Simultaneous Quantification of HDR and NHEJ Editing Events Induced by Site-Specific Nucleases Using ddPCR

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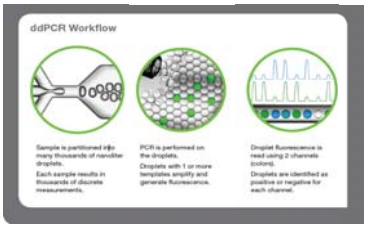
Introduction

Genome editing tools such as TALENs and the CRISPR/Cas9 system have revolutionized our ability to edit the genome of any cell including human induced pluripotent stem cells (iPSCs). Sequence-specific nucleases induce double strand breaks or nicks at target sites, activating the DNA repair pathways of non-homologous end joining (NHEJ) or homology-directed repair (HDR). Through the production of small insertions or deletions, error-prone NHEJ is useful for disrupting gene function. However, for many applications, HDR is more desirable than NHEJ, since HDR utilizes homologous donor DNA to produce precise gene repair, while NHEJ causes unpredictable damage. Unfortunately, cell populations edited by HDR often contain alleles damaged by NHEJ. Since NHEJ and HDR involve different repair enzymes, it is conceivable that conditions could be achieved with high HDR and low NHEJ. However, balance between NHEJ and HDR is elusive, since we lack a rapid sensitive assay to quantify NHEJ and HDR at endogenous genomic loci. To overcome this hurdle, we developed a method to detect NHEJ and HDR simultaneously based on droplet digital PCR (ddPCR). ddPCR is a digital PCR technology based on water-oil emulsions to partition a reaction into over 20,000 uniform droplets. Combination of ddPCR and fluorescent oligonucleotide probes specific to wild-type, NHEJ, and HDR alleles allows us to simultaneously detect NHEJ and HDR events induced by site-specific nucleases in a sensitive and quantitative manner. By using our ddPCR-based method, we are able to test multiple conditions for genome editing including different types of sequence-specific nucleases and donor DNAs in order to tilt the balance between NHEJ and HDR towards HDR. We are currently optimizing mutagenesis conditions to activate HDR with minimum NHEJ in human iPSCs. Our system will result in significant improvements to genome editing technology.

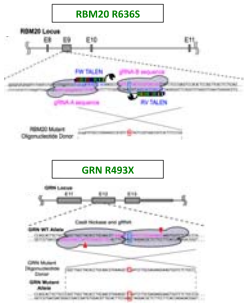
Materials and Methods

QX200 Droplet Digital PCR System



- Three disease-relevant loci (RBM20, ATP7B, GRN) were targeted for editing using TALEN, WT-Cas9, dCas9-FokI, or Cas9-Nickase strategies. TALENs were constructed using the Voytas laboratory's Golden Gate assembly system (Cermak *et al., Nucleic Acids Res.* 39, e82, 2011) and the MR015 backbone vector (gift from M. Porteus and M. Rahdar, Stanford University). Guide RNAs (gRNAs) were cloned into the Zhang laboratory's pX335 (nickase) and pX330 (nuclease) (Cong *et al.*, Science, 819-23, 2013; Ran *et al., Cell* 154, 1380–1389, 2013).
- QX100 & QX200 Droplet Digital PCR systems were used (Bio-Rad). ddPCR reactions were assembled using standard protocols: ddPCR Supermix for Probes (no dUTP) was combined with 5 ng – 125 ng sample, 1 uL of 20x assay (Each 1x primer at 900 nM, each 1x probe at 250 nM, and if necessary a dark non-extendible probe at 500 nM, all from IDT), 4 units of restriction enzyme (HaeIII for ATP7B and RBM20 or MseI for GRN targets) and water to 20 uL. Reactions were converted into ~20,000 1-nL droplets using the Droplet Generator and transferred to a 96 well plate for thermal cycling. After thermal cycling, droplets were read on the Droplet Reader and assigned as positive or negative based on fluorescence amplitude.

A. Targeting design for RBM20 & GRN loci

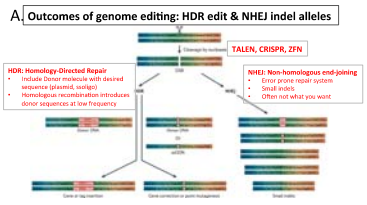


- **(A) Targeting strategy for introducing pathological point mutations in human induced pluripotent stem (iPS) cells and HEK293 cells for disease-relevant loci.** A pair of gRNAs (shown in magenta) for Cas9 nickase, CRISPR-Cas9 (singly) and dCas9-FokI as well as TALENs were designed to introduce the disease-relevant point mutation in the RBM20, ATP7B (not shown), and GRN loci. The wild-type and mutant residues are highlighted by a blue and a red rectangles, respectively. The nick/cut sites are indicated by red triangles.
  - **RBM20 R636S (1906C>A): Cardiomyopathy**
  - **ATP7B R778L (2333G>T): Wilson's Disease**
  - **GRN R493X (1477C>T): Frontotemporal dementia**
- **(B) Sequences of an isolated GRN R989X iPS cell clone aligned to the WT sequence.** This clone had the C>T point mutation and a 24-bp deletion. These results indicate that HDR and NHEJ can occur in single cells, resulting in compound heterozygous mutant cells.

