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Comparative Analysis of Cell Dilution Assays for Single-Cell Colony Formation in CRISPR-Mediated Knockout Screening

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Abstract

BACKGROUND/AIMS: The ribonucleic acid (RNA)-guided Cas9 nuclease from the microbial clustered regularly interspaced palindromic repeats (CRISPR) immune system enables genome editing in eukaryotic cells by using a 20-nucleotide target sequence guide RNA (gRNA). A key CRISPR technique is the ability to identify individual cells in 96-well plates, which highlights the major challenges of obtaining single-cell knockouts. Two distinct dilution strategies were applied to breast cancer cells-the standard dilution method and the two-gradual dilution method-to compare their efficiency in producing single-cell colonies.

MATERIALS AND METHODS: It began with target design, CRISPR-mediated gene modifications, and single-cell assays. Following genetic modifications, successful cloning was confirmed by Sanger sequencing. MDA-MB-231 cells were transfected with the constructed Cas9 plasmid. Forty-eight hours after transfection, the cells were passaged for a single-cell colony assay. Cells were seeded in 96-well plates using two different methods. In the standard cell dilution method, 100 µL of medium was added to each well. A cell suspension containing 100 cells in 100 µL was placed in the first column, and serially diluted across the plate by stepwise transfer of 100 µL from one column to the next to the final column. In the two-gradual cell dilution method, 100 µl of medium was added to all wells except A1, where 2×104 cells in 200 µl were seeded. Cells were diluted down the A1-H1 column, and then 100 µl was transferred sequentially from column to column to the 12th column, with the remaining volume discarded. Single-cell growth and colony formation were monitored by light microscopy at 24-hour intervals.

RESULTS: Comparison of the two-gradual and standard cell dilution methods demonstrated that each plate in the two-gradual method yielded 8-9 single colonies, whereas the standard method yielded only 1-2 colonies per plate (p=0.0192). This result revealed that the two-step gradual dilution method offers a statistically significant advantage in obtaining a greater number of candidate knockout single cells compared with the standard approach.

CONCLUSION: This methodological improvement substantially enhances the efficiency of genetic screening workflows and more robust experimental designs. Consequently, researchers are expected to achieve higher success rates in identifying and validating candidate knockout cells, thereby accelerating downstream functional studies and advancing the reliability of CRISPR-based applications.

Keywords: CRISPR, breast cancer, gRNA, single-cell colony

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INTRODUCTION

Many bacteria and archaea possess ribonucleic acid (RNA)-guided adaptive systems known as clustered regularly interspaced palindromic repeats (CRISPR) systems.[1] The Cas9 nuclease, along with suitable guide RNA (gRNA), can introduce a double-strand break at a specific location of interest in mammalian cells.[2] CRISPR/Cas9 technology offers reduced cost, greater efficiency, and increased simplicity compared with conventional gene-editing technologies such as meganucleases, zinc-finger nucleases, transcription activator-like effector nucleases.[3] The use of CRISPR/Cas9 for genome editing has indeed been widely adopted across various cell lines, including those relevant to breast cancer research, such as MDA-MB-231 and MCF-7. The application of CRISPR technology to breast cancer includes breast cancer modelling, oncogenes and tumor-suppressor genes, breast cancer therapy, diagnosis, drug sensitivity, and resistance.[4] After introduction of the gRNAs, single cells must be isolated to generate clonal lines that can be validated as knockouts.[5] Limiting dilution is a universally applicable and cost-efficient method. Limiting dilution does not require expensive equipment, unlike fluorescence-activated cell sorting (FACS); it involves no radiation, gives reproducible results, and is easily automated. As a future direction, the combination of limiting dilution with upstream enrichment techniques may increase the proportion of highly productive clones.[6] In knockout studies, the single-cell colony assay is both time-consuming and demanding, imposing a long, tiring schedule on the personnel conducting the study. At this stage, it is necessary to obtain potential single knockout colonies for both detection and expansion purposes. Following CRISPR-mediated knockout, obtaining a single colony is crucial for reproducibility, precise genotype-phenotype correlation, and clonal purity. The functional effects of the knockout may be obscured by remaining wild-type or partially edited cells when mixed populations are employed, producing unclear or deceptive results.[7,8] Specifically, single-colony isolation ensures that downstream tests (such as drug sensitivity, differentiation, or proliferation) reflect the behavior associated with a homogeneous genetic background, which is crucial for research on synthetic lethality or gene essentiality.[9]

In this study, two serial dilution methods for a single-cell colony assay are compared. For this purpose, we aimed to determine an effective method for the CRISPR single-cell colony assay. The goal is to identify which serial dilution method most reliably and efficiently isolates potential knockout single colonies. This will ultimately enhance the effectiveness of CRISPR technology in genetic studies.

