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TITLE:

Investigation of Genetic Dependencies using CRISPR-Cas9-based Competition Assays

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AML, CRISPR-Cas9, high-throughput assay, competitive growth assay, DOT1L, leukemia, cancer

SUMMARY:

This manuscript describes a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) CRISPR-Cas9-based method for simple and expeditious investigation of the role of multiple candidate genes in Acute Myeloid Leukemia (AML) cell proliferation in parallel. This technique is scalable and can be applied in other cancer cell lines as well.

ABSTRACT:

Gene perturbation studies have been extensively used to investigate the role of individual genes in AML pathogenesis. For achieving complete gene disruption, many of these studies have made use of complex gene knockout models. While these studies with knockout mice offer an elegant and time-tested system for investigating genotype-to-phenotype relationships, a rapid and scalable method for assessing candidate genes that play a role in AML cell proliferation or survival in AML models will help accelerate the parallel interrogation of multiple candidate genes. Recent advances in genome-editing technologies have dramatically enhanced our ability to perform genetic perturbations at an unprecedented scale. One such system of genome editing is the CRISPR-Cas9-based method that can be used to make rapid and

efficacious alterations in the target cell genome. The ease and scalability of CRISPR/Cas9-mediated gene-deletion makes it one of the most attractive techniques for the interrogation of a large number of genes in phenotypic assays. Here, we present a simple assay using CRISPR/Cas9 mediated gene-disruption combined with high-throughput flow-cytometry-based competition assays to investigate the role of genes that may play an important role in the proliferation or survival of human and murine AML cell lines.

INTRODUCTION:

The past few decades have seen numerous research efforts focused on identifying the contribution of key molecular pathways in acute myeloid leukemia (AML) pathogenesis. Traditionally, gene-disruption in AML cells has been performed using conditional knockout mice or short-hairpin RNA (shRNA). While knockout mice offer a sophisticated system for spatio-temporal control of gene-deletion, generating gene knockout mice is labor-intensive, time-consuming and expensive. Furthermore, gene-knockouts using recombination strategies is not easily scalable; these strategies do not lend themselves well to the interrogation of several genes in parallel. After the discovery of RNA interference methods to knock-down endogenous mRNAs using small interfering RNA (siRNA) or shRNA, many groups started using RNA interference techniques to investigate the role of specific genes in AML. Since both murine and human AML cells are notoriously difficult to transfect using traditional lipid-based transfection methods, most studies employed lentivirally or retrovirally-encoded shRNA for studying gene function in AML cells. The recent discovery of clustered regularly interspaced short palindromic repeats (CRISPR) and the associated Cas nucleases (CRISPR-Cas9) has revolutionized gene-targeting technologies¹⁻³. Using CRISPR-Cas9, specific genes or genomic regions can be deleted, edited or tagged with efficiency and ease. CRISPR-Cas9-based gene-editing is now emerging as the method of choice for investigating genotype-to-phenotype relationships in diverse cell types due to the simplicity, effectiveness, and broad applicability of this technique. CRISPR-Cas9-based methods are also becoming the method of choice in AML, not only for interrogating individual genes, but also as a way to target multiple genes in arrayed or pooled genetic screens aimed at investigating several genes in parallel as potential AML-dependencies⁴⁻⁶.

In this manuscript, we describe a simple competitive growth assay for measuring the impact of gene-disruption on the growth of AML cells, based on stable CRISPR-Cas9-mediated gene-editing followed by high-throughput flow cytometry. This method is simple, efficient, and scalable to medium-throughput experiments for investigating the role of several genes in parallel in AML cells.

PROTOCOL:

1. Generating AML Cell Line Clones with High Expression of Stable and Active Cas9

1.1. Production of Cas9 lentivirus

1.1.1. Day 0: Plate 4×10^6 293T cells in 10 mL of DMEM with 10% fetal bovine serum (FBS) and penicillin and L-glutamine in a 10 cm tissue culture dish in a biosafety Level 2 (BSL2) certified

cell culture hood. Place the dish in a 37 °C incubator.

1.1.2. Day 1: The plated 293T cells should be 70-80% confluent on day 1. Perform the transfection using the following protocol in the afternoon.

1.1.3. Warm the transfection medium, culture media and transfection reagent to room temperature. Thaw all the required plasmids for transfection.

1.1.4. Mix 9 µg of psPAX2, 0.9 µg of pMD2.G and 9 µg of pLenti-Cas9 plasmids with 500 µL of transfection medium in a 5 mL tube.

1.1.5. Add 1.7 mL of transfection medium to a 14 mL round bottom tube. Add 57 µL of transfection reagent directly into the transfection medium in the tube to avoid touching the wall of the tube.

1.1.6. Gently add the entire plasmid mix to transfection reagent solution and mix by gently tapping the tube on the side. Incubate the mixture at room temperature for 20-30 min. Meanwhile, change the medium from the seeded 293T plate.

1.1.7. Add the transfection mix dropwise to the plate and gently rock the plate sideways for efficient mixing. Place the plate in a 37 °C incubator.

1.1.8. Day 2: Aspirate the supernatant from the transfection plate gently such that the cells are not disturbed or dislodged from the plate. Discard the supernatant. Add 10 mL of fresh 10% DMEM medium by sliding down gently from the sides of the plate to avoid dislodging the cells.

1.1.9. Day 3: Collect the virus containing supernatant from the transfection plate slowly by drawing it into a 10 cm sterile syringe. After the supernatant is collected into the syringe, attach a sterile 0.45 µm filter to the syringe and hold the syringe and filter over a fresh, sterile 15 mL polypropylene conical tube. Gently plunge the syringe to filter the virus containing supernatant through the 0.45 µm filter into the 15 mL polypropylene conical tube.

1.1.10. Store the viral conditioned medium in aliquots of 2 mL each in 2 mL cryovials at -80 °C.

1.2. Transduction of AML cell lines

1.2.1. Day -1: Dilute 1 mg/mL recombinant human fibronectin fragment stock to 10 µg/mL with sterile phosphate-buffered saline (PBS). Coat a non-tissue culture treated 6 well plate with 2 mL of the 10 µg/mL recombinant human fibronectin fragment working solution in a BSL2 approved cell culture hood. Wrap the plate in cling wrap to avoid loss on evaporation and store at room temperature overnight.

1.2.2. Day 0: Thaw the viral supernatant containing Cas9. Aspirate the recombinant human fibronectin fragment solution completely from the coated plate just before spinfection. Add 2

mL of viral conditioned medium with Cas9 to the plate and spin it at 1300 x g for 90 min at 35 °C. This helps the viral particles attach to the spininfected well.

1.2.3. Meanwhile, count the cells to be transduced and spin down 2×10^6 cells in a 15 mL polypropylene conical tube. Aspirate the supernatant and resuspend the pellet in 2 mL of fresh culture media.

1.2.4. After spinfection, remove all the viral supernatant from the spininfected well and discard. Retroviral particles from the supernatant are attached to the bottom of the spininfected well. To this well, add the 2×10^6 cells resuspended in 2 mL of medium from the step above.

1.2.5. Spin the plate again at 1300 x g very briefly (1-2 min) sufficient to allow the cells to settle down at the bottom. Place the plate back into the incubator and leave overnight for transduction with the spininfected viral particles attached to the well.

1.2.6. Day 1: Depending on cell density, add more medium to the transduced cells to avoid overgrowth.

1.2.7. Day 2: Since the pLenti-Cas9 plasmid has a Blasticidin resistance marker, add Blasticidin at a 10 µg/mL dose to transduced and untransduced (control) MOLM13 or mouse MLL-AF9 leukemia cells for the selection of Cas9 expressing cells.

