Detecting allele-specific genome editing outcomes using a fluorescence-based screening method

Montse Morell, Tatiana Garachtchenko, Matthew Rowe, Baz Smith, Michael Haugwitz and Andrew Farmer*

Takara Bio USA, Inc., Mountain View, CA 94043, USA *Corresponding author: andrew_farmer@takarabio.com

Abstract

One of the most powerful applications of genome editing is the ability to introduce precise changes at genomic loci of interest. However, the success rate for these types of experiments is low since it relies on endogenous repair mechanisms. Therefore, optimization of the editing protocol as well as application of sensitive screening methods for identifying successfully edited clones are essential factors for success.

To address this need, we developed a simple fluorescence-based method that enables detection of successful homology-directed repair (HDR) events independent of their length (from single-nucleotide substitutions to longer insertions) or the sequence at the targeted genomic site. The assay consists of PCR amplification of the genomic target site, followed by an enzymatic assay with a dual-color fluorescence-based readout using a standard plate reader. A positive fluorescent signal from the assay indicates the correct introduction of the desired edit.

Generating human disease models with heterozygous SNPs (one allele encoding the SNP, the other wild-type) is particularly challenging due to the low frequency of successful HDR and the propensity towards non-homologous end joining (NHEJ); as most edited clones encode the SNP in one allele and an indel in the other. Developed with these challenges in mind, the dual-color capability of our assay can be used to positively and unequivocally identify these rarely occurring heterozygous clones. As a test case, we introduced a SNP in an endogenous gene in human pluripotent stem cells (hiPSCs). To achieve a higher percentage of heterozygous clones, we used a mix of HDR templates encoding the SNP or the wild-type allele, both with mutations at the PAM site to prevent recutting by the Cas9 endonuclease. With the help of the dual-color screening assay, edited clones were successfully identified and verified by Sanger sequencing.

Additionally, for scenarios involving knockin (KI) of longer sequences, the assay allows for the simultaneous detection of seamless insertions at both 5' and 3' ends of the recombinant sequence. As a test case, we engineered hiPSCs with a fusion of a myc tag with the *UGT1A9* gene (related to drug metabolism) and screened the clonal cell lines with the dual-color screening assay. We were able to discern cell lines with either partial or complete insertions due to the assay's ability to interrogate both the 5' and 3' ends of the insert.