MATERIALS AND METHODS

This study was conducted exclusively using established commercial cell lines and *in vitro* experimental methods. No human participants, patient-derived samples, or identifiable personal data were involved. Therefore, ethical committee approval and informed consent were not required for this study.

sgRNA Design and CRISPR/Cas9 Construction

First, a 20-bp gRNA specific to the microRNA-182 (miR-182) gene locus was designed and synthesized at a 50-nmol scale. The gRNA design was performed using the Benchling online platform.[10] To induce double-stranded breaks at the target locus while minimizing the likelihood of off-target effects, three distinct double-stranded gRNAs were designed (Table 1). gRNAs were diluted in nuclease-free, double-distilled water to prepare a 100 µM stock solution. For cloning gRNAs, pX330, a human codon-optimized SpCas9, and a chimeric guide RNA expression plasmid were provided. Oligonucleotides were annealed and phosphorylated in a polymerase chain reaction (PCR) reaction to form the gRNA template. The protocol described by Santos et al.[11] was adopted with minor modifications. A PCR reaction was performed using miR-182 gRNA forward and reverse oligos (Table 2). The obtained oligoduplexes were diluted 1:125. Then, the pX330 vector (100 ng) was digested with BbsI, and ligation was performed using Quick Ligase and Quick Ligation Reaction Buffer from New England Biolabs (NEB, Ipswich, MA, USA).

Bacterial Transformation and Sequencing

The expression plasmid carrying the designed gRNA was transformed into *Escherichia coli* (One Shot TOP10, Invitrogen, Carlsbad, CA, USA) following ligation. Transformants were selected on luria-bertani agar plates supplemented with 100 µg/mL ampicillin, and the plates were incubated overnight. Positive colonies were amplified, plasmid deoxyribonucleic acid (DNA) were isolated, and inserts were sequence-verified to confirm correct cloning of the gRNA. After incubation for 24 hours, plasmid DNA was isolated using the QIAprep Spin Miniprep Kit according to the manufacturer's protocol. After plasmid DNA isolation, PCR was performed with U6 F1 and U6 R1 (Table 3) using Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA, USA) to confirm the cloning.

Briefly, 10 µM U6 F1, 10 µM U6 R1, and 100 ng of plasmid DNA were mixed, and the volume was adjusted to 50 mL with Platinum High Fidelity Supermix. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Following purification, the DNA samples were also loaded onto an agarose gel to generate

Table 1. Designed gRNAs targeting miR-182

gRNA name	gRNA sequence (5'→3')
miR-182-gRNA-1	CCATTGCCAAAAACGGGGGG
miR-182-gRNA-2	CTACCATTGCCAAAAACGGG
miR-182-gRNA-3	TCTACCATTGCCAAAAACGG

RNA: Ribonucleic acid, gRNAs: Guide RNA, miR-182: microRNA-182.

Table 2. Designed cloning gRNAs for miR-182 knockout

gRNA	gRNA sequence (5'-3')
miR-182 gRNA1 F (25bp)	CACCGCCATTGCCAAAAACGGGGGG
miR-182 gRNA1 R (25bp)	AAACCCCCCCGTTTTTGGCAATGGC
miR-182 gRNA2 F (25bp)	CACCGCTACCATTGCCAAAAACGGG
miR-182 gRNA2 R (25bp)	AAACCCCGTTTTTGGCAATGGTAGC
miR-182 gRNA3 F (25bp)	CACCGTCTACCATTGCCAAAAACGG
miR-182 gRNA3 R (25bp)	AAACCCGTTTTTGGCAATGGTAGAC

RNA: Ribonucleic acid, gRNAs: Guide RNA, miR-182: microRNA-182.

