Optimizing CRISPR-Cas9 lentiviral transduction efficiency and antibiotic selection measurements

Introduction

The CRISPR-Cas9 system has revolutionized genome editing through its efficiency and simplicity of design and implementation. CRISPR-Cas9 is a two-piece system, comprising the Cas9 nuclease and a guide RNA (gRNA), which form a ribonucleoprotein (RNP) complex. The gRNA, as the name suggests, guides the complex to a specific site in the genome where the Cas9 nuclease cleaves the DNA, resulting in a double-strand break [1,2].

The introduction of the double-strand break is a critical step in any genome editing experiment, and the success of such an experiment hinges on being able to maximize the cleavage efficiency of the CRISPR-Cas9 complex. One of the keys to maximizing the cleavage efficiency is to achieve high-efficiency delivery of the CRISPR-Cas9 complex into the cells [2].

Methods of delivery

The CRISPR-Cas9 system can be delivered into cells in several formats, using three methods of delivery—electroporation, reagent-based transfection (e.g., Invitrogen™ Lipofectamine™ reagent), or viral transduction. Viral transduction, lentiviral transduction in particular, is one of the most high-efficiency delivery methods for introducing exogenous DNA constructs into nearly any cell type and is therefore an ideal option for delivering CRISPR-Cas9 tools [3].

To determine the optimum multiplicity of infection (MOI) for delivering CRISPR-Cas9 tools via lentiviral delivery, one can utilize control lentiviral particles that express a fluorescent protein and/or an antibiotic resistance gene. In this way, cells that express the fluorescent protein and/or antibiotic resistance can reliably indicate successful transduction [4].

Another method of optimizing specific lentiviral delivery of CRISPR-Cas9 tools is the use of control particles that contain a gRNA specifically targeting the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene [5]. HPRT is an enzyme that recycles purine nucleotides, and its knockout is easily confirmed by selection with 6-thioguanine (6-TG), a purine analog. When 6-TG is present, it is modified by HPRT and competes with guanine for incorporation into DNA [5,6]. Although 6-TG is a guanine analog, it does not function as a nucleotide and will cause cell death when enough of it is incorporated into the DNA. Cells that survive 6-TG selection are reliably indicative of *HPRT* knockouts [5].

Viability measurement with fluorescence

To effectively optimize lentiviral delivery of CRISPR-Cas9 tools, viability measurements during the above-mentioned procedures are critical. The Invitrogen™ Countess™ II FL Automated Cell Counter enables taking these measurements easily and accurately. In addition to quick and accurate counting of cells, viability and GFP expression can easily be quantitated using fluorescence-based cell counting techniques. Here we demonstrate methods for the use of the Countess II FL Automated Cell Counter in optimization of lentiviral gRNA delivery.

Materials

- Countess II FL Automated Cell Counter (Cat. No. AMQAF1000)
- Invitrogen™ Countess™ Cell Counting Chamber Slides (Cat. No. C10228) or Countess™ II FL Reusable Slide (Cat. No. A25750)
- Invitrogen™ EVOS™ Light Cube, GFP (Cat. No. AMEP4651)
- 6-thioguanine (6-TG)
- U2OS-Cas9 stable cell line
- Gibco™ McCoy's 5A Medium (Cat. No. 12330-031)
- Gibco™ Fetal Bovine Serum (Cat. No. 16000-036)
- Gibco™ Puromycin Dihydrochloride (Cat. No. A11138-03)
- Gibco™ TrypLE™ Express Enzyme (Cat. No. 12605-010)
- Invitrogen™ LentiArray™ CRISPR Positive Control Lentivirus, human HPRT gRNA, with GFP (Cat. No. A32060)
- Invitrogen™ LentiArray™ CRISPR Negative Control Lentivirus, scrambled gRNA sequence, with GFP (Cat. No. A32063)

Protocol: Quantitation of transduction efficiency with GFP

1. Plate U2OS-Cas9 stable cells in a 96-well plate at 2,500 cells per well. Incubate overnight under normal cell culture conditions in McCoy's 5A medium supplemented with 10% fetal bovine serum (complete medium).

2. Transduce four separate samples of cells expressing Cas9 protein with either a LentiArray CRISPR positive control (HPRT, GFP) or negative control (scramble, GFP) particles at MOI of 1 and 2. Use one well for each condition and one additional well as an untransduced control. In addition to the gRNA for the target of interest and GFP gene, the LentiArray controls also contain a puromycin resistance gene for positive selection of successfully transduced cells.