B.

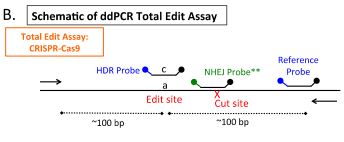
Figure 1: ddPCR assay for simultaneous single-well quantification of HDR and NHEJ

A. Outcomes of genome editing: HDR edit & NHEJ indel alleles



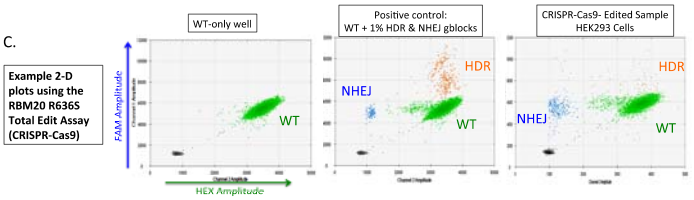
Modified from H.Kim & J.Kim, Nat Rev Genetics 2014

B. Schematic of ddPCR Total Edit Assay



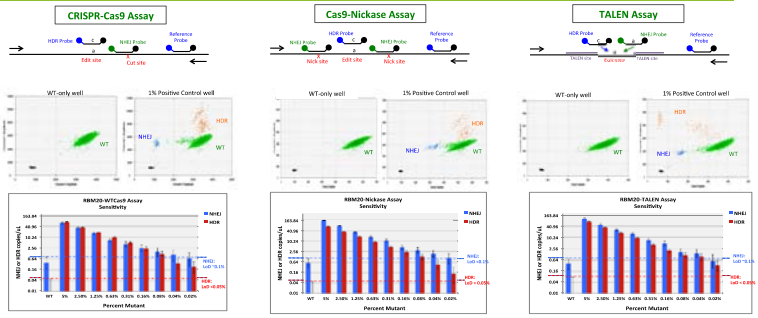
***Note that NHEJ probe position and number is dependent on the nuclease used. For example, a Nickase assay has 2 NHEJ probes, each overlapping one of the two nick sites. See Fig. 2.*

C.



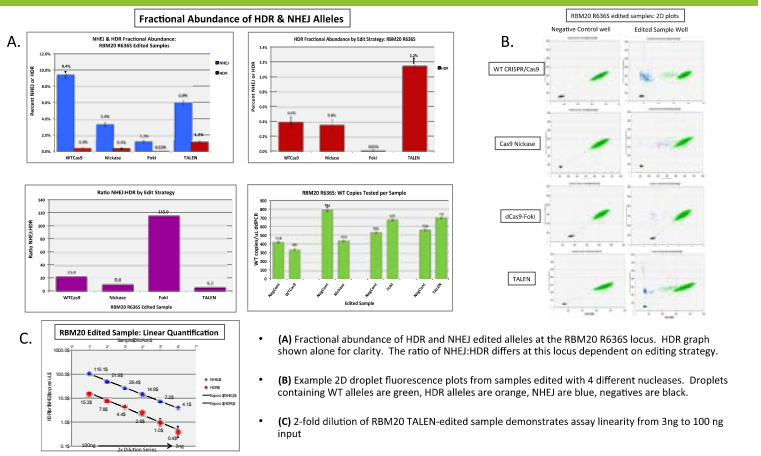
- **(A)** Outcomes of genome editing include both small indels caused by non-homologous end joining (NHEJ) and rare intended edits introduced by Homology Directed Repair (HDR) working off a donor DNA sequence. When HDR edits are desired, NHEJ alleles are considered collateral damage.
- **(B)** Schematic of a Total Edit Assay for ddPCR: a single amplicon comprises the predicted cut site (e.g. 3 bp upstream of NGG for WTCas9) and the intended edit site. One Reference probe (FAM) binds all alleles, and one HDR Edit Probe (FAM) binds edited alleles. A third NHEJ "Drop-off" probe (HEX) sits on top of the predicted cut site and binds only wild-type sequence. If insertions or deletions are introduced, the NHEJ probe fails to bind, revealed by a loss of HEX amplitude (C). Depending on NHEJ probe position, an additional unlabeled non-extendible oligo (dark probe) may be included (not shown).
- **(C)** 2-dimensional fluorescence amplitude droplet plots of the RBM20 R636S CRISPR-Cas9 Total Edit Assay run on WT only DNA (left), a Positive control (WT plus gblocks mimicking a 1-bp NHEJ deletion and the HDR C>A point mutation, middle), and HEK293 cells engineered with RBM20 R636S (right). Clusters of droplets containing WT template (green), HDR edited template (orange), NHEJ template (blue) or no template (black) are clearly distinguishable. The number of positive droplets in each cluster is used to determine the absolute concentration of edited alleles in this sample.