Table 3. Sequence primers

gRNA	Sequencing primers
gRNA1F	5' CAC CGC CAT TGC CAA AAA CGG GGG G 3'
gRNA2F	5' CAC CGC TAC CAT TGC CAA AAA CGG G 3'
gRNA3F	5' CAC CGT CTA CCA TTG CCA AAA ACG G 3'
U6F1	5' GAG GGC CTA TTT CCC ATG ATT C 3'
U6R1	5' GGG CCA TTT ACC GTA AGT TAT G 3'

RNA: Ribonucleic acid, gRNA: Guide RNA.

the documentation required by the sequencing provider. The purified PCR products, gel images, and primers were submitted to Medsantek (İstanbul, Türkiye) for Sanger sequencing using a Thermo Fisher Applied Biosystems 3500 Genetic Analyzer. The chromatogram files (.ab1) were analyzed using FinchTV (Geospiza, USA) to evaluate peak quality and were exported in FASTA format. Subsequent sequence alignment against the pX330 reference vector was performed using SnapGene Viewer (Dotmatics, USA). Both software tools were used complementarily to enhance the accuracy and reproducibility of sequence verification. Purified DNAs were sequenced with the primers shown in Table 3.

Cell Culture and Transfection

The triple-negative breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection and cultured in [Dulbecco's Modified Eagle Medium (DMEM), Gibco, Thermo Fisher Scientific, Waltham, MA, USA]. It was supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin (10,000 U/mL, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 in air. Cells with a confluency of 80-90% were detached with trypsin-ethylenediaminetetraacetic acid. Prior to seeding and experimentation, cell viability was assessed using 0.4% trypan blue solution. Transfection was performed using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions with minor modifications. On the first day of the experiment, 1×10^6 cells were seeded in 6-well plates containing complete medium. After 24 hours, the medium was replaced with OPTI-MEM reduced serum medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) without serum to optimize transfection efficiency. Twenty-four hours later, MDA-MB-231 cells were transfected with clones that had been confirmed by sequencing. Two hours after transfection, the medium was replaced with complete DMEM supplemented with serum to support cell growth. After 48 hours of culture, the transfected cells were harvested for the single-cell colony assay.

Single-Cell Colony Assay

Cells in the 6-well plate were collected and counted 48 hours after transfection. Cell counting was performed with a Thoma slide, and the total cell number was calculated to be 600,000 cells. Serial dilutions were made using two different methods[12,13] using the 96-well plate in Figure 1A and 1B. For each dilution method, three independent 96-well plates were prepared (six plates in total). Wells were categorized into two groups: (1) empty wells or wells with more than one cell, and (2) wells containing a single-cell. After the dilution process was completed, the next day and every day thereafter, the plates were scanned for a single colony. After approximately one week, rounded colonies radiating from a central point begin to form.

Steps of the Standard Cell Dilution Method

1. 100 µl of medium was added to each well.
2. Cell suspension was added to each well of the "1st column", 100 cells in 100 µl, then 100 µl of cell suspension was taken from the wells in the "1st column" with a multi-pipette and placed in the 2nd column, and this process was continued until the last column.

Steps of the Two-Gradual Cell Dilution Method

1. In serial dilution method 1, 100 µL of medium was first added to each well of each 96-well plate, except A1.
2. Then 200 µl of 2x104 cells were added to the "1st column".
3. From A1 to H1, cells in A1 were diluted along the "1st column" and reached H1.
4. 100 µl of medium was taken with a multiple pipette and added to the "1st column" and the take-and-give process was performed. The 100 µl samples were transferred to the 2nd column, and the same process was repeated until the 12th column. The remaining 100 microliters in the pipette were discarded.

Statistical Analysis

All experimental data were presented as mean ± standard deviation, and statistical analyses were conducted using a trial version of GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk test was used to check the distribution of the data. Comparisons among multiple groups were performed using one-way analysis of variance, followed by Tukey's multiple comparisons test to assess statistical significance. $p<0.05$ was considered to indicate a statistically significant difference.