Note: Blasticidin selection of the Cas9-transduced MOLM13 or MLL-AF9 leukemia cells is considered complete when all the untransduced control cells have been eliminated. For cell lines other than MOLM13 and MLL-AF9 leukemia, a dose response curve must be performed prior to this experiment so that an optimal dose can be employed. Titration of the viral supernatant can also be performed in case of low-transduction rates.

1.3. Clone selection of high-Cas9 expressing cells.

Note: We have noticed that in some AML cell lines, clone selection might not be necessary: the bulk Cas9-Blasticidin selected population already has high genome-editing efficiency. In this case, it is possible to skip Step 1.3 and move on to Step 1.4 to assess Cas9 expression in the bulk Cas9-blasticidin AML cells by western blotting. It would still be important to evaluate genome-editing efficiency in those bulk Cas9-AML cells (Step 1.5) before proceeding to the competition assays. We surmise that the selection of single high-Cas9 clones reduces the genome-editing variability, which is especially important when testing a number of different single guide RNAs (sgRNAs).

1.3.1. Perform a single-cell sort of the Cas9-Blasticidin selected MOLM13 and MLL-AF9 leukemia cells henceforth called MOLM13-Cas9 and MLL-AF9-Cas9 cells, respectively, using a FACS sorter contained in a BSL2 approved hood. Sorting can be performed into 5-10 round bottom non-tissue culture treated 96 well plates.

1.3.2. Once single MOLM13-Cas9 and MLL-AF9-Cas9 clones have been picked and individually named, expand 10-20 clones with 10 µg/mL of Blasticidin in culture media to ensure the maintenance of Cas9 expression.

1.4. Expression check of stable Cas9 protein by immunoblotting.

1.4.1. Make nuclear extracts of all the Blasticidin selected clones of MOLM13 and MLL-AF9 leukemia using Nuclear Extraction Kit as per the manufacturer's protocol. Check Cas9 protein expression using anti-Flag M2 antibody since the Cas9 in the pLenti-Cas9 plasmid is linked to an N-terminal Flag epitope.

1.4.2. Load 50 µg of total protein from each nuclear extract onto 10% Bis-Tris gel and run the gel at 120 V till upper bands are well separated.

1.4.3. Block the membrane with 5% milk solution in TBST buffer for 1 h.

1.4.4. Incubate the membrane with 1:1000 dilution of anti-Flag M2 antibody at a final concentration of 1 µg /mL at 4 °C overnight.

1.4.5. Next day, wash the membrane with TBST buffer and incubate with HRP conjugated anti-mouse secondary antibody at a dilution of 1:5000 (final concentration of 0.16 µg/mL) at room temperature for 1 h.

1.4.6. Wash the membrane thoroughly with TBST and develop the blot using ECL substrate.

1.4.7. Pick 3-4 clones with the highest protein expression of Cas9 as seen by Western blotting for further functional analysis (**Figure 1**).

1.5. Ensuring high Cas9 activity in selected clones

1.5.1. Prepare lentiviral supernatant from an sgRNA encoding vector with sgRNAs targeting the human or mouse AAVS1 safe-harbor locus as described in the Step 1.1.

1.5.2. Transduce the top Cas9-expressing MOLM13 or MLL-AF9 leukemia clones with the anti-AAVS1 sgRNA lentiviral supernatant using the spinfection method described above. Select with 2.5 µg/mL Puromycin for 4 days.

1.5.3. Purify genomic DNA from the MOLM13-Cas9 or MLL-AF9-Cas9 leukemia clones after Puromycin selection together with respective wild-type controls using the Genomic DNA extraction kit as per manufacturer's instructions. Use 50 ng of DNA as a template to perform a PCR with the AAVS1_test_primers using 2x master mix of Taq Polymerase and the PCR program listed in **Table 1**.

1.5.4. Run the PCR product on a 2% agarose gel and gel-purify the 268 base pair band using a

gel extraction kit as per the manufacturer's protocol. Sanger sequence the gel purified PCR product using the AAVS1_target-frag_F primer.

1.5.5. Assess editing efficiency by the comparison of the AAVS1 edited and wildtype sequences using online web-based tools (**Figure 2**).

2. Cloning and Transduction of sgRNAs in AML-Cas9 Cells

2.1. Medium-throughput cloning of sgRNAs

2.1.1. Design 4-6 sgRNAs for the gene of interest using a web-based CRISPR design software. There are a number of CRISPR design tools available online such as <http://crispr.mit.edu/> which generate sgRNA sequences based on an input DNA sequence.

2.1.1.1. Fill in an appropriate information in fields with asterisks. Click on the target genome for sgRNA design. Paste the target sequence in the **Sequence box and click on **Submit** button.**

2.1.2. For cloning in the pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W sgRNA expression plasmid, prepend the nucleotides "CACC" to the sense oligo and "AAAC" to the reverse complemented antisense oligo before ordering. Order premixed sense and anti-sense oligos in a 96-well plate labelled **Oligo-Mix** from an oligonucleotide synthesis company.

Note: We have made use of the pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W plasmid, which has the Bbs1 restriction site for sgRNA cloning. In case other sgRNA expression plasmids are used, the overhangs used for the sgRNA oligonucleotides need to be changed accordingly.

2.1.3. Take a separate U bottom 96-well plate labelled **Annealing Plate. Make a master mix of 1 μ L T4 PNK, 1 μ L of 10x T4 DNA ligation buffer and 6 μ L of water per annealing reaction.**

2.1.4. Add 8 μ L of the master mix to each well of the annealing plate. Add 1 μ L of 100 μ M each, sense and antisense oligo (or 2 μ L of mixed sense-anti-sense oligos) to the master mix. Pipette 2-3 times gently to mix well, spin the plate briefly to get the mixes to the bottom of wells.

2.1.5. Use the following annealing program on a PCR machine: 30 min at 37 $^{\circ}$ C, 2 min 30 s at 95 $^{\circ}$ C followed by slow cooling to 22 $^{\circ}$ C at the rate of 0.1 $^{\circ}$ C/s. After annealing, take a fresh 96-well plate labelled **Diluted OligoMix. In this plate, dilute the phosphorylated and annealed oligos from the above reaction with water (1:1000) using multichannel pipettes.**

2.1.6. Digest 5 μ g of pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W vector with 1.5 μ L of BbsI restriction enzyme (10,000 units/mL) using an appropriate buffer at 37 $^{\circ}$ C for 2 h to linearize it for ligation later.

2.1.7. Take a fresh 96 well plate labelled **Ligation Plate and add 20 ng of the BbsI digested**

pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W vector to one well per desired sgRNA ligation. To this, add 2 μ L of phosphorylated, annealed oligos from the **Diluted OligoMix** plate.

2.1.8. Add 1 μ L of 10x T4 ligase buffer and 1 μ L of the T4 DNA Ligase enzyme. Gently pipette with a multichannel pipette and incubate the ligation mix at room temperature for 2 h.

Note: Ligation mixes can be stored at -20 °C for the transformation step later.

2.1.9. Meanwhile, thaw 90 μ L of chemically competent *E. Coli* cells on ice 10 min before the end of the ligation step.

2.1.10. Using a multichannel pipette, make 10 μ L aliquots of the competent cells into each well of a separate 96 well plate.

2.1.11. Add the ligation mixture from Step 2.1.8 into the well containing the competent cells, pipette up and down gently and incubate for 10 min at room temperature.

2.1.12. Pipette 5 μ L of the bacteria-DNA mix directly from the above reaction into a 6 well plate containing LB-agar with 100 μ g/mL Ampicillin. Repeat for each of the transformation reactions.

2.1.13. Plate each transformation reaction into a separately labelled well of a 6-well plate. Add approximately 5-8 glass beads to each well and shake the whole 6-well plate 8-10 times in a circular motion.

