# Direct Quantification of Genome Editing Efficiency from Whole Cells Using SingleShot Cell Lysis Buffer and ddPCR Genome Edit Detection Assays

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Droplet Digital PCR

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**Abstract**

Genomic DNA extraction from transfected cell populations and single-cell clones for the assessment of genome editing efficiency is time consuming and a common pain point in gene editing workflows. Here, we report a rapid, automation-compatible method that eliminates the need for DNA extraction prior to determining gene-editing efficiency. Intact, whole cells are lysed using diluted SingleShot Cell Lysis Buffer with proteinase K to release genomic DNA from transfected cells. The cell lysate can be used directly to determine gene editing fractional abundance using Droplet Digital PCR (ddPCR).

## Introduction

With the advancement of CRISPR and other genome editing technologies, modification of genomes to induce targeted changes is opening up vast possibilities in gene therapy, agriculture, and bioenergy. Though various methods of gene editing reagent delivery and methods can be automated using robotics, there remains a bottleneck where the induced genetic changes are rapidly characterized. Currently, genomic edit characterizations are performed on column-purified genomic DNA (gDNA) from up to thousands of single-cell clones, greatly reducing the efficiency and scalability of gene editing workflows. Therefore, the need for a rapid, quantitative workflow to assess genome edits at the population level to quickly provide metrics for optimization of transfection and in vivo efficiency is critical to gene editing scale-up and manufacturing.

## Materials and Methods

### Cell Culture

eHAP cells (catalog #C669; Horizon Discovery, Cambridge, UK) were maintained in IMDM (#1244053; Thermo Fisher Scientific, Waltham, MA) plus 10% FBS (#26140-079; GIBCO, Gaithersburg, MD) supplemented with penicillin-streptomycin (#15070063; Thermo Fisher Scientific). Cells were trypsinized and passaged at ~70% confluence. All transfections were performed on cells at ~40% confluence.

### crRNA and ddPCR NHEJ Assay Design

CRISPR RNA (crRNA) targeting the earliest exon common among all splice variants of STAT5A was designed using the Broad Institute's Genetic Perturbation Platform. ddPCR NHEJ assays targeting the crRNA putative cut sites were designed according to manufacturer's instructions using the automated assay design portal found at **bio-rad.com/digital-assays**.

### RNP Transfection

crRNA, transactivating RNA (tracrRNA) (#1073189; IDT, Skokie, IL), and Cas9 nuclease (#1074181, IDT) were transfected using Lipofectamine RNAiMax Reagent (#13778075, Invitrogen, Carlsbad, CA). eHAP cells were thawed by diluting at 1:10 into warmed growth media 24 hr prior to transfection. Transfection conditions were 40,000 cells/well in 96-well cell culture plates. Briefly, crRNA and tracrRNA were combined 1:1 and diluted to 1 µM in NFDB buffer (#1072570, IDT). The 1 µM crRNA/tracrRNA solution was heated to 95°C for 5 min, then cooled to room temperature (RT). Cas9 enzyme was diluted to 1 µM in OptiMEM (#31985070, Thermo Fisher Scientific) and combined 1:1 with the crRNA/tracrRNA complex to generate the ribonucleoprotein (RNP). The RNP was transfected at 40 nM with Lipofectamine concentrations at 1.5 µl/well. The transfected cells were incubated in a 5% $CO_2$ incubator for 48 hr prior to harvesting for determination of NHEJ fractional abundance by ddPCR.

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#### Cell Lysis and Proteinase K Digestion of Transfected Cells Using a Modified SingleShot Protocol

SingleShot Cell Lysis Buffer without DNase (#1725080, Bio-Rad Laboratories, Hercules, CA) was diluted 1:6 using sterile DI water. Proteinase K was added to a final concentration of 40 µg/ml. Each well of transfected cells was washed with 200 µl of PBS and incubated with 30 µl of diluted SingleShot Cell Lysis Buffer with proteinase K for 10 min at RT. After incubation, the cell lysates were transferred to a clean 96-well PCR plate and incubated for 5 min at 37°C. The proteinase K was then inactivated at 75°C for 5 min. The resultant cell lysates were used immediately for ddPCR analysis at 2 µl/reaction. The cell lysates can also be stored at –20°C to be used at a later time. See Figure 1 for a detailed workflow using diluted SingleShot Buffer.