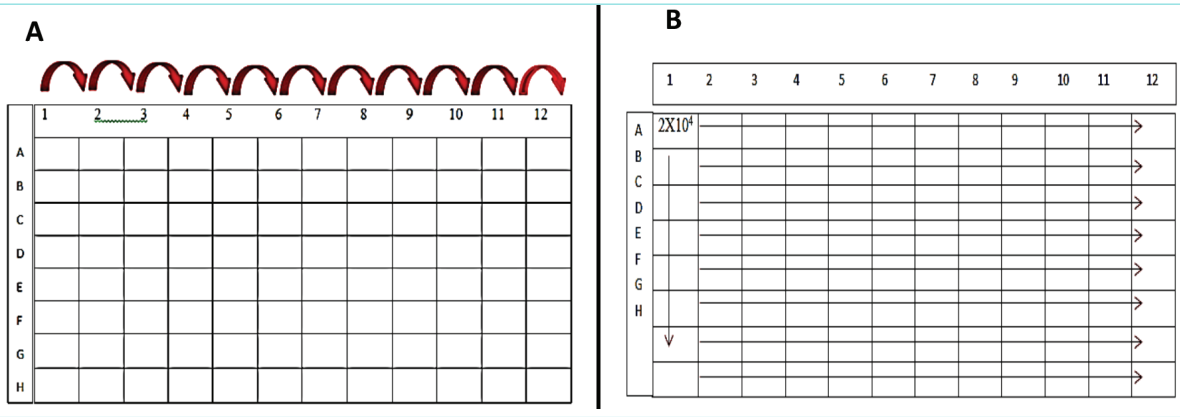

Figure 1. Cell dilution approaches in 96-well plates. A) Plate setup for the standard cell dilution method. B) A plate setup for the two-gradual cell dilution method. A twofold serial dilution is performed across the plate.

RESULTS

PCR of Plasmid DNA and Sequencing

Three different single gRNA (sgRNAs) targeting the miR-182 gene were designed for knockout experiments. After bacterial transformation, three colonies were randomly selected for each sgRNA, each designated S1K1-S3K3. Successful cloning of the gRNAs into the pX330 vector was confirmed by agarose gel electrophoresis, which showed the expected 478-bp product (Figure 2A). Sequence analysis (Figures 2B-D) confirmed correct insertion of the designed sgRNAs into the cloning region of the pX330 vector. Cloning verification was performed by Sanger sequencing; chromatogram files (.ab1) were first inspected in FinchTV (Geospiza, USA) to assess peak quality and then exported in FASTA format. The resulting sequences were then aligned with the pX330 reference in SnapGene Viewer (Dotmatics, USA), enabling precise localization of the designed 20-nt spacer. The reverse complement of the spacer (5'-CCATTGCCAAAAACGGGGGG-3'→5'-CCCCCCGTTTTTGGCAATGG-3') was detected immediately upstream of the sgRNA scaffold motif within the U6-sgRNA cassette, excluding BbsI overhangs. Clean chromatographic peaks without ambiguous signals confirmed the correct insertion. Representative chromatogram (Figure 2B), a zoomed-in view of the spacer region (Figure 2C), and the alignment with the pX330 vector (Figure 2D) are presented to demonstrate accurate integration. In the SnapGene alignment output, the designed spacer sequence (CCATTGCCAAAAACGGGGGG) was detected in the sequencing read but was aligned opposite the "----" gaps in the reference pX330 vector. This alignment gap indicates that the parental vector contains an empty BbsI cloning site at this position, whereas sequencing confirms successful insertion of the spacer at this site.

Single-Cell Colony Assay

The two-gradual cell-dilution method yielded a markedly higher number of single colonies: 8-9 wells per 96-well plate showed clonal growth, compared with only 1-2 wells using the standard cell-dilution method. Figures 3A-D show the proliferation of colonies obtained by the two-step gradual dilution method in cell culture, as observed under the microscope. Figure 3E shows an image of a colony obtained by the standard dilution method under the microscope on the 6th day, and Figure 3F shows two different colonies obtained by the same method on the 7th day of culture. Single colonies appeared in wells with no consistent positional pattern. However, as an observed trend, colonies were more frequently detected in the latter half of the vertical axis and in the lower half of the horizontal axis of the plates. This pattern is consistent with the stochastic nature of limiting dilution.[14] The ImageJ software was used for colony size quantification. Figure 3G shows that the two gradual cell-dilution methods may yield significantly more candidate knockout single cells than the standard cell-dilution method ($p=0.0192$).