Figure 2: Design and Validation of RBM20 R636S Total Edit Assays



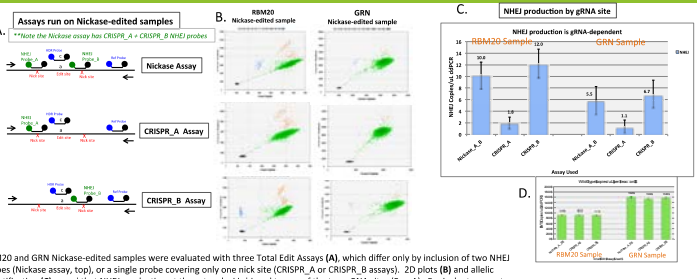
- To quantify the relative contributions of HDR and NHEJ alleles under different editing conditions, 4 Total Edit assays were designed for RBM20 R636S editing: CRISPR-Cas9, Cas9-Nickase, TALEN, and dCas9-FokI (not shown). A schematic of probe position is shown for each assay, and a representative 2D plot on a WT-only and positive control (gblock) sample.
- Limit of Detection (LoD) for HDR and NHEJ alleles was empirically determined by assaying 100ng WT DNA (Promega) plus a 2-fold serial dilution of gblocks containing the point mutation edit (HDR positive control) or a 1-bp deletion at the cut site (NHEJ positive control). LoD was <0.05% for HDR and ~0.1% for NHEJ. Data merged from 2 technical replicate wells is shown. 95% confidence interval is displayed. The dotted lines represent the top of the 95% CI for the WT-only negative control well.

Figure 3: Absolute quantification of HDR and NHEJ alleles in edited samples



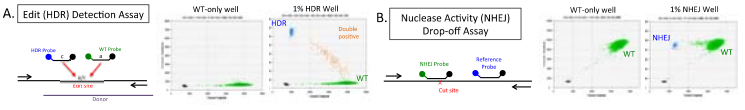
- **(A)** Fractional abundance of HDR and NHEJ edited alleles at the RBM20 R636S locus. HDR graph shown alone for clarity. The ratio of NHEJ:HDR differs at this locus dependent on editing strategy.
- **(B)** Example 2D droplet fluorescence plots from samples edited with 4 different nucleases. Droplets containing WT alleles are green, HDR alleles are orange, NHEJ are blue, negatives are black.
- **(C)** 2-fold dilution of RBM20 TALEN-edited sample demonstrates assay linearity from 3ng to 100 ng input

Figure 4: Differential NHEJ production at paired gRNA sites in Nickase-edited samples



- RBM20 and GRN Nickase-edited samples were evaluated with three Total Edit Assays **(A)**, which differ only by inclusion of two NHEJ probes (Nickase assay, top), or a single probe covering only one nick site (CRISPR_A or CRISPR_B assays). 2D plots **(B)** and allelic quantification **(C)** reveal that NHEJ production at these two loci is biased to one of the two gRNA sites (B vs A). Equivalent amounts of sample was loaded per assay test **(D)**.

Figure 5: Additional ddPCR assay strategies for counting HDR edits & NHEJ alleles



- **(A)** Quantification of HDR Edits alone is possible with an allelic discrimination assay. In this scheme, one primer sits outside the donor sequence, and two probes compete for binding to Edited (FAM) or WT (HEX) alleles. See Miyaoka *et al, Nature Methods* 2014.
- **(B)** Quantification of NHEJ indel alleles alone is possible using a Drop-off assay. In this scheme, one probe (FAM) counts all alleles, and one NHEJ "Drop-off" probe (HEX) sits on top of the predicted cut site (e.g. across 3bp upstream of NGG). The HEX NHEJ probe will not bind an indel allele, causing loss of HEX signal and assignment of the droplet to a FAM+HEX- position (see 2D plot).

Conclusions

- Droplet digital PCR is a high-throughput, ultra-sensitive method for rapid and inexpensive quantification of genome editing events.
- Using the Total Edit Assay, HDR (targeted edit), NHEJ (indel), and WT alleles can be counted simultaneously in a single well. NHEJ and HDR can also be counted independently with a Drop-off (NHEJ) or Edit Detection (HDR) assay. The NHEJ assay provides a quantitative readout of nuclease activity, and could provide a readout of gRNA efficacy.
- The ddPCR Total Edit Assay offers a rapid readout for identification of cell pools with a high HDR:NHEJ ratio, and for optimization of genome editing protocols.

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