1 Workflow for the detection of specific homologous recombination events in clonal cell lines

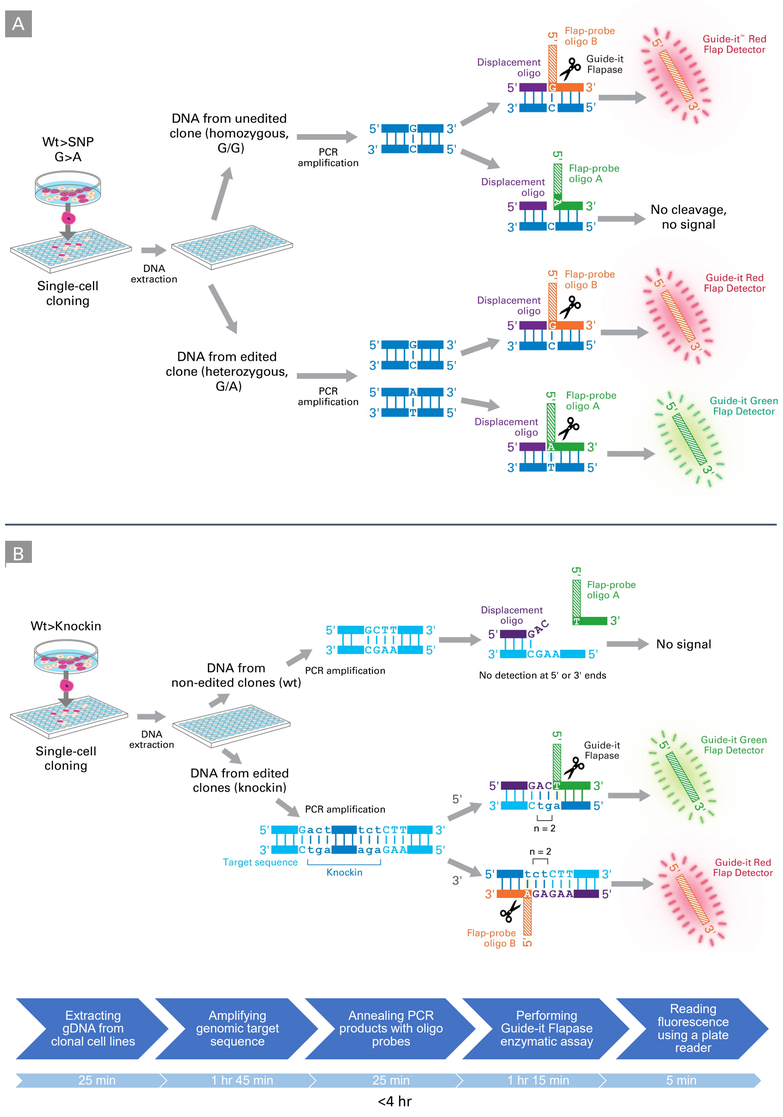

Figure 1. Workflow for the detection of single base substitutions or longer knockin insertions. Panel A. This example workflow demonstrates analysis of a G>A substitution, where G is the wild-type base edited to an A. After genome editing, single cells are isolated via FACS or limiting dilution and then expanded to clonal cell lines that can have one of several different genotypes at the site of interest: G/G, G/A, A/A, G/Indel, A/Indel, or Indel/Indel (for the sake of demonstration, only examples of the former two genotypes are represented above). After PCR amplification of the target site, the PCR product is annealed simultaneously with different oligo probes: a displacement oligo (purple) in combination with either flap-probe oligo A (green; encoding the SNP allele, A) or flap-probe oligo B (orange; encoding the WT allele, G). After annealing of the oligos to the PCR products, the Guide-it Flapase enzyme (scissors) recognizes and cleaves a portion of the flap-probe oligo from structures in which there is complete base-pairing between the oligo probes and the PCR products. The cleaved flaps from the respective flap-probe oligos are then detected by corresponding Guide-it Flap detectors, which yield red and green fluorescent signals, respectively. In the example above using the Guide-it assay, analysis of a clonal population that is homozygous WT (G/G) at the site of interest yields only a red signal, while analysis of a heterozygous population carrying both edited and WT alleles (G/A) yields both red and green signals. **Panel B.** For the detection of longer knockins, the PCR product is annealed with two different sets of displacement and flap-probe oligos: one set that hybridizes with the 5' end of the insert, and the other with the 3′ end. If the HR event has been successful and seamless, the full hybridization of the probes at both termini (5' and 3') will generate both green and red fluorescence signals after the cleavage of the respective flap oligos by the Guide-it Flapase. Detection of only one signal (red or green) indicates an insertion truncated on either the 5′ or 3′ end, respectively. The lack of fluorescence is indicative of the presence of the wild-type sequence or an indel at the target site. Estimated time for each workflow step is indicated at the bottom.

2 Simultaneous screening for WT and SNP alleles in gDNA samples

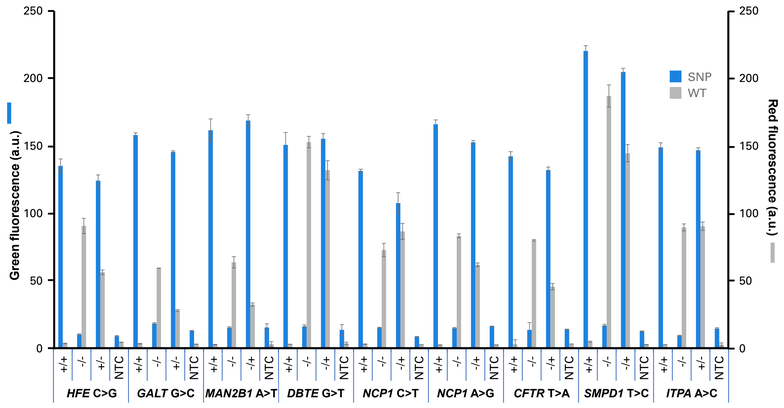

Figure 2. Simultaneous detection of WT and SNP alleles in previously characterized genomic DNA samples. Genomic DNA samples carrying WT and SNP alleles in homozygous or heterozygous configurations at a variety of different loci were obtained from the Coriell Institute and analyzed using the Guide-it Knockin Screening Kit (Figure 1, Panel A). Legend: (+/+) homozygous for SNP allele; (–/–) homozygous for WT allele; (+/–) heterozygous; (NTC) no-template control. For each sample analyzed, the output from the Guide-it assay corresponded with the predetermined genotype at the locus of interest. Samples homozygous for the WT allele yielded only a red signal (gray bar), samples homozygous for the SNP allele yielded only a green signal (blue bar), and heterozygous samples yielded both red and green signals in the assay. For each sample and locus analyzed, the resulting fluorescent signals were of sufficient intensity to be detected above the background signals observed for the no-template control.