DISCUSSION

CRISPR-Cas9 is a powerful cell genetic editing tool, but learning and perfecting this revolutionary technology is still advancing.[15] The traditional selection and verification processes, despite the CRISPR-Cas9 system's high efficacy, remain an indispensable part of current cloning workflows.[16]

Handling single cells is critical in applications such as cell line development and single-cell analysis, for example in cancer research and emerging diagnostic methods.[17] Currently available single-cell isolation technologies are classified based on their major technical characteristics. The most prominent technologies are limiting dilution, FACS, single-cell printing, hydrodynamic trapping, droplet microfluidics, and cell manipulation.[18] Among these, flow cytometry and random seeding/dilution accounted for 33% and 15% of usage, respectively. However, Ye et al.[19] claimed that, compared with FACS of single cells, limiting dilution cloning is more widely used because of its lower cost, independence from specialized instrumentation, and minimal cellular stress. In addition to its use in CRISPR research, limiting dilution is employed to investigate the generation of monoclones,[20] obtain mesenchymal stem cells derived from a single colony,[21] assess the viral titer,[22] and clone hybrid cells in fusion experiments.[23] Thus, it is a conventional yet important approach.

In this study, the limiting dilution method, notable for its cost-effectiveness, was performed in two ways. The results we found show that a two-step dilution is advantageous, especially for obtaining a colony from a single cell. Because no studies compare these two traditional approaches in the literature, this study will make a significant contribution to the field.

Limiting dilution is also used in CRISPR studies in different cell lines. Reported that they obtained single cells using a limiting dilution strategy in deletion assays in the Neuro2A cell line using CRISPR/Cas9.[24] In a different study, single-cell clones were obtained from human pluripotent stem cells by the limited-dilution method following CRISPR-Cas9 editing.[25] This study, which compares two alternative approaches in breast cancer cell lines, was expected to make a unique contribution to the literature. Furthermore, single colonies were scanned under a light microscope every 24 hours, and colonization was observed on day 6. This result closely parallels another study by Hong et.,[13] in which the cells were monitored under a microscope for 3 days and counted within 7 days. Obtaining a potential single-cell knockout count sufficient to sustain the study during the serial dilution phase is necessary to continue the research and is strategic given possible colony losses during the expansion phase.

An examination of the literature reveals that no studies have compared these two methods in CRISPR knockout research, highlighting a gap that should inform future studies. In addition, the absence of single cells in any well of the 96-well plate with standard serial dilution, or a statistically lower number of single cells compared with two-gradual serial dilution, will guide the selection of the methodology for future studies.

Study Limitations

The study's limitation is the need for further functional testing to support its broad applicability. Specifically, this conclusion can be supported by various molecular tests.

CONCLUSION

In conclusion, the colonies obtained using the two-gradual cell dilution method were more numerous. These data make this method more attractive, allowing screening for CRISPR knockouts and increasing the probability of detecting a single knockout cell. In addition, attempting two different methods for single-cell assays in CRISPR experiments is both costly and burdensome to laboratory personnel. Rather than employing two distinct techniques to maximize effectiveness, the two-

