccacttt ttc
6	1	aatgatacggcgaccaccgagatctacacgatcgaagagcacacgtctgaactccagtcacATCACGcgactcgggtgccacttt ttc

Common 3' Primer CAAGCAGAAGACGGCATACGAGATCGACTCGGTGCCACTTTTTC

Common 5' Primer CAAGCAGAAGACGGCATACGAGATGCACAAAAGGAACTCACCCT

5' Sequencing Primer GTGTGTTTTGAGACTATAAGTATCCCTTGGAGAACCACCTTGTTG

3' Sequencing Primer CCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATGCTGT