Video Article

Using a Fluorescent PCR-capillary Gel Electrophoresis Technique to Genotype CRISPR/Cas9-mediated Knockout Mutants in a High-throughput Format

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Abstract

The development of programmable genome-editing tools has facilitated the use of reverse genetics to understand the roles specific genomic sequences play in the functioning of cells and whole organisms. This cause has been tremendously aided by the recent introduction of the CRISPR/Cas9 system—a versatile tool that allows researchers to manipulate the genome and transcriptome in order to, among other things, knock out, knock down, or knock in genes in a targeted manner. For the purpose of knocking out a gene, CRISPR/Cas9-mediated double-strand breaks recruit the non-homologous end-joining DNA repair pathway to introduce the frameshift-causing insertion or deletion of nucleotides at the break site. However, an individual guide RNA may cause undesirable off-target effects, and to rule these out, the use of multiple guide RNAs is necessary. This multiplicity of targets also means that a high-volume screening of clones is required, which in turn begs the use of an efficient high-throughput technique to genotype the knockout clones. Current genotyping techniques either suffer from inherent limitations or incur high cost, hence rendering them unsuitable for high-throughput purposes. Here, we detail the protocol for using fluorescent PCR, which uses genomic DNA from crude cell lysate as a template, and then resolving the PCR fragments via capillary gel electrophoresis. This technique is accurate enough to differentiate one base-pair difference between fragments and hence is adequate in indicating the presence or absence of a frameshift in the coding sequence of the targeted gene. This precise knowledge effectively precludes the need for a confirmatory sequencing step and allows users to save time and cost in the process. Moreover, this technique has proven to be versatile in genotyping various mammalian cells of various tissue origins targeted by guide RNAs against numerous genes, as shown here and elsewhere.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55586/>

Introduction

Reverse genetic approaches have allowed scientists to elucidate the effects of specific alterations in the genome on the cell or whole organism. For example, the expression of a particular gene can be attenuated by gene knockdown^{1,2} (partial reduction) or gene knockout^{3,4} (complete ablation) in order to determine the effect that this has on the function of the cell or on the development of the organism.

Gene knockout experiments have become easier since the introduction of sequence-specific programmable nucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). However, the relatively recent characterization of the clustered regularly interspersed short palindromic repeat (CRISPR)/Cas9 system has made it extremely easy for any laboratory around the world to perform gene knockout experiments. In essence, the CRISPR/Cas9 system consists of two essential components—a single guide RNA (sgRNA), which recognizes and binds via base complementarity to a specific sequence in the genome, and an endonuclease called Cas9. The aftermath of the specific binding and action of the sgRNA-Cas9 complex on genomic DNA is the double-strand cleavage of DNA. This, in turn, triggers the DNA damage response mechanism in the cell, which is subsequently repaired via the non-homologous end-joining (NHEJ) or homologous recombination (HR) pathways. Since the NHEJ repair mechanism (but not the HR mechanism) often results in the random insertion or deletion of nucleotides at the site of repair, resulting in insertion/deletion (indel) mutations, it may cause the reading frame of an exon to shift. This may then result in the knockout of the gene due to premature termination of translation and nonsense-mediated decay^{5,6,7}.

Despite the convenience afforded by the introduction of the CRISPR/Cas9 system in knocking out a gene, the genotyping of clones of targeted cells remains a bottleneck, especially in a high-throughput setting^{8,9}. Existing techniques either suffer major inherent limitations or are financially costly. For example, the SURVEYOR or T7E1 assay, which is an enzymatic assay that detects mismatches in DNA duplexes¹⁰, is not able to distinguish between wildtype clones and homozygous mutants (clones whose alleles are mutated identically), since these clones have identical alleles and thus do not present mismatches in their DNA sequence¹¹. In addition, the use of Sanger sequencing, which is considered the gold standard in genotyping mutant clones, in a high-throughput setup is undesirable due to its high cost. Here, we present a detailed protocol of the fluorescent PCR-capillary gel electrophoresis technique, which can circumvent the limitations of the other existing genotyping techniques and is

particularly useful in performing a high-throughput screen of nuclease-mediated knockout clones. This method is technically simple to perform and saves time and cost.

Protocol

1. Obtaining CRISPR/Cas9-targeted Single-cell Clones

- Seed HEPG2 cells on a 6-well plate at 500,000 cells per well in 2 mL of antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Incubate for 24 h at 37 °C and 5% CO₂.
- Transfect cells with plasmid co-expressing Cas9 and specific sgRNA against the gene of interest using an appropriate transfection reagent as per the manufacturer's instructions.
NOTE: For example, sgRNA can be cloned into the pSpCas9(BB)-2A-GFP vector, as described previously⁴.
- Replace the culture medium 4 - 16 h after transfection with 2 mL of fresh antibiotic-free medium.
- About 48 h after transfection, collect single-cell suspension by trypsinizing the cells.
 - Trypsinize the cells in 0.2 mL of 0.25% trypsin-EDTA and incubate at 37 °C for 5 min. Add 1 mL of medium and resuspend thoroughly.
- Sort the cells for GFP-positive clones, as described previously¹², and collect about 3,500 cells.
- Plate the GFP-positive sorted cells on 10-cm dishes at 500, 1,000 and 2,000 cells per dish in 8 mL of penicillin/streptomycin-supplemented culture medium. Incubate the cells at 37 °C and 5% CO₂.
- Maintain the cells at 37 °C and 5% CO₂, replacing the medium every five days, until they grow into single-cell colonies large enough to be visible to the naked eye. For most cancer cell lines, this takes about two weeks from day of plating.
- When the colonies are of the appropriate size (*i.e.*, visible to the naked eye), transfer individual colonies to wells of a 96-well culture plate containing 200 µL of DMEM supplemented with 10% FBS.
 - Aspirate the single-cell colonies using a 200-µL pipette with a small volume of medium. Resuspend the cells thoroughly in individual wells by triturating several times.
- Maintain the cells at 37 °C and 5% CO₂, replacing the medium every five days, until they reach 50 - 90% confluence. For most cancer cell lines, this takes about 24 - 48 h.

2. Extracting Crude Genomic DNA Using a Direct Lysis Method

- When the cells reach 50 - 90% confluence, remove as much of the culture medium from the wells as possible using multi-channel vacuum suction or a multi-channel pipette.
- Add 25 µL of 0.05% trypsin-EDTA (without phenol red) into each well and incubate at 37 °C for 7 min.
- Resuspend the trypsinized cells thoroughly by pipetting up and down several times. Check the cells under a microscope to make sure that they are detached from the plastic surface.
- Create a replicate of the individual clones by transferring approximately 5 µL of the single-cell suspension to an empty 96-well culture plate. Add 200 µL of culture medium to each well and maintain the cells until positive clones are identified using fluorescent PCR-capillary gel electrophoresis (see below). Serially expand the cells to 10-cm dishes or any other scale of choice (see section 7).
- Add 5 µL of the single-cell suspension from step 2.3 to 10 µL of homemade direct-lyse buffer (10 mM Tris pH 8.0, 2.5 mM EDTA, 0.2 M NaCl, 0.15% SDS, and 0.3% Tween-20)¹² in a 96-well PCR plate and mix thoroughly by pipetting up and down several times. Centrifuge briefly (to bring the liquid down to the bottom of the wells).
- Add 200 µL of culture medium to the remaining ~ 15 µL of cell suspension from step 2.3 and incubate at 37 °C and 5% CO₂, together with the replicate from step 2.4.
- Subject the lysates from step 2.5 to the following thermal cycling program to ensure complete lysis of the cells and release of genomic DNA: 65 °C for 30 s, 8 °C for 30 s, 65 °C for 1.5 min, 97 °C for 3 min, 8 °C for 1 min, 65 °C for 3 min, 97 °C for 1 min, 65 °C for 1 min, and 80 °C for 10 min. Centrifuge the lysates briefly.
- Dilute the lysates by adding 40 µL of nuclease-free water and mix thoroughly using a vortex mixer. Centrifuge briefly. The diluted lysates can be used immediately or stored at -20 °C for several months without significant loss of quality.

3. Performing Fluorescent PCR to Amplify CRISPR/Cas9 Target Regions

- Design two fluorophore-labeled forward primers (both labeled at the 5' end) for each CRISPR/Cas9 target region; cutting sites that are further than 300 bp from each other should be considered as two separate target regions (*e.g.*, green fluorophore-labeled primer for untargeted wildtype control and blue fluorophore-labeled primer for CRISPR/Cas9-targeted clones; see the **Table of Materials**).
 - Procure these labeled forward primers and an unlabeled reverse primer accordingly. Note that primers can be designed using any tool of choice and that the amplicons should be 200 - 500 bp long.
- Perform PCR as described previously¹² to amplify target regions using the labeled primers.
 - Use 3 µL of the diluted lysates from step 2.8 in a 20-µL reaction (see **Table 1**) and the following thermal cycling program: 94 °C for 10 min (1 cycle); 94 °C for 10 s, 64 °C for 30 s, 68 °C for 1 min (4 cycles); 94 °C for 10 s, 61 °C for 30 s, 68 °C for 1 min (4 cycles); 94 °C for 10 s, 58 °C for 30 s, 68 °C for 1 min (4 cycles); 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 1 min (35 cycles).
- Resolve 5 µL of the PCR amplicons on a 1% agarose gel to check for size and relative amount of amplicons¹².
NOTE: Performing this step for all the samples is encouraged but not necessary; resolving a selected number of samples is sufficient to estimate the amount of amplicons present in the samples in general.

4. Preparing Samples for Capillary Gel Electrophoresis

1. Dilute amplicons of wildtype (untargeted parental cells) and CRISPR/Cas9-targeted DNA in nuclease-free water to approximately 2.5 ng/ μ L. Make sure to dilute enough wildtype DNA sample to be added to each targeted DNA sample (a minimum of 0.5 μ L of diluted wildtype sample per targeted sample is required).
2. Mix the diluted wildtype and targeted DNA samples in equal ratio (e.g., mix 1 μ L of wildtype sample with 1 μ L of targeted sample).
3. Add 1 μ L of the mixed amplicons to 8.7 μ L of deionized formamide and 0.3 μ L of dye-labeled size standard in a 96-well PCR plate that is compatible with the genetic analyzer.
NOTE: The use of a master mix of the formamide and the size standard (i.e., a preparation of a premix of the formamide and the size standard in a 29:1 ratio prior to the addition of amplicons to ensure standardized amounts) is recommended.
4. Tightly seal the plate and heat the samples at 95 °C for 3 min using a PCR thermocycler.
5. Place the plate on ice immediately after the heating step and incubate for at least 3 min.

5. Performing Capillary Gel Electrophoresis on a Genetic Analyzer

1. Set up assay parameters, instrument protocol, and size-calling protocol on the capillary gel electrophoresis software connected to a genetic analyzer.
NOTE: This step is only required for the first electrophoresis run; the program can be saved for future use. For subsequent runs, go straight to step 5.2.
 1. Click on the "Create New Plate" icon on the software dashboard.
 2. Give the run a dummy name and select the following options: Number of wells, 96; Plate Type, Fragment; Capillary Length, 50 cm; and Polymer, POP7. Click on the "Assign Plate Content" button.
 3. Under "Assays," click "Create New Assay"; a new panel will appear.
 4. Name the assay "FPCR-CGE Assay" and check that "Application Type" is correctly set as "Fragment."
 5. Set up instrument protocol by clicking on the "Create New" button under "Instrument Protocol."
 1. Set or choose the following options and parameters in the appropriate areas: Application Type, Fragment; Capillary Length, 50 cm; Polymer, POP7; Dye Set, G5; Run Module, FragmentAnalysis; Protocol Name, FPCR-CGE Instrument Protocol; Oven Temperature, 60 °C; Run Voltage, 19.5 kV; Pre-run Voltage, 15 kV; Injection Voltage, 1.6 kV; Run Time, 1,330 s; Pre-run Time, 180 s; Injection Time, 15 s; and Data Delay, 1 s.
 6. Click on the "Apply to Assay" button and then the "Save to Library" button to save the program. Close the panel to continue.
 7. Set up a size-calling protocol by clicking on the "Create New" button under "Sizecalling Protocol."
 1. Set or choose the following options and parameters in the appropriate areas: Protocol Name, FPCR-CGE Sizecalling Protocol; Size standard, GS500(-250)LIZ; Size-caller, SizeCaller v1.1.0; Analysis Settings,-; Analysis Range, Full; Sizing Range, Full; Size Calling Method, Local Southern; Primer Peak, Present; Blue, Green, Orange Channels, (Check); Minimum Peak Height, 175 for all channels; Use Smoothing, None; Use Baseline (Baseline Window (Pts)), (Check) 51; Minimum Peak Half Width, 2; Peak Window Size, 15; Polynomial Degree, 3; Slope Threshold Peak Start, 0.0; Slope Threshold Peak End, 0.0; QC Settings, -; Size Quality, -; Fail if Value is <0.25; Pass if Value is <0.75; Assume Linearity from 0 bp to 800 bp; and Actuate Pull-Up flag if Pull-Up Ratio ≤ 0.1 and Pull-Up Scan ≤ 1 .
 8. Click on the "Apply to Assay" button and then the "Save to Library" button to save the program. Close the panel to continue.
 9. Ensure that the assay is saved by clicking on the "Save to Library" button once more and exit the panel by clicking the "Close" button.
 10. Back at the "Assign Plate Contents" page, click on the "Create New File Name Convention" link under "File Name Conventions."
 11. Name the program "FPCR-CGE File Name."
 12. Under "Available Attributes," select the desired attributes that will appear in the file name (such as "Date of Run," "Time of Run," "Well Position," and "Sample Name"). Choose the desired file location where the results of the run will be stored.
 13. Click on the "Apply to Assay" button and then the "Save to Library" button to save the program. Close the panel to continue.
 14. Back at the "Assign Plate Contents" page, click on the "Create New Results Group" link under "Results Groups."
 15. Name the program "FPCR-CGE Results Groups."
 16. Under "Available Attributes," select the desired attributes that will appear in the file name (such as "Assay Name"). Choose the desired file location where the results of the run will be stored.
 17. Click on the "Apply to Assay" button and then the "Save to Library" button to save the program. Close the panel to continue.
2. Set up the program for an electrophoresis run by following the steps below.
 1. Go to the dashboard and click on the "Create New Plate" icon.
 2. Name the run as desired (for easy reference, include the date, cell line, and gene name). Select the following options: Number of wells, 96; Plate Type, Fragment; Capillary Length, 50 cm; and Polymer, POP7. Click on the "Assign Plate Content" button.
 3. Label each well of the sample as desired (e.g., a sample can be named "NC" to indicate that the well is a negative control).
 4. Under the "Assays," "File Name Conventions," and "Results Groups" boxes, click the "Add from Library" link and select the programs created in step 5.1.3 to 5.1.17.
 5. Highlight all the wells to be analyzed and select the relevant programs under "Assays," "File Name Conventions," and "Results Groups" by checking the boxes next to them.
3. Before loading the plate on the tray of the genetic analyzer, apply the rubber seal on the 96-well plate and put the sealed plate in the plastic case that comes with the genetic analyzer.
4. Push the "Tray" button at the front of the genetic analyzer, and when the tray reaches the front of the equipment, open the door and load the encased plate on the tray. Ensure that the plate is locked in place and then close the door.
5. Click on the "Link Plate for Run" button.
6. In the "Load Plates for Run" page, do a final check to ensure that all the reagents and conditions are correct and in order.

7. Click on the "Start Run" button. Each run of 24 samples (the first 3x8 wells of the 96-well plate) takes less than 55 min to complete.

6. Analysis of the Electropherogram to Determine Base-pair Differences

1. When the capillary gel electrophoresis run is complete, open the analysis software to analyze the results.
2. Click on the "Add Samples to Project" icon and search for the folder containing the run files. Recall from step 5.1.12 the assigned location of the results files.
3. Select all the results files of each injection in the completed run and click on the "Add to List" button; the names of these files begin with "Inj" and contain the details of the run.
4. Click the "Add and Analyze" button to proceed with the analysis.
5. Select all the samples in the run by clicking on the first sample and dragging the cursor down to the last sample and then click the "Display Plots" icon.
6. To check the quality of the size standard, open the orange channel of the results by checking the orange icon and unchecking the rest of the colored icons; this is important to ensure that the size-calling is accurate and reliable.
7. To view the peaks corresponding to the fragments derived from the untargeted control and the targeted clones, check the blue and green icons to open readings from these channels.
8. Place the cursor over the horizontal axis of the first results plot and right-click to select "Full View." Scroll down to view all the results at a glance to determine if there is any major problem with the run. To zoom in on a specific range of values, right-click the mouse on the relevant axis of the plot, choose "Zoom To...", and key in the range of values of interest.
NOTE: One potential major problem is that all the samples give low intensity peaks. This is usually due to the prolonged storage of fluorophore-labeled amplicons prior to the capillary electrophoresis run or the over-dilution of amplicons, which can easily be rectified by repeating the PCR step or by diluting the amplicons using a lower dilution factor, respectively.
9. To save the results, follow the steps described below.
 1. Ensure that the blue and green channels are selected and click on the "Sizing Table" icon.
 2. Save the table of values in tab-delimited text (.txt) format by opening "File" and choosing "Export Table." Name the file accordingly and select the file location of choice.
 3. To save the plots of the run in PDF format, go to "File" and click on the "Print" option. Choose the relevant PDF writer and press "Print." Name the file accordingly and select the file location of choice.
10. To calculate the difference in the size of the fragments derived from untargeted wildtype control and the CRISPR/Cas9-targeted clones, follow the steps described below.
 1. Open the tab delimited text (.txt) file from step 6.9.2 with a spreadsheet program. The table should include these four important columns: "Dye/Sample Peak" (indicating a blue or green channel), "Sample File Name" (the first characters indicate the well or sample name), "Size" (indicating the size of the fragment called), and "Height" (indicating the fluorescence intensity of the peak).
 2. To single out relevant peaks, exclude those that are not in the expected size range.
NOTE: Typically, indel mutations rarely result in more than a 100-bp difference in size; thus, peaks whose size differs by more than 100 bp from the wildtype control peak (dominant peak in the green channel) are excluded. This can be easily done by using the "Between" option under the "Conditional Formatting" function in the spreadsheet.
 3. To remove non-specific peaks, which are indistinguishable from background level, use the "Less Than" option under the "Conditional Formatting" function in the spreadsheet program to exclude peaks whose heights are lower than 2,000 units. This cut-off value has been empirically determined and can therefore be adjusted where deemed fit.
 4. Calculate the difference in fragment size between each of the resulting peaks in the blue channel (CRISPR/Cas9-targeted clones) and the sole peak in the green channel (untargeted wildtype control) by subtracting the latter from the former.
NOTE: It is important to use values attributed to each capillary electrophoresis sample, as there may be inter-sample differences in the fragment size of the wildtype control.
 5. Round off the values to the nearest integer to determine the number of base pairs that have been inserted into or deleted from, if any, the genomic sequence in question. When knocking out a gene is of interest, select clones whose indel mutations are not of multiples of 3 bp to ensure that there is frameshift in the coding sequence.

7. Verification of the Knockout Status of Clones

1. Expand individual knockout clones from the 96-well plate (from steps 2.4 and 2.6) to a 24-well plate by trypsinizing the cells using 25 μ L of 0.25% trypsin-EDTA and incubating at 37 °C for 5 min.
2. Add 125 μ L of medium (DMEM supplemented with 10% FBS) to the trypsinized cells and resuspend thoroughly.
3. Transfer the cell suspension to a 15-mL tube and centrifuge at 400 x g for 5 min at room temperature.
4. Remove as much of the medium as possible using vacuum suction or a pipette, resuspend the cell pellet in 500 μ L of medium, and transfer the cell suspension to a well of a 24-well plate. Incubate at 37 °C and 5% CO₂ until the cells reach 80-90% confluence.
5. Expand the individual clones serially from the 24-well plate to a 6-well plate and from the 6-well plate to a 10-cm dish when they reach 80 - 90% confluence by repeating steps 7.1 to 7.4, using the following volumes: 50 μ L of 0.25% trypsin-EDTA and 150 μ L of medium (for expanding from the 24-well plate to the 6-well plate) and 200 μ L of 0.25% trypsin-EDTA and 1 mL of medium (for expanding from the 6-well plate to the 10-cm dish).
6. Harvest the clones when they reach 80 - 90% confluence by trypsinizing the cells using 1 mL of 0.25% trypsin-EDTA and incubate at 37 °C for 5 min.
7. Add 5 mL of medium (DMEM supplemented with 10% FBS) to the trypsinized cells and resuspend thoroughly.
8. Transfer the cell suspension equally into two 15-mL tubes, centrifuge at 400 x g for 5 min at room temperature, and remove the medium as much as possible. One portion of the cells is for genomic DNA extraction and the other is for total protein extraction.
9. For verification via Sanger sequencing, follow the steps described below.
 1. Extract genomic DNA from the clones as described previously¹².

2. Perform PCR amplification of the region spanning the CRISPR/Cas9 target site, as described in section 3.2, but use unlabeled primers. Do not use labeled primers for this step, as the fluorescence from the tag will interfere with the subsequent Sanger sequencing step.
 3. Purify the amplicons using a PCR clean-up kit, as described previously¹².
 4. Sequence the purified amplicons using the PCR primers used in step 7.9.2 to determine the genotype of the clones, as described previously¹².
10. For verification via Western blot analysis, extract total protein from the wildtype cells and targeted clones, as described previously¹².
1. Perform Western blot analysis using appropriate antibodies against the protein of the target gene, as described previously¹²; a true knockout clone is devoid of the expression of the protein.

Representative Results

The fluorescent PCR-capillary gel electrophoresis technique described here is anticipated to be applicable to any targetable region in the genome in virtually any cell line that is amenable to foreign DNA delivery. We have previously demonstrated its application by targeting three genes in a colorectal cancer cell line¹². Here, we show its efficacy in genotyping a hepatocellular carcinoma cell line, HEPG2, targeted with a CRISPR/Cas9 construct against the Nucleosome Assembly Protein 1 Like 1 (NAP1L1) gene. In fact, we have successfully utilized the fluorescent PCR-capillary gel electrophoresis technique to genotype various other cells, including non-human mammalian cell lines, targeted at numerous other genes^{12,13}.

Experiment-wise, the fluorescent PCR-capillary gel electrophoresis technique is easy and fast to perform. After the introduction of Cas9 and sgRNA expression constructs into the cells and the selection of positive clones, the individual clones are lysed directly from the 96-well culture plate using our homemade lysis buffer, Direct-Lyse Buffer¹². In our experience, the resulting lysates can be stored at -20 °C or lower for several months without significant loss of genomic DNA quality. The lysis step is followed by the PCR amplification step, which involves the production of fluorophore-tagged amplicons that span the CRISPR target region. The fluorescent PCR protocol provided has been optimized for this purpose and has consistently produced ample PCR products, regardless of the region being amplified. **Figure 1** shows a representative result of the resolution of the amplicons derived from the fluorescent PCR step, with each lane corresponding to an individual CRISPR/Cas9-targeted clone and the various bands corresponding to the amplified regions of individual alleles in the clones. In our experience, the use of other polymerases and their concomitant protocols resulted in a lower amplicon yield. Although this can be easily rectified by adjusting the dilution factor during sample preparation for capillary gel electrophoresis, the background or noise level may consequently be higher when amplicons of lower yield are used. After the PCR step, the amplicons are diluted, and those of the wildtype sample and the targeted clones are mixed in equal ratio before they are added to deionized formamide buffer and a size standard, denatured, and resolved on a capillary gel.

After the completion of the capillary gel electrophoresis, the genotyping results are ready to be analyzed. Two important sets of results are required from the electrophoresis run: the electropherograms containing the peaks corresponding to individual fluorescence signals and the result table containing all the necessary values required for calculation. **Figure 2** shows the results for the genotyping of two clones targeted by sgRNA against the NAP1L1 gene in HEPG2 cells. The green peaks correspond to the amplicons of the untargeted wildtype allele in the parental HEPG2 cells, whereas the blue peaks correspond to amplicons of the indel mutation-containing alleles of the CRISPR/Cas9-targeted clones. As clearly shown, the two clones are homozygous mutants (mutants with identically mutated alleles), with the deletion of one and ten nucleotides on both alleles. It is important to note that the electropherograms are used for visualization purposes only, whereas the peak values are important in determining the number of base pair differences between the amplicon of the targeted clones and that of the wildtype cells.

To validate the authenticity of the knockout status, we recommend performing Sanger sequencing and Western blot analysis to confirm the abolition of gene expression in the cells. The two clones identified above gave Sanger sequencing results consistent with the fluorescent PCR-capillary gel electrophoresis results (**Figure 3A**). They also displayed complete ablation of NAP1L1 protein expression (**Figure 3B**), as expected of knockout clones.

Figure 1: PCR Amplicons of HEPG2 Clones Targeted by CRISPR/Cas9 Against the NAP1L1 Gene. Amplicons of the fluorescent PCR step were resolved on two separate agarose gels (top and bottom), and each lane corresponds to an individual targeted clone. L: DNA ladder (the sizes of individual bands are given next to the diagram). [Please click here to view a larger version of this figure.](#)

Figure 2: Plots of Fluorescence Signals from Two Samples Resolved via Capillary Gel Electrophoresis. The horizontal axis represents the fragment size and the vertical axis represents the fluorescence signal intensity. The blue peaks correspond to fragments derived from CRISPR/Cas9-mediated targeted clones, while the green peaks correspond to fragments derived from wildtype cells. Magenta lines correspond to automatic peak-calling positions determined by the analysis software, which marks positions that consistently show peaks across samples. The values provided next to individual peaks correspond to the sizes of the fragments (in bp) and are obtained from the analysis software. The values in parentheses depict the calculated difference in size between individual fragments from a targeted clone and the wildtype fragment. [Please click here to view a larger version of this figure.](#)

Figure 3: The Results of Assays to Confirm the Knockout of the targeted gene in two clones. (A) Sanger sequencing results and **(B)** Western blot analysis using antibodies against the indicated protein. Nucleotides in blue represent the sgRNA target sequence, the ones in red represent the protospacer adjacent motif (PAM), the ones in brown represent base-substituted nucleotides, and the dashes represent the positions where the nucleotide are deleted in the allele. The values in parentheses next to the individual clone names represent the genotype of the alleles of the clone; "-1" and "-10" means that the alleles contain a deletion of one and ten nucleotides, respectively. Approximate size of proteins detected in the Western blot analyses: ~ 54 kDa for NAP1L1 and ~ 84 kDa for p84 (loading control). [Please click here to view a larger version of this figure.](#)

Reagent	Volume for one reaction (µl)
Kit specific solution	4
10x PCR Buffer	2
10 µM forward primer (labeled)	1
10 µM reverse primer (unlabeled)	1
25 mM dNTP mix	0.4
Taq DNA Polymerase	0.2
Water	8.4
Diluted lysate	3
Total	20

Table 1: Reagents for the Fluorescent PCR Reaction.

Discussion

The knocking out of a specific gene in a model cell line of choice has become routine for elucidating the role that the gene plays in that particular cellular context. In fact, several genome-wide screens are currently available that use the CRISPR/Cas9 system to target virtually all known human genes in the genome^{14,15,16}. With these large-scale screens (or even small-scale targeting of individual genes of interest), it is important to design and utilize sgRNAs targeting different loci of the same gene. Consistent results across the different sgRNAs used would unambiguously capitate the true effect of the depletion of the gene. As such, the targeting of one single gene would require a high-volume genotyping step to accurately genotype a multitude of clones from each of the individual sgRNA used. The fluorescent PCR-capillary gel electrophoresis technique described here can precisely perform this task.

Whilst most of the existing genotyping methods are amenable to high-throughput purposes, each of them suffers from certain inherent limitations that render them less than ideal. The SURVEYOR, or T7E1, assay, which detects mismatches in DNA duplexes¹⁰, is not able to differentiate between wildtype cells and homozygous mutants (clones with two identically mutated alleles) due to the fact that both these clones yield duplexes devoid of mismatches¹¹. The restriction fragment length polymorphism (RFLP) assay, which reports the disappearance of restriction sites due to indel mutations in the CRISPR target region¹⁷, is limited by the availability of suitable restriction sites at the target region¹⁸. The DNA melting analysis technique, which discerns the different genotypes based on their melting curves^{19,20}, suffers from a lack of consistency. In addition, all three of these methods are not particularly informative in that they do not report the occurrence of a frameshift in the genetic sequence. In contrast, Sanger sequencing - which is presently the most popular genotyping technique - is extremely informative, since it provides the exact sequence of the genomic region being targeted. However, this technique is costly, especially in large-scale experiments. Thus, the characterization of the fluorescent PCR-capillary gel electrophoresis technique described here is vital because it can circumvent all the limitations faced by the other techniques described above.

The fluorescent PCR-capillary gel electrophoresis technique enables users to distinguish between all possible genotypes a clone may exist in-wildtype, heterozygous mutant (characterized by a wildtype allele and a mutant allele), homozygous mutant (characterized by two identical mutant alleles), and compound heterozygous mutant (characterized by two non-identical mutant alleles). These genotypes are easily differentiable by the peak patterns in the electropherogram. Furthermore, this genotyping technique reports the difference between the fragment size of the wildtype amplicon and that of the targeted clones and is accurate to a single base pair. This effectively reports the presence or absence of a frameshift in the genetic sequence, allowing the users to reduce their validation to only a handful of clones, saving them time and cost. On top of that, this technique also allows for the multiplexing of gene targeting (*i.e.*, it can concurrently genotype more than one target), and it enables the detection of a heterogeneous cell population from the presence of aberrant peak patterns (*e.g.*, the presence of three or more peaks in the electropherogram of diploid cells). In addition to the cell lines used here and previously^{12,13}, we have also successfully genotyped various other human and non-human cell lines, including stem cells and neuronal cells (unpublished data), using the fluorescent PCR-capillary gel electrophoresis technique, and we anticipate that this technique is applicable to any cells that are amenable to CRISPR/Cas9 targeting. Moreover, since this technique reports the genotype of individual alleles in the cell, it is extremely useful in genotyping multi-allelic cells, such as cancer cells that have aneuploidy or genetic amplifications.

The protocol described here (including all the material used) has been optimized for the versatile and reproducible genotyping of CRISPR/Cas9-targeted clones. Nevertheless, there are some parts that warrant modification. They include, but are not limited to: 1) the use of a different sgRNA expression vector (or cloning strategy), which may necessitate the use of a different selection strategy (*e.g.*, the antibiotic selection of clones instead of FACS); 2) the use of a different polymerase for the fluorescent PCR step; and 3) the use of different fluorescent labels. Moreover, it is worth noting that a couple of essential steps in the protocol may prove problematic. For one, the fluorescent PCR amplicons may yield unspecific bands in the agarose gel electropherogram, or the peak pattern in the capillary gel electropherogram may be "noisy." This is likely due to the sub-optimal quality of the PCR primers and therefore can be rectified by re-designing these primers. We recommend testing the quality of the primers using unlabeled oligonucleotides prior to procuring the fluorophore-labeled ones to ensure the consistency, reproducibility, and specificity of the amplification step. The other important factor to note is the efficiency of the CRISPR guide RNA, which may determine the rate of obtaining true positive knockout clones. Since none of the sgRNA search programs that are currently available are foolproof, the efficacy of each individual sgRNA can only be determined empirically. Thus, it is important to design and utilize more than one sgRNA (preferably three or more) for each targeted gene to reduce the chance of not obtaining a successful knockout clone.

Whilst the fluorescent PCR-capillary gel electrophoresis technique is informative, sensitive, and easy to use, it comes with some caveats. First, this technique requires the use of a genetic analyzer, which may not be readily available. However, since the genetic analyzer used for this purpose is the same one used for Sanger sequencing experiments, the capillary gel electrophoresis protocol may be outsourced to existing Sanger sequencing service providers. In fact, we have previously arranged with our sequencing service provider to perform the capillary gel electrophoresis protocol at a cost comparable to when done in-house. Second, the accuracy of the technique may suffer when indel mutations longer than 30 bp are involved. In our experience, the fluorescent PCR-capillary gel electrophoresis technique tends to underestimate the size of the indels when large indels (>30 bp) are observed. However, in our experience, a vast majority of mutants displayed very short indel mutation lengths (most are less than 5 bp). Nonetheless, this is highly dependent on the CRISPR target site and cell line used. Third, the fluorescent PCR-capillary gel electrophoresis technique is not able to detect base substitutions upon NHEJ repair at the CRISPR/Cas9 cut site. Nevertheless, it has been reported that base substitution as a result of the NHEJ repair of double-stranded breaks is a rare event²¹, and they do not deleteriously alter the reading frame of the gene. Fourth, this technique only detects changes in the target region amplified in the fluorescent PCR step and thus does not report any off-target aberrations (genetic changes outside the amplified region). If such a comprehensive survey of the off-target effects of individual CRISPR sgRNA is required, we recommend whole genome sequencing to accurately detect any changes in the genome of the targeted clones. However, this rather costly experiment is not necessary if more than one sgRNA is used per targeted gene, as discussed above. Last, the fluorescent PCR-capillary gel electrophoresis technique described here has a fragment size limit of 600 bp. This may pose a problem if the target region consists of repeated sequences or has exceptionally high guanine-cytosine (GC) content, which may affect PCR amplification efficiency and specificity. This easily preventable problem stresses the importance of careful sgRNA target design, which must include proper consideration for the appropriate amplification of the targeted region. Thus, considering the strengths and caveats of the

fluorescent PCR-capillary gel electrophoresis technique, this facile method of genotyping may ease the burden of high-volume genotyping of knockout clones, which remains a bottleneck to this day.

Disclosures

The authors declare that they have no competing financial interests.

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