2.1.14. Pick 1-2 single colonies from each well with a sterile 20 μ L pipette tip and streak it into a carefully labelled spot on a sterile 10 cm Petri-dish with LB agar and 100 μ g/mL Ampicillin. After streaking into the LB plate, simply eject the tip used for clone streaking into 3 mL of LB-Ampicillin containing medium in a 14 mL round bottom tube marked with the corresponding bacterial clone number.

2.1.15. Send the bacterial plate directly for Sanger sequencing with the Human U6 promoter forward primer.

2.1.16. After sequence confirmation of cloned sgRNAs, purify the DNA from the corresponding 14 mL tube using a mini-prep kit according to the manufacturer's instructions.

2.1.17. Measure the concentration and quality of each of the mini-preps with a Spectrophotometer. Normalize each mini-prep to a concentration of 15 ng/ μ L and pipette into a 96 well plate labelled **sgRNA Clones Plate**.

Note: This plate can be stored at -20 °C or used directly for virus preparation.

2.2. Viral production of sgRNA constructs and transduction

2.2.1. For the production of sgRNA lentiviral particles in the 96-well format, follow the “shRNA/sgRNA/ORF High Throughput Viral Production (96 well)” protocol from the Genetic Perturbation Platform (GPP web Portal) of the Broad Institute (URL: <https://portals.broadinstitute.org/gpp/public/resources/protocols>).

2.2.2. Transfer 200 µL of viral supernatant to sterile tubes and freeze at -80 °C immediately.

2.2.3. Day -1: Coat a flat bottom Non-tissue culture treated 96 well plate with 100 µL of recombinant human fibronectin fragment at a concentration of 10 µg/mL in a BSL2 cell culture hood. Wrap the plate using cling wrap to avoid evaporation loss and leave it at bench overnight.

2.2.4. Day 0: Remove the recombinant human fibronectin fragment from each well. Thaw the viral supernatant of each sgRNA at room temperature. Add 50 µL of viral supernatant from each tube to each coated well of the transduction plate. Spin the transduction plate at 1300 x g for 90 min at 35 °C.

2.2.5. Towards the end of spinfection, count MOLM13-Cas9 (clone B3) cells from the culture flask. Use 10,000 cells per well in 100 µL volume. Count the cells for all the transduction wells, taking dead volume into consideration.

2.2.6. After the 90 min spinfection, remove the supernatant from all the wells using a multichannel pipette adjusted to 50 µL slowly by tilting the plate. Add 100 µL of the culture of MOLM13-Cas9 clone B3 cells to each well slowly sliding down from the rim.

2.2.7. Spin the plate at 1300 x g for 2 min at 35 °C to let the cells settle onto the bottom. Transfer the plate to the 37 °C tissue culture grade incubator.

2.2.8. Day 1: Add 100 µL of fresh medium to each well containing sgRNA transduced Cas9-MOLM13 or Cas9-MLL-AF9 leukemia cells such that the final medium volume is 200 µL.

3. Competitive Growth Assay

3.1. Day 3: 72 h (day 3) post transduction, check the percentage of sgRNA containing BFP positive cells in each well by flow cytometry (FACS). Use a cell viability dye to mark and exclude dead cells from the analysis.

3.2. Continue re-plating a proportion of the cells into new wells with fresh medium after every FACS analysis to avoid overgrowth during the assay.

3.3. Repeat the FACS analysis every 2-3 days to check the relative proportion of BFP positive (BFP+ve) cells compared to the BFP negative (BFP-ve) counterparts (**Figure 3**). Analyze the percentage of BFP positive cells for each time point (using the FlowJo or similar FACS analysis software).

3.3.1. Drag Fcs files for each sample to FlowJo software. Double click on any one sample file and plot a dot blot of forward scatter (FSC) vs. Side scatter (SSC) with FSC on X axis and SSC on Y axis. Gate all the cells.

3.3.2. Double click on the gated cells and plot them on Viability stain (on Y axis) vs. FSC height (H) or Area (A) dot blot. Gate the viability stain negative (live) cells.

3.3.3. Double click on gated live cells and plot BFP (on Y axis) vs. FSC (A) on X axis. Gate BFP+ve cells. Apply all these gates to all the samples by dragging the selected sample to **All Samples** under **Group** tab.

3.3.4. Click on **Table Editor** to make an analysis table of all the samples. Drag **BFP+ve** count from one sample to the table and click on the **Display** button in the **Table Editor** to create a batch report of all the samples with a table displaying the percentage of **BFP+ve** cells. Save the table as an xls.

3.4. Calculate the proportional increase or decrease in % BFP positive cells within the live cell population over time and compare this ratio to the ratio of BFP positive cells in the control sgRNAs (such as luciferase, scrambled or GFP sgRNA).

3.5. Plot the ratio of BFP positive cells for the different sgRNAs including the gene(s) of interest as well as controls using day 3 as the baseline.

REPRESENTATIVE RESULTS:

In our study, we first transduced the MOLM13 human AML cell line that bears the MLL-AF9 translocation with high-titer virus encoding the Cas9-blasticidin lentiviral plasmid. In our hands, bulk unsorted MOLM13-Cas9 cells did not display high level Cas9 expression by Western blotting and also did not perform well when assayed for efficient gene editing-using the method described previously⁷. Therefore, we proceeded to establish single cell clones and only select the clones with high levels of Cas9 as seen by Western blotting (**Figure 1**). We picked 2 distinct clones and transduced them with sgRNAs targeting a site in the AAVS1 safe-harbor locus as described earlier⁷. Using genomic DNA extracted from the Puromycin-selected MOLM13-Cas9-AAVS1 sgRNA clones, we performed a PCR using primers spanning the AAVS1 sgRNA cut site and sanger sequenced a specific 268 bp PCR product from 10 isolated colonies for each MOLM13 clone. After alignment with the parental sequence, we found that MOLM13-Cas9 clone B3 and MLL-AF9-Cas9 clone 8 had an efficiency of 100% (data not shown). We then selected these clones displaying high Cas9-mediated genome-editing capability for our sgRNA competition assays. While these studies were being conducted, web-based tools for rapidly testing genome-editing efficiency from Sanger sequences were developed by different groups such as TIDE⁸ (<https://www.deskgen.com/landing/tide.html>) or ICE (<https://ice.synthego.com/#/>). These tools make it extremely easy and cost-effective to assess genome-editing efficiency without having to clone individual fragments and perform sequencing of multiple clones. Therefore, bulk Cas9-expressing cells should first be tested for genome editing efficiency using these methods. In case the genome-editing efficiency is high,

then single-cell cloning is not needed. Also, in case that single cell cloning is needed as seen in our studies, these web-based mutational frequency estimation tools offer a much faster method for testing several clones in parallel.

For sgRNA cloning, we made use of the sgRNA cloning plasmid pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W, which harbors a blue fluorescent protein (BFP) for tracking sgRNA transduced cells and an RNA Polymerase III-driven human U6 promoter that drives the expression of the cloned sgRNAs together with the tracer RNA (tracRNA) scaffold. We used this system to clone 2 sgRNAs targeting DOT1L, a protein known to play an important role in the epigenetic regulation of gene expression. DOT1L is a histone methyltransferase that deposits mono, di and tri-methylation at target genes^{9,10}. Studies have shown that DOT1L plays an important role in AML driven by MLL-fusion oncogenes. Therefore, DOT1L knockout using CRISPR-Cas9 is expected to lead to significantly impair mouse MLL-AF9 leukemia cell proliferation in line with previous studies¹¹⁻¹⁴. We used two sgRNAs targeting the AAVS1 safe harbor locus, which is in the intron of the PPP1R12C gene. sgRNAs producing genomic alterations in this site are not likely to have any effects on the proliferative capacity of MLL-leukemia cell lines. As a positive control, we cloned sgRNAs targeting the DNA replication-associated gene RPA3. RPA3 is a pan-essential gene that has been shown to be important for the proliferation of several AML cell lines^{4,15,16}. Anti-RPA3 sgRNAs therefore display strong anti-proliferative effects in AML cells and are typically used as a positive control. After the transduction with anti-AAVS1 and anti-RPA3 sgRNA plasmids, we measured the relative proportion of BFP+ve sgRNA transduced cells in comparison to their BFP-ve counterparts every 2-3 days in culture (**Figure 3**). With the protocol described above, the transduction of MOLM13 or MLL-AF9 AML cells resulted in a 60-70% transduction efficiency with our viral preparations, leaving the remaining 30-40% cells untransduced or BFP-ve in every well. This allows for the study of relative proliferation of genome-edited cells with wild-type cells in the same well. The proportional increase or decrease in percentage of sgRNA transduced cells was used as a measure to reflect the functional effect of sgRNA mediated gene-deletion in the MOLM13-Cas9 cells. If your gene of interest is important for the proliferation of the test AML cells, then sgRNA-mediated disruption of this gene will lead to the relative diminution of the sgRNA-expressing BFP+ve cells in comparison to the BFP-ve cells during the course of the assay, whereas sgRNAs targeting luciferase, GFP or non-essential genes will retain the BFP+ve/-ve ratio over time.

Using 2 separate anti-RPA3 sgRNAs, we observed a progressive and significant decline in the percentage of BFP+ve cells compared to the BFP-ve untransduced counterparts. In contrast, the percentage of anti-AAVS1 sgRNA expressing BFP cells remained relatively constant over time, demonstrating that AAVS1 targeting has no effect on the proliferation of MOLM13 cells (**Figure 4a**). Similarly, in the mouse MLL-AF9-Cas9 cells, we tested the effects of sgRNAs targeting Rhodopsin (Rho1), the eye pigmentation gene as a negative control and Dot1l, an epigenetic regulator known to be required for the proliferation of MLL-AF9 leukemia cells over time. In this study, BFP+ve mouse MLL-AF9-Cas9 cells transduced with 2 separate anti-DOT1L sgRNAs showed a dramatic and progressive loss over time compared to BFP-ve sgRNA non-transduced cells (**Figure 4b**). In contrast, the ratio of anti-Rho1 sgRNA remained relatively unchanged over

time. These results demonstrate the vulnerability of the MLL-AF9 expressing mouse leukemia cells to Dot1l depletion, confirming previously published results.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative Western blot results showing different Cas9 levels. Single cell clones of the MOLM13 AML cell line transduced with CAS9 were probed for flag-Cas9 expression levels using anti-Flag antibodies. Clones showing the highest expression of Cas9 were selected for further studies. HEK293-T cells transfected with a Cas9 expression plasmid were used as a positive control and Cas9 non-transduced MOLM13 cells as negative controls.

Figure 2: Representative analysis of genome editing at the Dot1l locus assessed by ICE analysis. A PCR amplicon centered around the Dot1l sgRNA target site was Sanger sequenced and analyzed using ICE analysis. A comparison of the sgDot1l sequence (orange line) to non-targeted DNA sequence (green line) demonstrates the high level of editing efficiency in sgDot1l targeted cells around the sgRNA target site.

Figure 3: Schematic workflow of the proposed methods. sgRNAs are cloned in sgRNA expression vector co-expressing a fluorescent protein such as BFP. Target cells are transduced at 30-60 percent transduction rates and followed by flow-cytometry every 2-3 days. sgRNAs targeting genes required for AML cell proliferation will show relative depletion over time as shown.

Figure 4: Representative results from competition assays in human and mouse AML cells. (a) Results show a statistically highly significant progressive decline in RPA3 transduced MOLM13-Cas9 clone B3 cells. In contrast, sgRNAs targeting the AAVS1 site have no effect. **(b)** sgRNAs targeting Dot1l show a remarkable and progressive decline in competitive proliferation, in contrast to sgRNAs targeting Rhodopsin (Rho). * $p > 0.05$. ** $p < 0.05$. Error bars represent standard deviation of mean (SD).

Figure 5: General outline of the protocol. (a) Time for each step in the production of Cas9 virus and sgRNA cloning described. Design and cloning of sgRNAs can be performed while generating single clones of AML cell lines with stable Cas9 expression. **(b)** General protocol for the transduction of AML cell lines with sgRNAs and competitive growth assay using FACS is shown.

Table 1: PCR program for AAVS1 locus amplification from Genomic DNA. PCR program used for the amplification of AAVS1 locus from genomic DNA for testing cutting efficiency of Cas9 in Cas9 expressing clones.

Supplementary File: sgRNA oligo sequences.

DISCUSSION:

In this manuscript, we describe a detailed protocol for conducting a CRISPR-Cas9-based competitive growth assay to investigate the role of candidate genes in AML cell lines using flow-cytometry in human/murine AML cells (**Figure 5**). The goal of the assay is to identify the effect

of gene deletion on maintenance of AML cell proliferation over two to three weeks on a medium-throughput scale. Some critical steps need to be followed carefully to facilitate the scaling up of the described protocol. In the production of Cas9 lentivirus, it is necessary to filter the virus conditioned medium through a 0.45 μ M filter to avoid the carryover of 293T cells to the virus containing supernatant. During spinfection, wrapping the spinfection plate with cling wrap or parafilm helps avoid potential contamination during centrifugation. However, the wrap should be removed before placing the spinfected plate back in tissue culture incubator. It is very important to assess Cas9-expressing clones for editing efficiency. Clones with low-genome editing efficiency reflect inadequate Cas9 activity and may affect the success-rate of the experiment. In our experiments, we first transduced human AML Cas9 clones with sgRNAs targeting the AAVS1 locus and sequenced their genomic DNAs for editing efficiency. Comparison of the AAVS1 edited and wildtype sequences can be assessed using online web-based tools such as TIDE⁸ (<https://www.deskgen.com/landing/tide.html>) or ICE (<https://ice.synthego.com/#/>). These programs compare Sanger sequence traces of potentially edited PCR fragment to the unedited wildtype or reference sequence and estimate the frequency of changes. This is a rapid way of assessing mutational changes brought about by the test sgRNA, which is the measure of cellular Cas9 activity. Alternatively, cloning of the PCR product into a PCR-cloning plasmid such as the TOPO blunt cloning vector can be performed, followed by Sanger sequencing of individual colonies to assess genome-editing efficiency by sequence alignment of each clone to the wild-type. We prefer the former method, given its ease and cost-effectiveness compared to the cloning method. Typically, the discovery of base pair changes such as point mutations, indels, *etc.*, at or around the sgRNA cutting site in a vast majority of sequenced clones indicate the presence of a highly active Cas9. Thus, we chose MOLM13 or MLL-AF9 leukemia clones B3 and 8, respectively, with the highest editing efficiency for further experiments.

We designed sgRNAs targeting different genes mentioned in the protocol using <http://crispr.mit.edu/>, a web-based software that gives a list of sgRNAs. It is recommended to select top-scoring sgRNAs from the list in order to eliminate those with predicted off-target effects. The designed sgRNAs can be cloned using any of the published protocols such as the one on (<http://www.addgene.org/67974/>) website. Sense and antisense oligos are first phosphorylated and annealed as per the Step 2.1.15. The first step at 37 °C is important for phosphorylating the oligos with the T4 kinase and subsequent PCR cycles are important for annealing of the sense and anti-sense oligonucleotides into dsDNA. It is important to have a fluorescent protein in the sgRNA cloning vector which is crucial for tracking sgRNA transduced cells later in the competition assays. sgRNA cloning is scaled up with the use of 96 well plate at all the steps including annealing and ligation. For ligation, clean PCR microtube strips can also be used since they are compatible with multichannel pipettes. In case that the transformation of a larger set of sgRNA clones is needed, a 96-well plate in which 10 μ L of the competent cells are pre-aliquoted in each well and frozen at -80 °C can be used to expedite the entire process. The purpose of plating the ligation reactions is to pick individual colonies, which is difficult to perform in the 96-well format. Hence, for bacterial transformation, there is a slight loss in scalability. Still, even for the plating of transformation reactions from an entire 96-well plate, it will only require a total of sixteen 6-well plates for the entire project. One has to be careful in

the next step of picking colonies from the transformation plates. While streaking a colony on a labelled spot, ensure that the spot is clearly isolated from the other spots. Marking a square grid on the bottom of the Petri dish with a dark permanent marker helps prevent cross-contamination of bacterial clones.

Once the minipreps of sgRNA constructs are ready, the virus can be made in the 96-well format. At this level of throughput, it is impractical to filter 200 μ L of virus containing supernatant. Hence, the viral supernatant should be frozen at least overnight to avoid cross contamination from any 293T cells carried over into the supernatant. It can also be stored in 50 μ L aliquots in sterile PCR tube strips to avoid freeze thawing of the supernatant. Further, these virus conditioned supernatants are used for transducing AML cells. In case that low titers of viral transduction are observed, as assessed by low frequency of BFP+ve cells, then it may be important to determine the viral titer and testing transductions at different multiplicity of infections (MOI) to ensure 40-60 percent transduction rates. Viral titer determination and MOI calculation is described in detail here¹⁷. In the competition assay, as described in Step 3 of the protocol, it is highly important to exclude non-viable cells to prevent spurious artefacts from auto-fluorescence while analyzing the BFP to non-BFP ratio. At the time of FACS, before analyzing test samples, the use of BFP negative and BFP positive controls is highly recommended to accurately set voltages. At each re-plating time point, the volume of the cells to be re-plated depends on the concentration of cells at the given time-point. We typically re-plated 20 μ L from a total of 200 μ L at each time-point into a fresh well with 180 μ L of fresh medium. Thorough mixing of the cells by gently pipetting up and down just before re-plating is highly recommended. Typically, we have observed that the assay needs to be carried out over 15-20 days to notice strong changes in the BFP+ve/-ve ratios, although this varies from gene to gene.

This experimental set-up is well suited to testing several genes in parallel in multiple AML cell lines to expeditiously identify the role of candidate genes in the survival or proliferation of AML cells. One caveat of the competitive proliferation assay described here is that it does not consider potential cell-extrinsic factors such as paracrine signaling events from gene-targeted cells that may influence the non-targeted cell population within the same well. Even though this might be a rare occurrence, this factor should be borne in mind when conducting this assay. Although we have used AML as an example for the CRISPR-Cas9-based competition assays described in this manuscript, this method can be used for any cancer cell line to identify the role of multiple genes in parallel. There are various advantages of gene-deletion using CRISPR-Cas9 over gene-knockdown using RNA interference. Firstly, in contrast to shRNAs, which typically show a broad range of target mRNA inhibition, sgRNAs coupled with the Cas9 nuclease effectuate complete knockout of target genes. This may result in more dramatic and consistent phenotypes with CRISPR-Cas9-based methods, even though it must be cautioned that the targeting efficiency of individual sgRNAs as well as shRNAs may vary widely.

The flow-cytometry-based competition assay offers several advantages over traditional proliferation assays in which a fixed number of cells are plated to measure the relative proliferative activity of gene-perturbed cells. One advantage is that the relative proliferative

activity of cells can be easily and rapidly measured by flow-cytometry for several days, bypassing the need for cell counting at every plating step. This is useful when testing a large number of candidate sgRNAs targeting several genes, as counting all of the wells is cumbersome and may lead to inaccuracies. Unlike, ATP-based measurement assays for cell growth, flow-cytometry can be performed on a sampling of live cells, which makes this method helpful for long-term analysis. This is especially beneficial in the study of factors such as epigenetic modulators, which may show late-acting effects on proliferation of AML cells and may require long-term assays. Secondly, the presence of non-transduced cells within the same well allows for a well-controlled cell population that can be used to set the baseline for the assay. Thirdly, when set-up in a 96-well format, the assay can almost completely be conducted using multi-channel pipettes, substantially speeding up the entire process from cloning of sgRNAs to virus preparation and eventually flow-cytometric assessment of fluorescence.

The method described here can be efficiently scaled-up for the investigation of up to 96 sgRNAs in parallel in an arrayed screen. Assuming 4-6 sgRNAs per gene, this method can therefore be used for the rapid interrogation of at least 16-24 genes in parallel. In case of larger number of genes, such as an entire molecular pathway that needs to be interrogated in AML cells, pooled CRISPR-Cas9-based screens will be more useful.

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DISCLOSURES:

A.J.D is a consultant to A2A pharmaceuticals (New Jersey) and Salgomed Therapeutics (San Diego). Other authors have no conflicts to declare.

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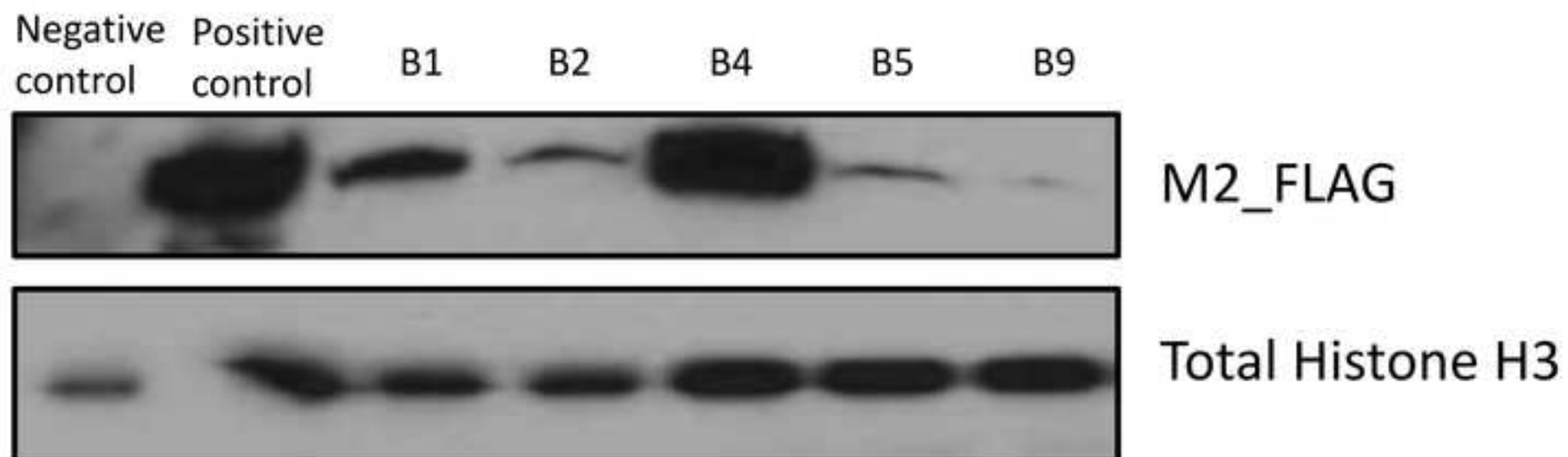
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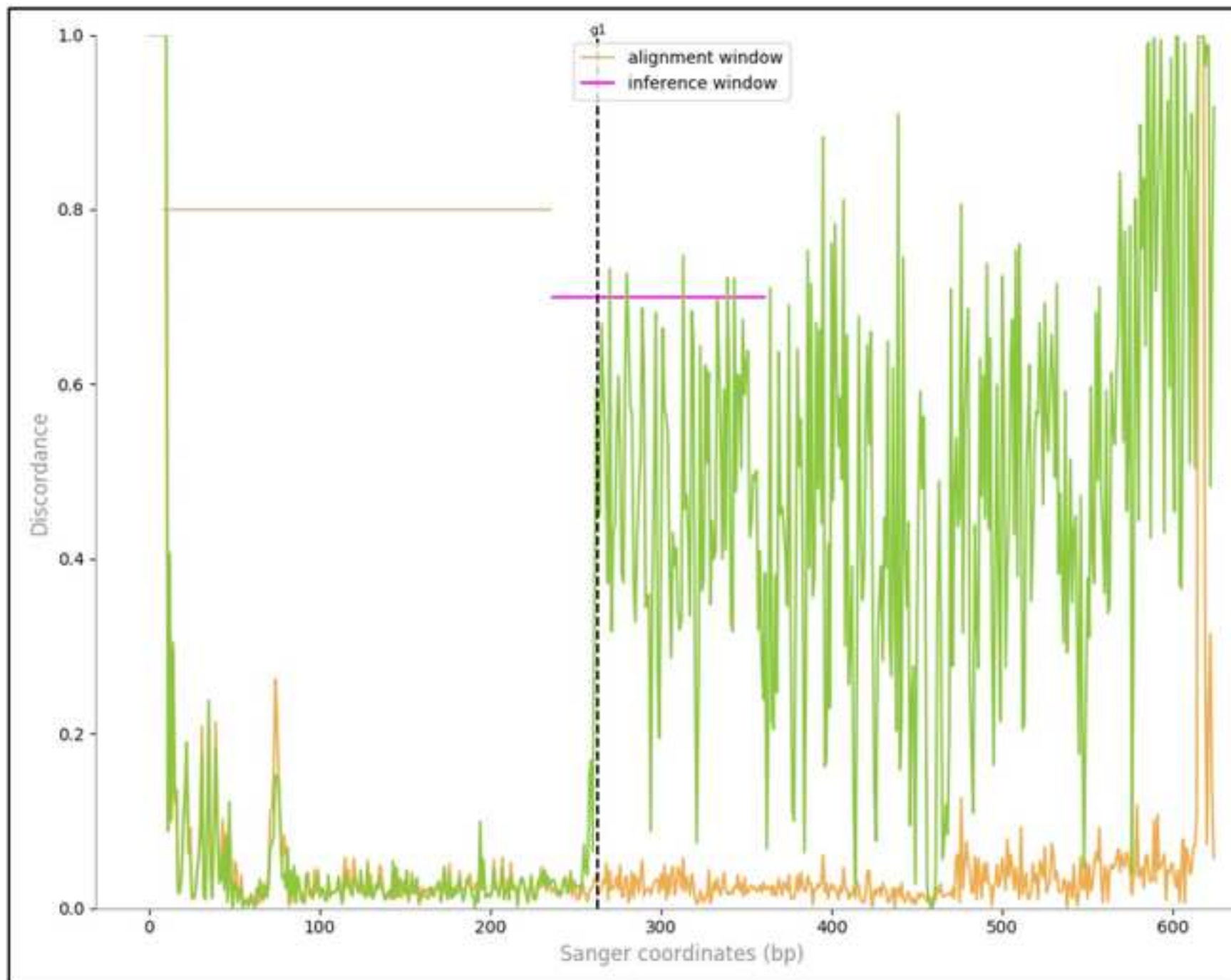
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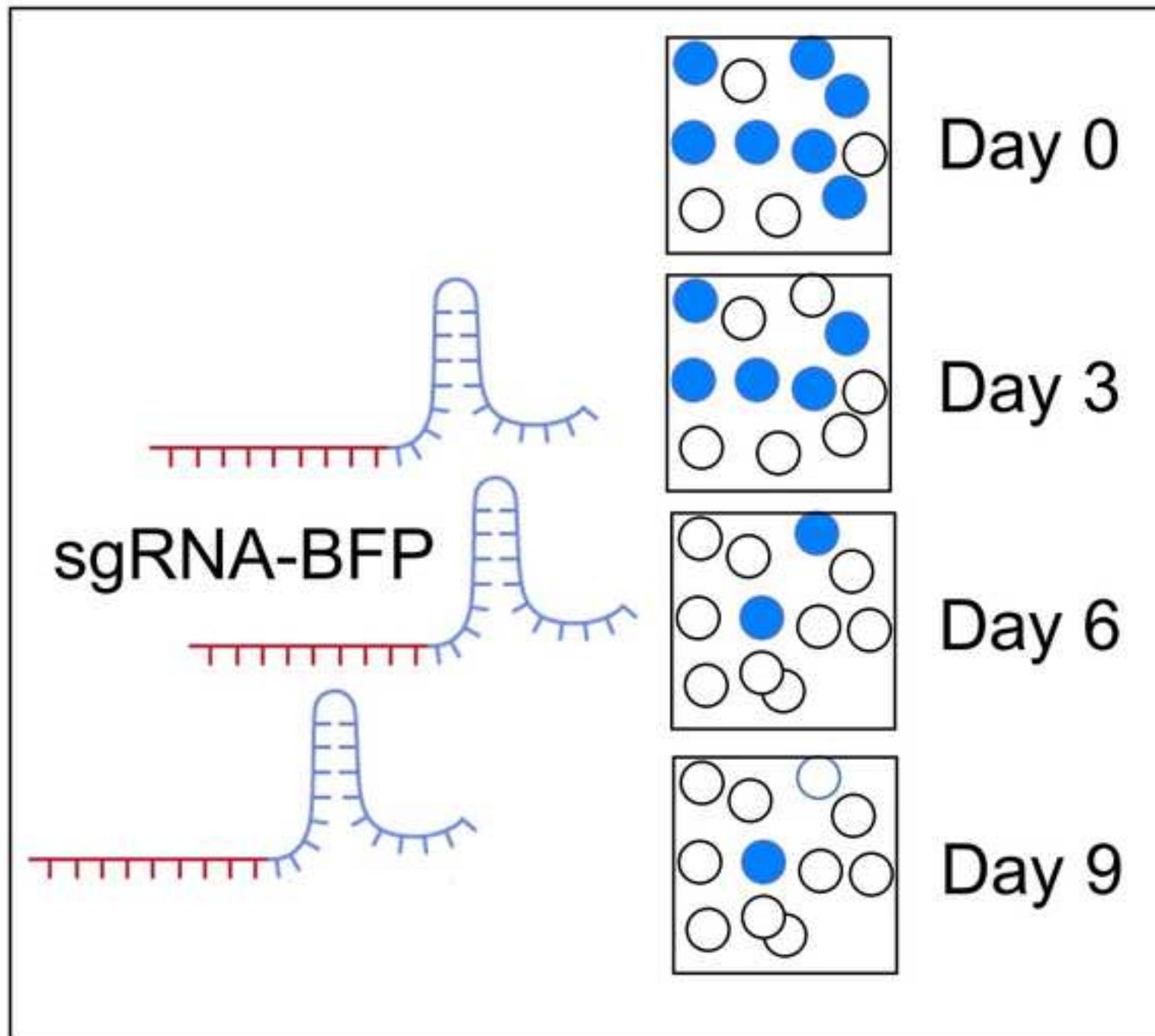
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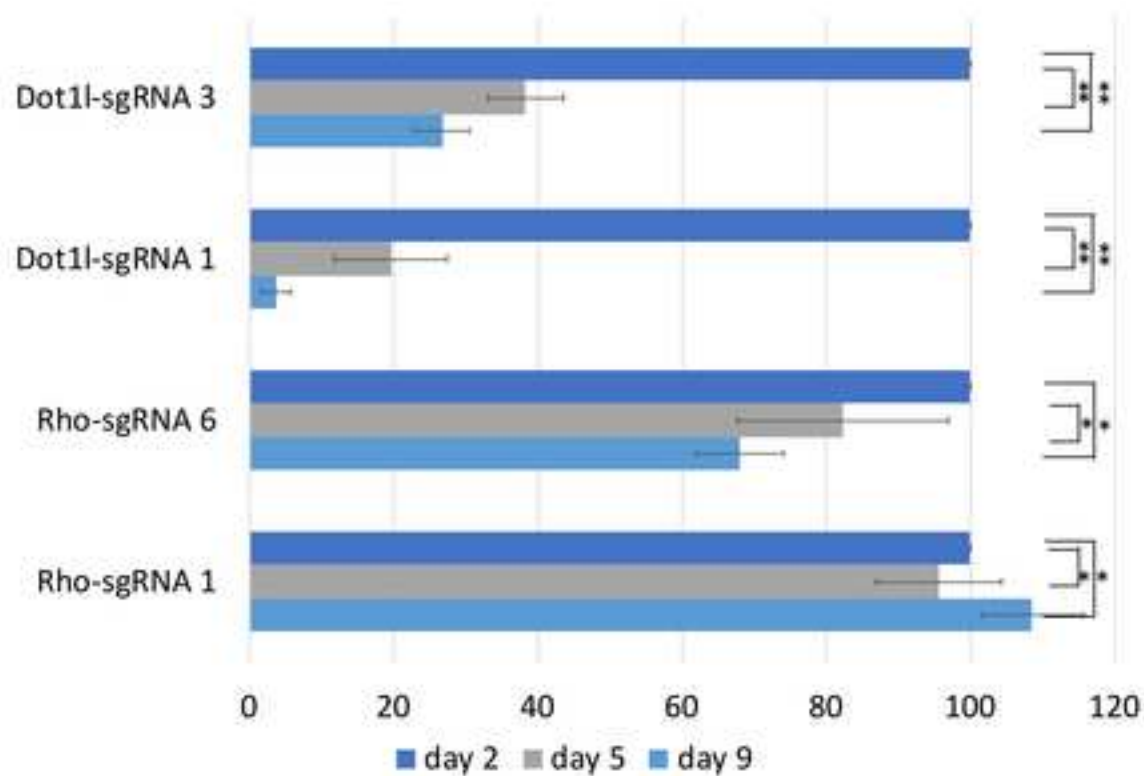
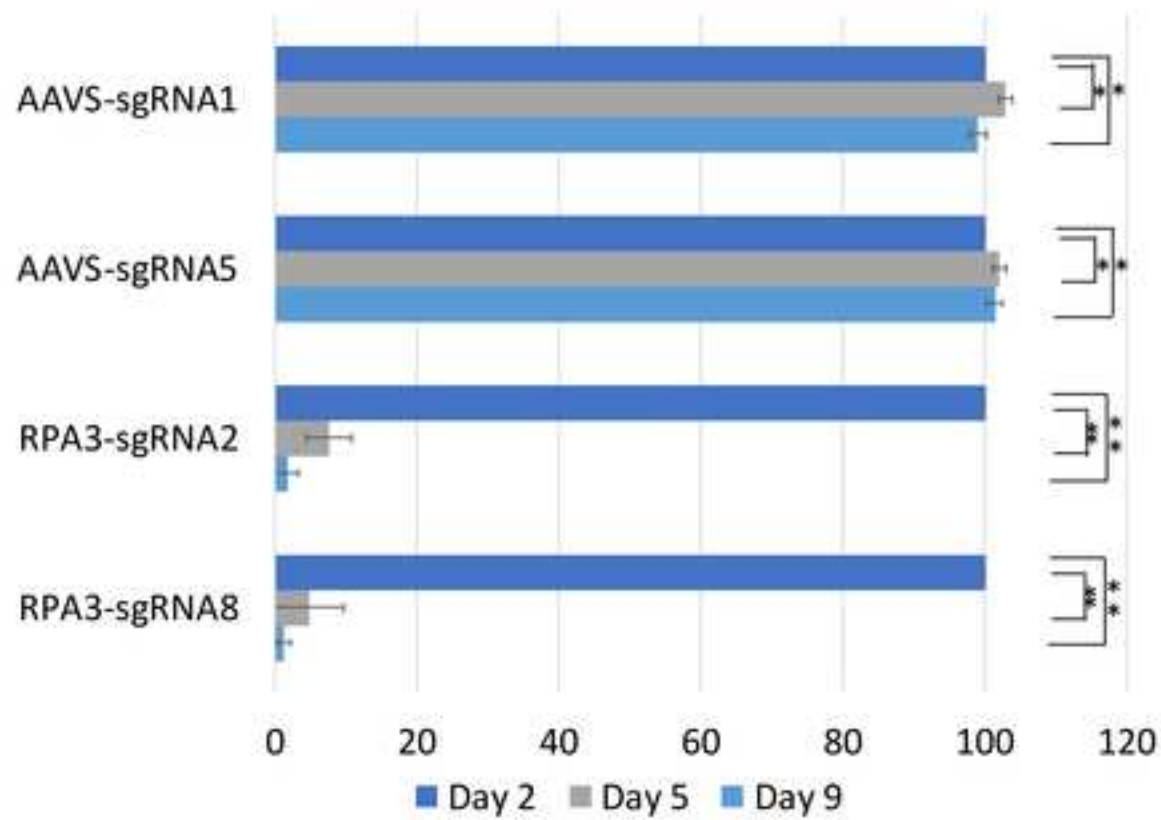