#### Population NHEJ Editing Assessment by ddPCR (Column Purification vs. SingleShot Lysate)

From the same STAT5A RNP transfection experiment, half of the transfected wells were subjected to column-based gDNA purification using the Quick-DNA Plus Kit (#D4074, Zymo Research, Irvine, CA) and eluted into 30 µl DI water. The other half were lysed using the modified SingleShot protocol detailed previously. Two microliters of lysate and 2 µl of purified gDNA were used for each NHEJ genome edit probe assay. SbfI HF (1:4,000 final concentration) was added to each reaction to increase access to gDNA. The NHEJ assays were performed using the ddPCR Supermix for Probes (no dUTP) (#1863024; Bio-Rad) in the T100 Thermal Cycler (Bio-Rad) using ddPCR 96-Well Plates (#12001925; Bio-Rad). Cycling parameters were as recommended by the manufacturer. After thermal cycling, the droplets were analyzed on the QX200 Droplet Digital PCR System (#1864100, Bio-Rad). The fractional abundance of NHEJ DNA was determined using QuantaSoft Analysis Pro Software (visit **bio-rad.com** for free software download). The percent fractional abundance of NHEJ events was calculated as: a/(a+b)*100, where a is the number of FAM positive droplets (NHEJ) and b is the number of FAM/HEX double positive droplets (WT).

## Results

We designed a modified protocol using the SingleShot Cell Lysis Buffer, originally designed for performing bulk RT-qPCR in whole cells as input (Figure 1), to enable ddPCR assessment of population gene editing efficiency from whole cells immediately after transfection. To validate our method, the STAT5A gene was subjected to CRISPR/Cas9 RNP–mediated NHEJ editing. At 48 hr post-transfection, the transfected cells were lysed using the diluted SingleShot Cell Lysis Buffer and the fractional abundance of the edited cells was quantified using a ddPCR Drop-Off Assay designed using the assay design portal (**bio-rad.com/digital-assays**). Briefly, NHEJ assays are duplexed primer probe-based ddPCR Assays (Figure 2). A FAM-labeled probe is designed to bind a reference sequence distant from the nuclease target site but still within the amplicon. A second HEX NHEJ/drop-off probe binds the WT sequence at the nuclease target site, such that NHEJ-induced indels block the probe from binding. In a 2-dimensional view of the ddPCR analysis, droplets with signal from both the FAM and HEX probes contain wild-type amplicons while droplets that are FAM-positive but HEX-negative contain amplicons with mutations at the target site (Figure 3). The ratio of FAM positive to FAM/HEX double positive droplets provides the fractional abundance of NHEJ mutations.

In a mixed population of cells containing both WT and edited cells, the WT cells exhibit two fluorescent signals and the NHEJ edited population exhibits the reference probe signal only (Figure 2). The 2-D plot of the ddPCR NHEJ Drop-Off