3. Following incubation for 48 hours, trypsinize cells with TrypLE Express Enzyme. Count the number of cells in a 10 µL sample on the Countess II FL Automated Cell Counter using the bright-field and fluorescence GFP channels. Use the GFP channel to gate populations for percentage of cells positive for GFP expression.

Protocol: Puromycin selection

1. Replate the remaining cells in complete medium supplemented with 1 µg/mL puromycin.

2. Grow the cell populations in complete medium supplemented with 1 µg/mL puromycin for 14 days, splitting the cultures every 2–3 days.

3. With every split, add 10 µL of the culture to 10 µL of trypan blue stain (provided with the Countess Cell Counting Chamber Slides). Load 10 µL of each stained cell sample into either a disposable or reusable slide, and count the number of cells in the bright-field channel of the Countess II FL counter and gate for the percentage of viable cells. This measurement will enable the tracking of cell transduction efficiencies over time.

Protocol: 6-TG selection

1. Replace the puromycin-containing complete medium with HPRT selection medium (complete medium containing 1 µg/mL puromycin and 0.6 µg/mL 6-TG).

2. Split the cultures in each well every 2–3 days, and with every split add 10 µL of each transduced cell population to 10 µL of trypan blue stain (provided with the Countess Cell Counting Chamber Slides). Read the number of cells in 10 µL of each stained cell sample in the bright-field channel of the Countess II FL counter, and gate for the percentage of viable cells.

Results

Using the fluorescence mode and the EVOS GFP light cube, the Countess II FL Automated Cell Counter accurately detected changes in the percentage of GFP-positive cells during MOI optimization of lentiviral gRNA delivery (Figure 1). When using a lentiviral gRNA construct that includes an antibiotic resistance gene, the Countess II FL counter easily monitored changes in viability associated with antibiotic selection in cell culture, as shown here with puromycin selection (Figure 2). In addition, the Countess II FL counter enabled simple viability measurements during 6-TG selection for *HPRT* null mutations when optimizing lentiviral gRNA delivery for editing efficiency (Figure 3).

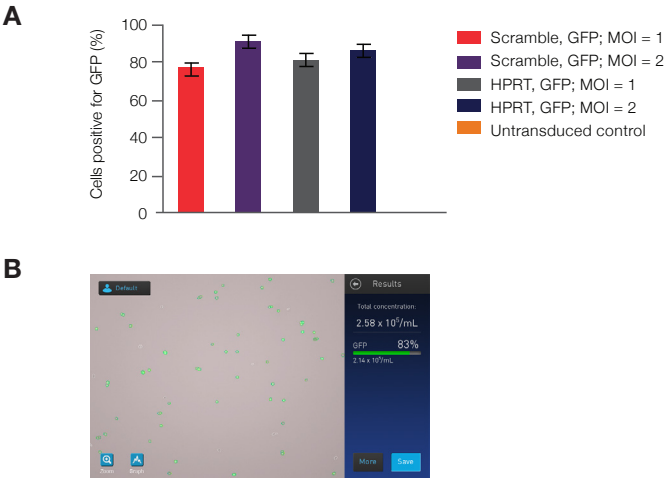

Figure 1. Lentiviral transduction efficiency as measured by GFP on the Countess II FL Automated Cell Counter. U2OS cells expressing the Cas9 protein were transduced with a LentiArray positive control lentivirus (HPRT, GFP) and a negative control lentivirus (scramble, GFP) at MOI of 1 and 2. Two days later, cells were counted on the Countess II FL cell counter for GFP expression. **(A)** Counts show the percentages of cells positive for GFP, indicating successful transduction. **(B)** Display of representative counting results on the Countess II FL instrument.

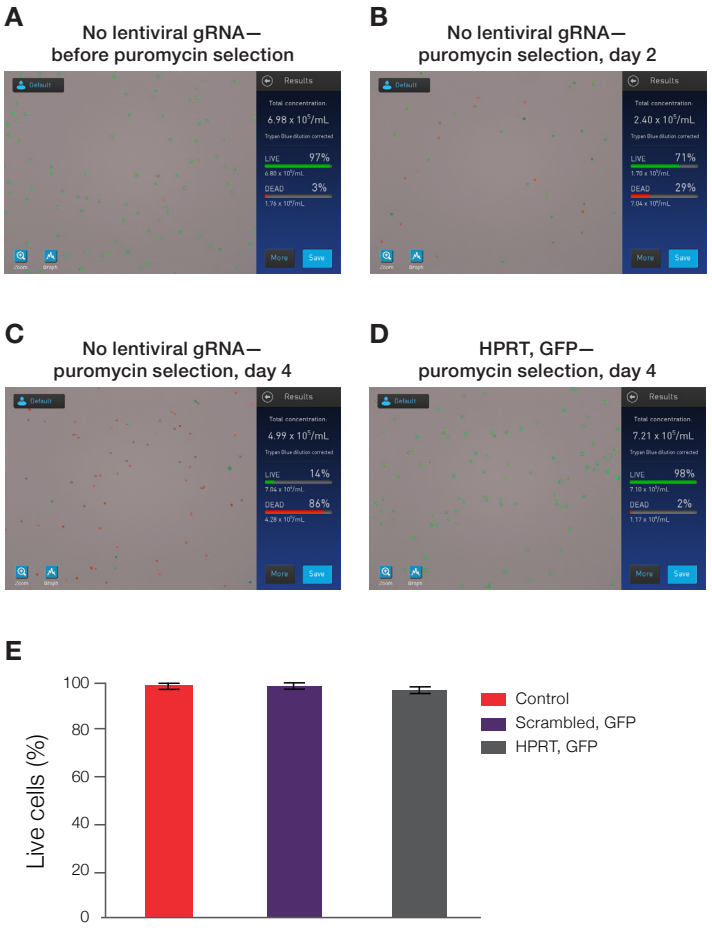

Figure 2. Viability of U2OS-Cas9 stable cells stained with trypan blue under puromycin selection measured on the Countess II FL cell counter. U2OS-Cas9 stable cells without lentiviral gRNA **(A)** before puromycin selection, **(B)** on day 2 under puromycin selection, and **(C)** on day 4 under puromycin selection, showing decreased viability because the cells do not possess the puromycin resistance gene. **(D)** Cells transduced with LentiArray Positive Control gRNA (HPRT, GFP) showed normal viability after 4 days under puromycin selection. **(E)** After 14 days under puromycin selection, cells transduced with a LentiArray positive control lentivirus (HPRT, GFP) and negative control lentivirus (scramble, GFP) show viability levels similar to that of U2OS-Cas9 stable cells (control) not under puromycin selection.

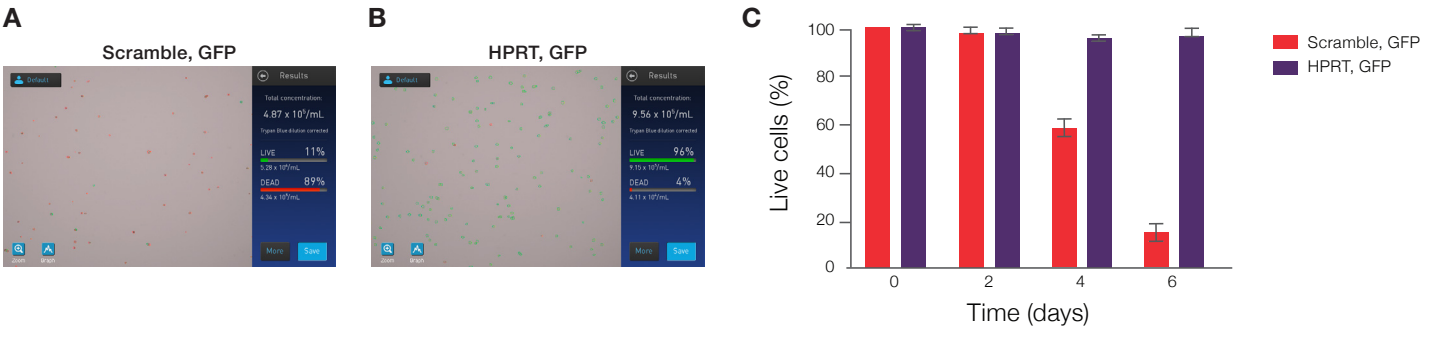

Figure 3. Measuring editing efficiency by 6-TG selection for *HPRT* knockouts, using the Countess II FL cell counter with trypan blue stain. **(A, C)** After 6 days under selection from 0.6 µg/mL 6-TG, cells with scrambled gRNA showed decreased viability, indicating 6-TG was being incorporated into the DNA instead of purine nucleotides. **(B, C)** Cells with HPRT-targeting gRNA showed normal viability, indicating knockout of the *HPRT* gene, preventing 6-TG from being incorporated into the DNA, thereby maintaining cell survival.

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Conclusion

The Countess II FL Automated Cell Counter provides an effective way to measure, based on GFP expression, the efficiency of lentiviral transduction to deliver CRISPR-Cas9 tools into cells. Additionally, the Countess counter could be used to monitor changes in viability of cell cultures under antibiotic selection, offering a quick and easy way to get both cell viability and transduction efficiency data.

References

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