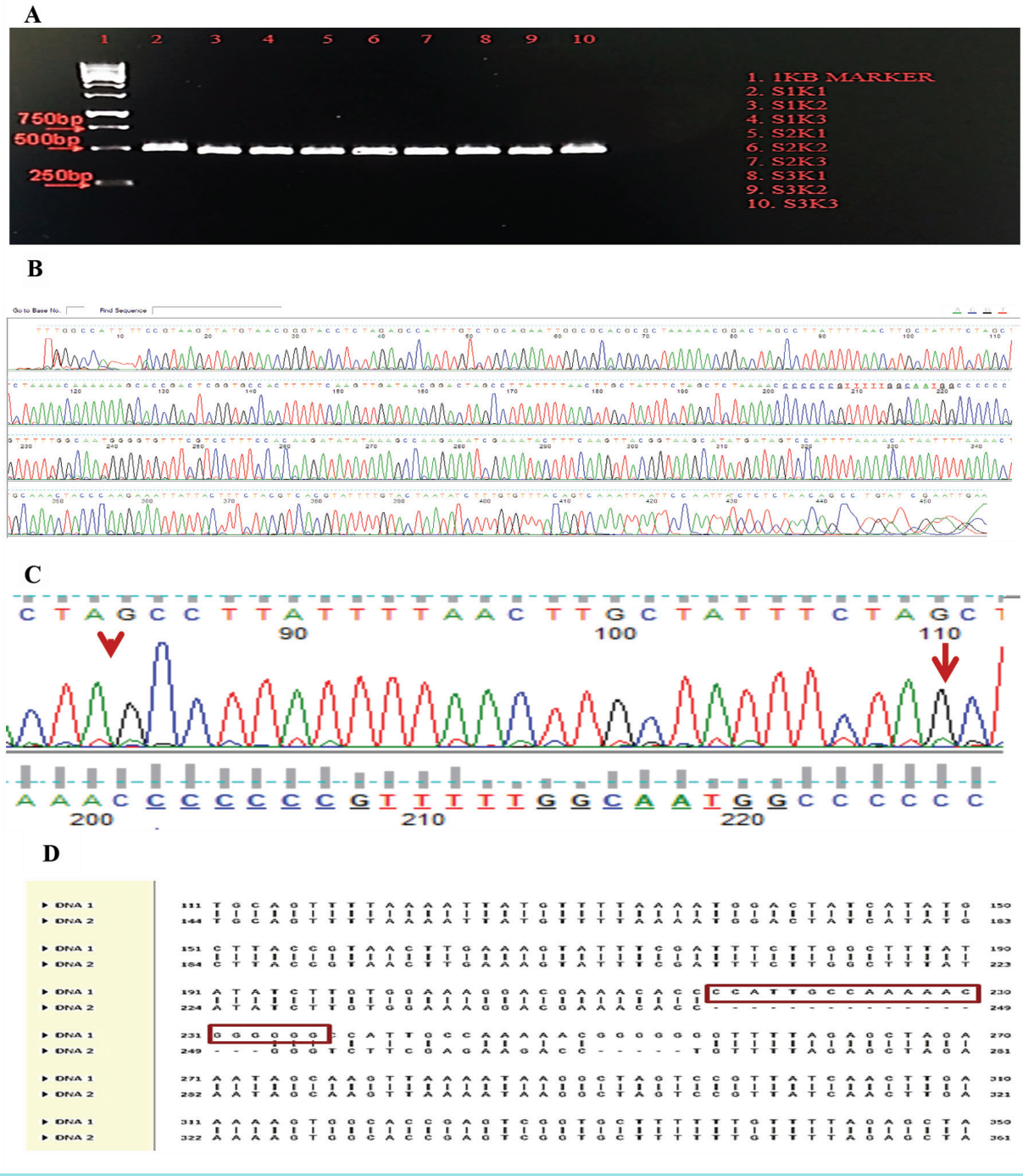

Figure 2. Agarose gel electrophoresis and verification of sgRNA cloning into the pX330 vector. A) Agarose gel image of PCR products obtained from transformed plasmids carrying the designed sgRNAs, amplified using the primers listed in Table 2. A high amount and high purity of the product were obtained. The obtained product was approximately 478 bp. B) Representative Sanger sequencing chromatogram confirming correct cloning of the designed 20-nt miR-182 gRNA1 (clone S1K1). C) Zoomed-in view of the cloned region. Because the sequencing reaction was performed in the reverse orientation, the reverse complement of the designed gRNA sequence is highlighted. D) SnapGene alignment showing integration of the designed gRNA sequence into the pX330 vector.

DNA: Deoxyribonucleic acid, RNA: Ribonucleic acid, sgRNAs: Single guide RNA, PCR: Polymerase chain reaction, gRNA: Guide RNA.