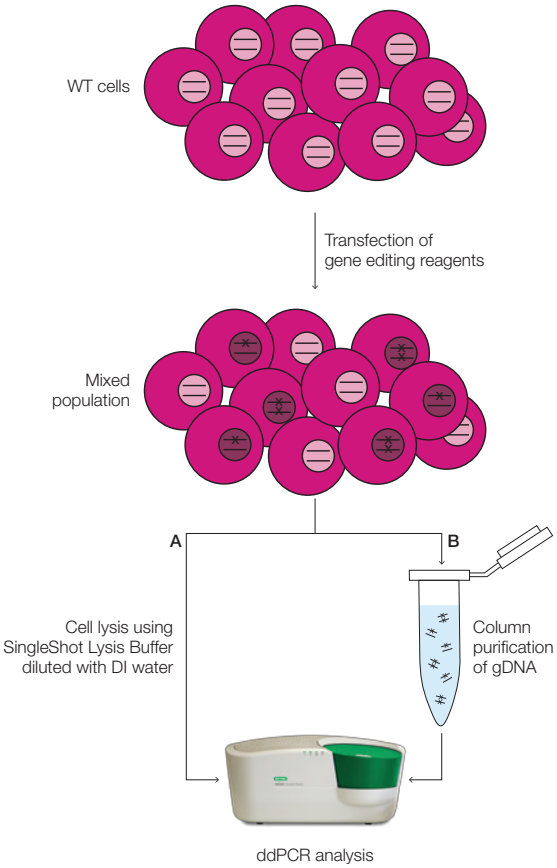


**Fig. 1. Experimental workflow comparing the quantification of genome editing efficiency between direct cell lysis and column-purified DNA.** **A**, gene edited cells were lysed directly into diluted SingleShot Lysis Buffer with proteinase K, followed by incubation at 37°C for 5 min and heat inactivation at 75°C for 5 min. The lysate was used at 2 µl per 20 µl reaction of ddPCR genome editing assay. **B**, gDNA from the gene edited cells was first extracted by column purification and then analyzed by Droplet Digital PCR using a ddPCR NHEJ Drop-Off Assay.

Assays shows WT cells in the top-right corner, with positive signals in both FAM (Y-axis) and HEX (X-axis) channels, and the NHEJ-edited cell population shifts to the left, with only the FAM reference signal remaining (Figure 3). The column-purified gDNA from the same transfection experiment was used as a benchmark for comparing the two workflows (Figures 3 and 4). The substitution of diluted SingleShot Lysis Buffer for column gDNA purification did not significantly alter the NHEJ fractional abundance measurement (Figure 4A) nor did it impact the droplet number (Figure 4B).

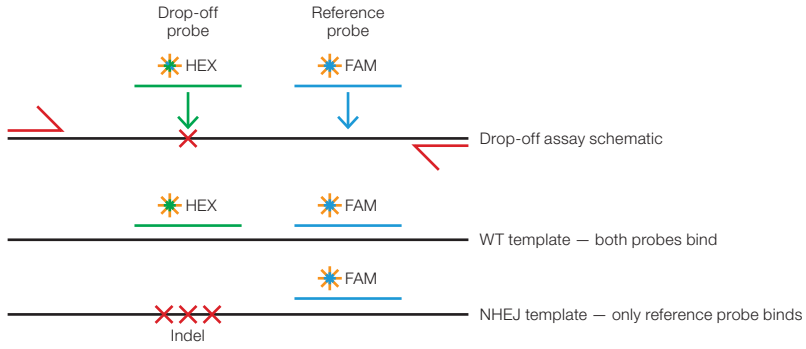


**Fig. 2. Assay schematic for ddPCR NHEJ Drop-Off Assay.** The ddPCR NHEJ assay comprises a pair of primers flanking the putative edit site and two fluorescent probes, one reference probe that binds to wild-type (unedited) DNA, and a drop-off probe that will not bind if an editing event occurs at the Cas9 cut site. The NHEJ drop-off assay is designed using the web assay design portal **bio-rad.com/digital-assays**.