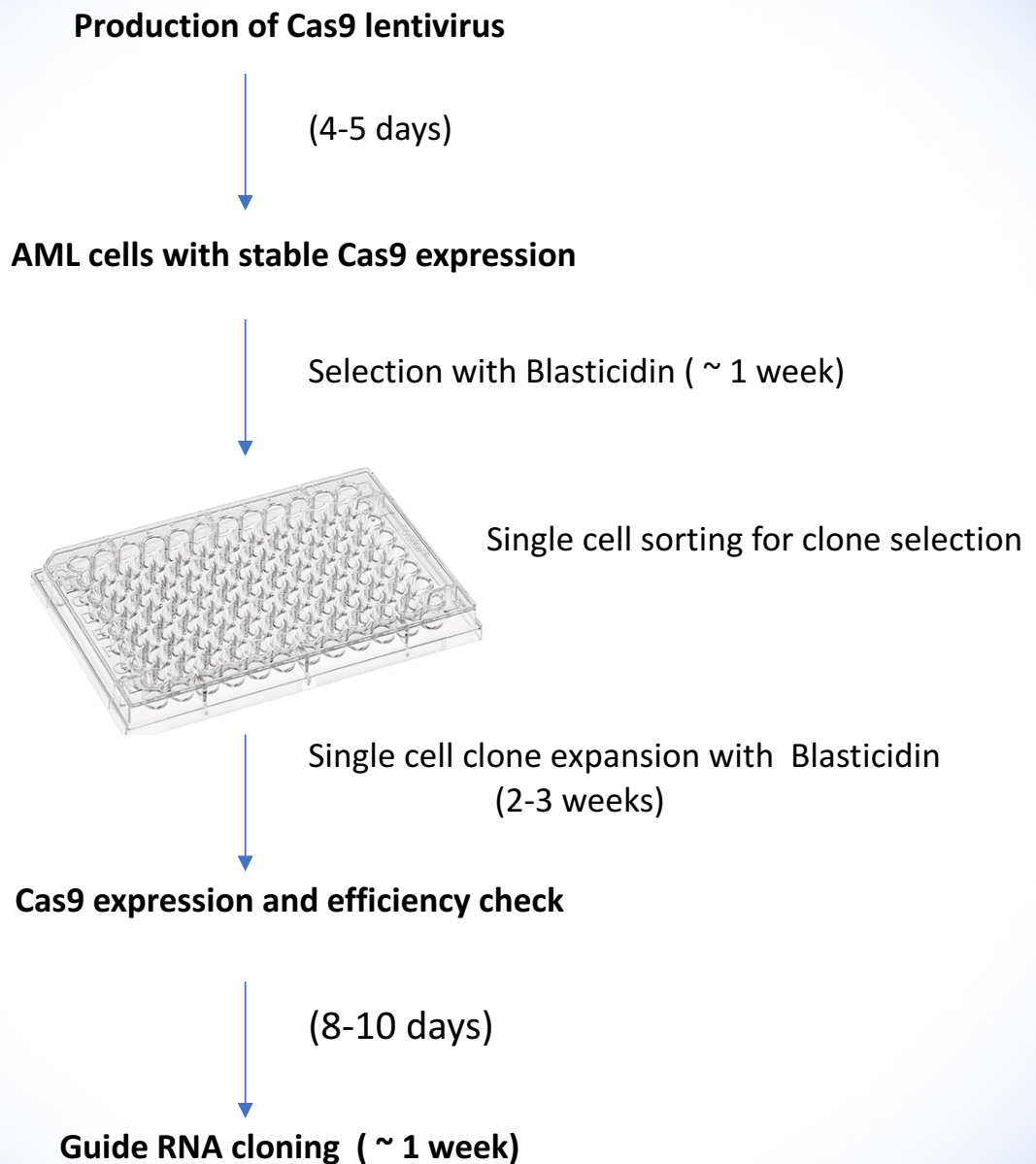
Fig. 5a

Fig. 5b

Production of sgRNA virus

(4-5 days)



Transduction of AML-Cas9 cells

(~ 3 weeks)



**Competitive growth assay using
flow cytometry**

Table 1

Cycles	Duration of Cycle	Temperature
35	1 2 min	95 °C
	30 s	95 °C
	30 s	55 °C
	30 s	72 °C
	1 5 min	72 °C
Hold	infinite	4 °C

Name of Material/ Equipment	Company
FLAG-M2 Antibody	sigma-aldrich
Anti-mouse Antibody	Invitrogen
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Fisher
ChemiDoc Imaging System	BIO RAD
Sorvall Legend RT centrifuge	Thermo Scientific
Blasticidin	Thermo Fisher
SYTOX Red	Thermo Fisher
Opti-MEM	Thermo Fisher
DMEM	Thermo Fisher
RPMI	Thermo Fisher
Penicillin-Streptomycin	Thermo Fisher
L-Glutamine (200 mM)	Thermo Fisher
Fetal Bovine Serum (FBS)	SAFC
single gRNA vector	Addgene #67974
CellLytic Nuclear extraction kit	sigma-alorich
XtremeGENE 9	sigma-alorich
Retronectin	Takara
Flow cytometer	BD Biosciences
T4 PNK	NEBioLabs
T4 DNA ligation buffer	NEBioLabs
T4 DNA Ligase enzyme	NEBioLabs
Ampicillin	Fisher scientific
LB agar	Fisher scientific
LB Broth	Fisher scientific
Qiagen mini-prep kit	Qiagen
NanoDrop Spectrophotometer	Thermo Fisher
Recombinant Murine IL-3	Peprotech
Recombinant Murine IL-6	Peprotech
Recombinant Murine M-CSF	Peprotech
Stable competent cells	NEBiolabs
10 cm Tissue Culture dishes	Fisher Scientific
Cell lysis solution	Qiagen
Protein precipitation solution	Qiagen
DNA hydration solution	Qiagen
QIAquick Gel Extraction Kit	Qiagen
BbSI	New England BioLabs
APEX 2.0 X Taq Red Master Mix Kit	Genessee Scientific
Puromycin	Fisher scientific
50 mL polypropylene conical tubes	Fisher scientific
15 mL polypropylene conical tubes	Fisher scientific

Catalog Number

F3165, lot # SLBS3530V

31446, lot # TA2514341

34095

17001401

R21001

S34859

31985062

11965-092

11875-093

15140122

25030081

12303C

pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W

NXTRACT

6365787001

T100B

M0201S

B0202S

M0202S

BP1760-25

BP9724-500

BP9731-500

27104

NanoDrop One

213-13

216-16

315-02

C3040I

353003

158906

158910

158914

28704

R0539S

42-138

BP2956100

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We have now proofread the entire manuscript and made changes.

2. The highlighted protocol steps are over 2.75 page limit (including headings and spacing). Please reduce the amount of the highlighted protocol steps.

We have reduced the highlighted protocol to less than 2.75 pages.

3. Please do not highlight a step without highlighting any of the sub-steps (Step 3.3, Step 2.1.1.).

All commercial names have now been removed from the manuscript. Please see lines 97,101, 103, 120 and 127 in section 1.1 and 1.2.

4. Step 1.4.5: Please specify the antibody.

This has now been fixed. Please see line 196.

5. 1.5.1: How to prepare? Please specify.

This has now been done. Line 208.

6. 1.5.2: Please describe all details about the spinfection method. All details need to be included in order to film this step.

This has now been done, see lines 321- line 341.

7. 2.1.1: Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This has now been corrected. Please see line 233.

8. 3.4: Calculation steps cannot be filmed.

Step 3.4 is now removed from the highlighted protocol.

9. 3.5: Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This has now been corrected. Please see line 380.

10. Please do not abbreviate the journal titles for all references.

Can you please provide information on how to do this? We downloaded the JoVE style from your submission link and formatted citations accordingly as mentioned in our last rebuttal. Please do not provide a single line request to abbreviate journal titles repeatedly. Please tell us exactly how. We followed instructions from your journal website.

11. Please specify all primers.

All the primers are listed in a separate excel file "Oligonucleotide sequences".

sgRNA oligo sequences:

Oligo name
RPA3 gRNA2 sense
RPA3 gRNA2 antisense
RPA3 gRNA8 sense
RPA3 gRNA8 antisense
AAVS gRNA1 sense
AAVS gRNA1 antisense
AAVS gRNA5 sense
AAVS gRNA5 antisense
Dot1l gRNA1 sense
Dot1l gRNA1 antisense
Dot1l gRNA3 sense
Dot1l gRNA3 antisense
Rho gRNA1 sense
Rho gRNA1 antisense
Rho gRNA6 sense
Rho gRNA6 antisense

Primer sequences:

Primer Name
Humer U6 promoter forward
AAVS1_target-frag_F
AAVS1_target-frag_R

Sequence
caccGCAGGTCGCGCATCAACGC
aaacGCGTTGATGCGCGACCTGC
caccGCCAGGTCGCGCATCAACGC
aaacGCGTTGATGCGCGACCTGGC
caccGGGGGGTTAGACCCAATATC
aaacGATATTGGGTCTAACCCCCC
caccGAGAGGTGACCCGAATCCAC
aaacGTGGATTCGGGTACCTCTC
caccGAGCGGCCACGGGTAGACGGC
aaacGCCGTCTACCCGTGGCCGCTC
caccGCGTCTACCCGTGGCCGCTGCG
aaacCGCAGCGGCCACGGGTAGACGC
caccGGTACGGTGACGTAGAGCGTG
aaacCACGCTCTACGTCACCGTACC
caccGCCGATGAGCAACTCCGCTT
aaacAAGCGGAAGTTGCTCATCGGC

Note: sgRNA sequences are in capital letters, nucleotides added to oligos for generating Bbs1 compatible

Sequence
GACTATCATATGCTTACCGT
CACTTCAGGACAGCATGTTTGCTG
TCCAGGAAATGGGGGTGTGTC

e overhangs are in lowercase