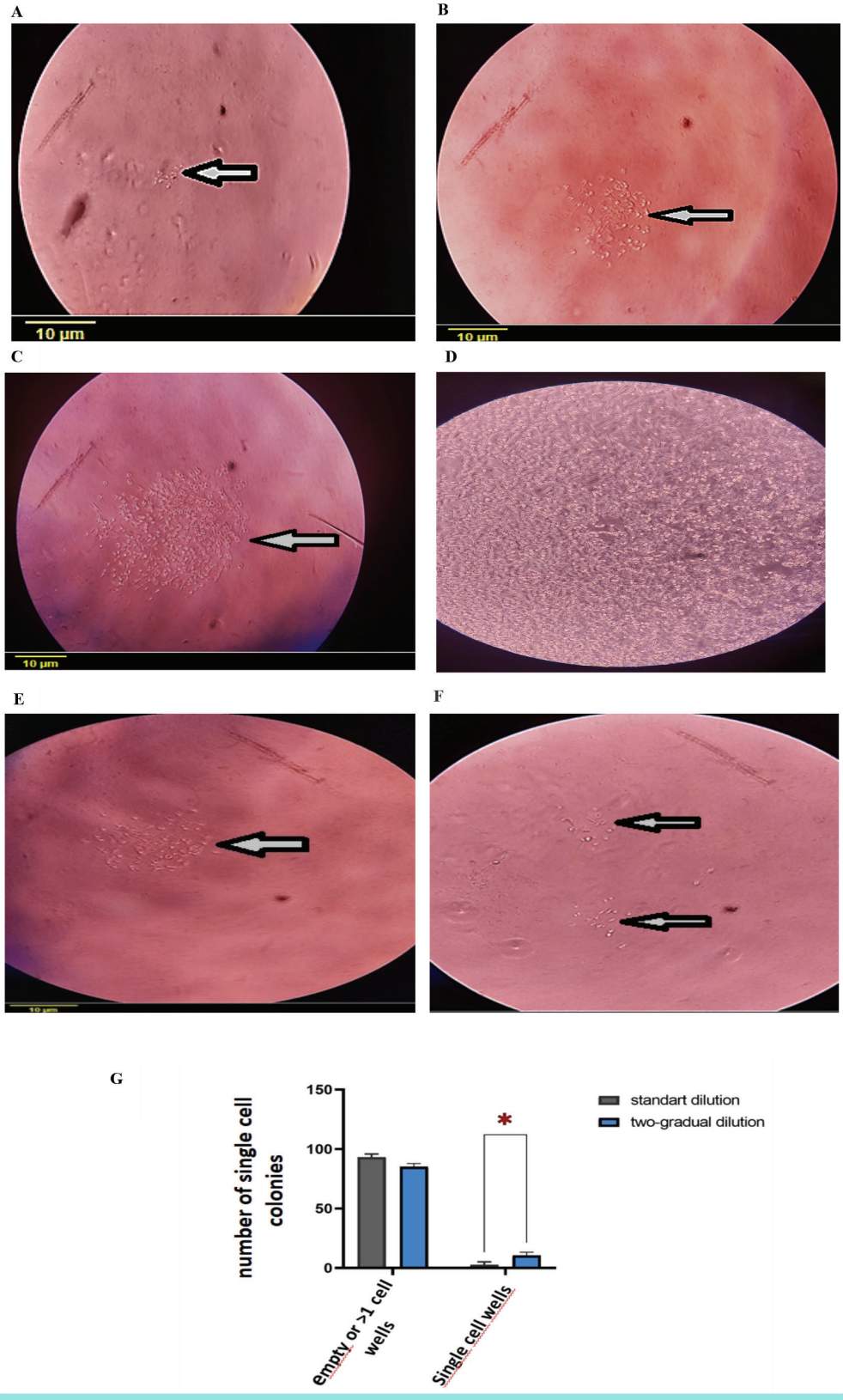

Figure 3. Single colonies detected under the light microscope. Scale bars (10 µm) were generated using ImageJ software (NIH, Bethesda, MD, USA). A) Two-gradual cell dilution; day 6 in cell culture. B) Two-gradual cell dilution; day 10 in cell culture C) Two-gradual cell dilution; day 14 in cell culture D) Two-gradual cell dilution; day 20 in cell culture E) Standart cell dilution; day 10 in cell culture F) Standart cell dilution; day 6 in cell culture G) Single-cell colonies number and empty or multiple cell colonies in 96-well plates with two different dilution methods (At least three independent experiments were performed; *p=0.0192).

step gradual cell-dilution method saves time and yields many single cells with knockout potential.

MAIN POINTS

- Two different dilution-based seeding approaches were compared in clustered regularly interspaced palindromic repeats (CRISPR)-edited MDA-MB-231 cells to determine which method more reliably generates single-cell-derived colonies.
- The two-step, gradual dilution method consistently produced more true single-cell colonies per 96-well plate (8-9 colonies) than the standard cell dilution method (1-2 colonies).
- This improvement offers a statistically significant advantage ($p=0.0192$) and increases the likelihood of obtaining candidate knockout clones after CRISPR editing.
- Implementing the two-step gradual dilution method can streamline CRISPR screening workflows by reducing time, labor, and plate usage while improving downstream validation success.

ETHICS

Ethics Committee Approval: The authors of this article declare that the materials and methods used in this study do not require approval from an ethics committee or special legal permission.

Informed Consent: An informed consent statement has been added. As the study did not involve human participants, patient samples, or identifiable personal data, informed consent was not applicable.

Footnotes

Authorship Contributions

Concept: H.D., B.B., Design: H.D., B.B., Data Collection and/or Processing: H.D., B.B., Analysis and/or Interpretation: H.D., B.B., B.T., Literature Search: H.D., B.T., Writing: H.D., B.B., B.T.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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