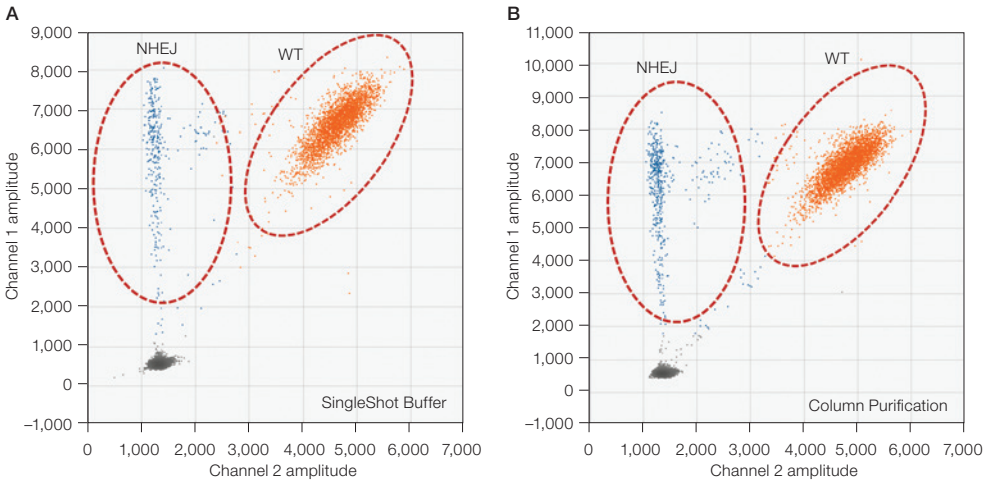


**Fig. 3. 2-D plots of SingleShot lysate versus column-purified gDNA.** The WT population is shown in orange and is positive in both FAM and HEX channels, whereas the edited NHEJ population is shifted to the left of the plot (blue). Empty droplets are shown in grey. **A**, 2-D plot from SingleShot lysate ddPCR reaction; **B**, data from column-purified gDNA obtained from the same transfection.

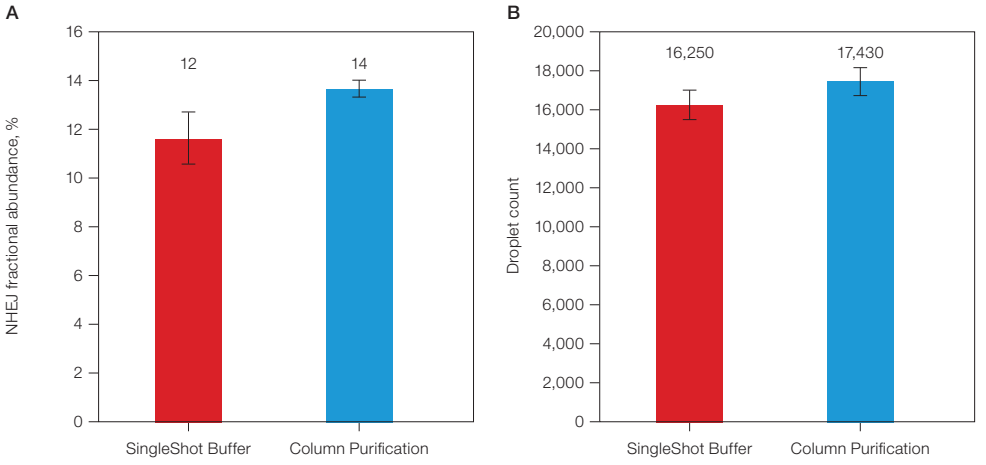


**Fig. 4. Absolute quantification of NHEJ events. A**, NHEJ fractional abundance was calculated using QuantaSoft Pro Analysis Software with the formula a/(a+b)*100 where a = NHEJ population, b = WT population. There was no significant difference in the fractional abundance observed between the SingleShot and column-purification workflows. **B**, the number of droplets was also not impacted when using SingleShot lysate.

## Conclusion

Here, we present a workflow that combines the SingleShot Cell Lysis Buffer with ddPCR Genome Edit Detection Assays for a streamlined workflow that can directly assess population-level gene editing efficiency from whole cells. The workflow bypasses the need for limiting dilution of single cells and column purification for gDNA extraction. The entire process can be performed immediately upon conclusion of transfection and provide quantitative measurement of genome edit events in the population in less than four hours, with less than an hour of hands-on time.

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