3 Generation of heterozygous clones with a single base mutation at a specific genomic site in hiPSCs

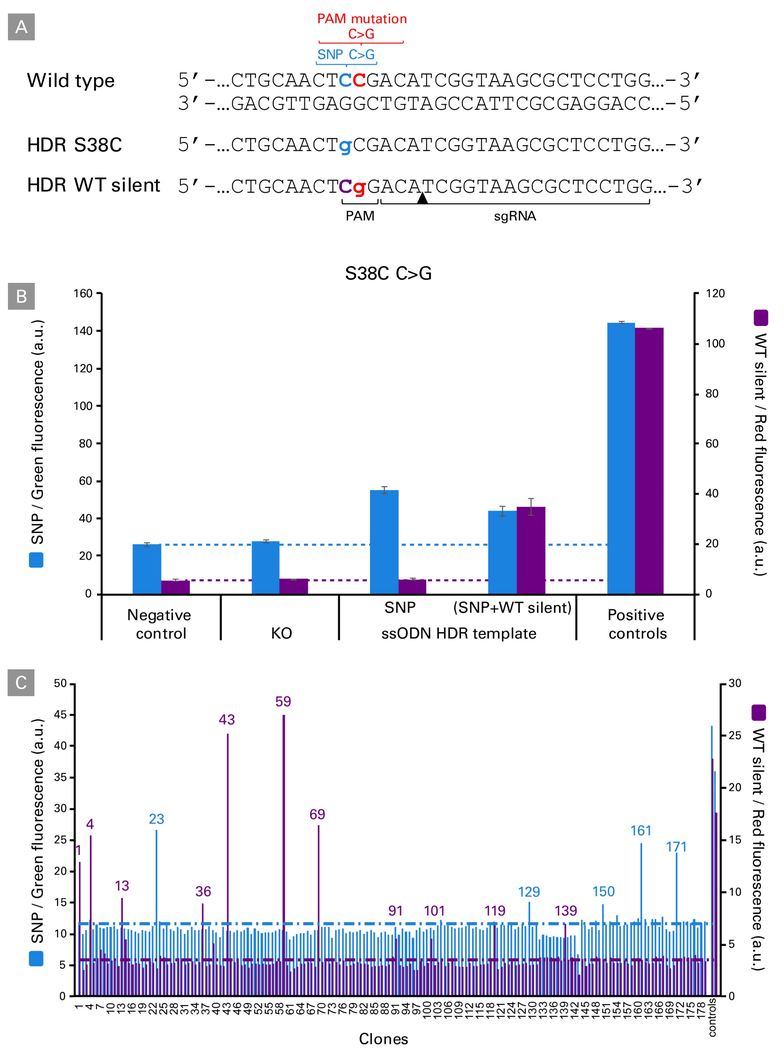

Figure 3. Detection of precise editing at an endogenous locus in bulk-edited and clonal iPSC populations. One of the strategies to maximize the percentage of heterozygous clones (WT/SNP) is to use an equimolar mixture of ssODNs encoding the SNP or WT alleles and silent mutations in the PAM site (if possible) as HDR templates**.** Mutation of the PAM site prevents Cas9 from recutting and generating indels at the genomic target site following successful HDR with either template, indirectly increasing the likelihood of obtaining heterozygotes. We applied this method to generate an iPSC line heterozygous for a C>G substitution (S38C) in an endogenous gene of interest (unidentified for reasons of confidentiality). **Panel A.** Editing outcomes following successful HDR at an anonymous locus of interest. Following successful HDR, the edited locus will encode either a SNP (in blue, lowercase) or a WT base (in purple) combined with a silent PAM mutation (in red, lowercase). **Panel B.** Detection of successful HDR in bulk-edited iPSCs. Displacement and flap-probe oligos were designed to detect WT silent or SNP alleles, yielding red and green fluorescent signals, respectively. In independent experiments, cells were electroporated with Cas9 protein alone (negative control), Cas9-sgRNA RNP complexes (KO), or RNP complexes combined with antisense SNP or SNP/WT silent ssODN mixtures. Synthetic oligos encoding the WT silent or SNP sequences were assayed in parallel as positive controls. For each editing scenario in which ssODNs were included in the electroporation mixture, successful HDR could be detected in the bulk population using the Guide-it Knockin Screening Kit, as indicated by the resulting fluorescent signals. **Panel C.** Detection of successful HDR in clonal cell lines. Clones obtained from single cells isolated by flow cytometry were screened for both edits (SNP and WT silent). While successful incorporation of either edit could be detected in separate clonal cell lines, no heterozygous clones carrying both edits were identified within the limited number of clones tested in this experiment.

4 Screening for full-length, seamless insertion of a myc tag at the C-terminus of the *UGT1A9* gene in hiPSCs

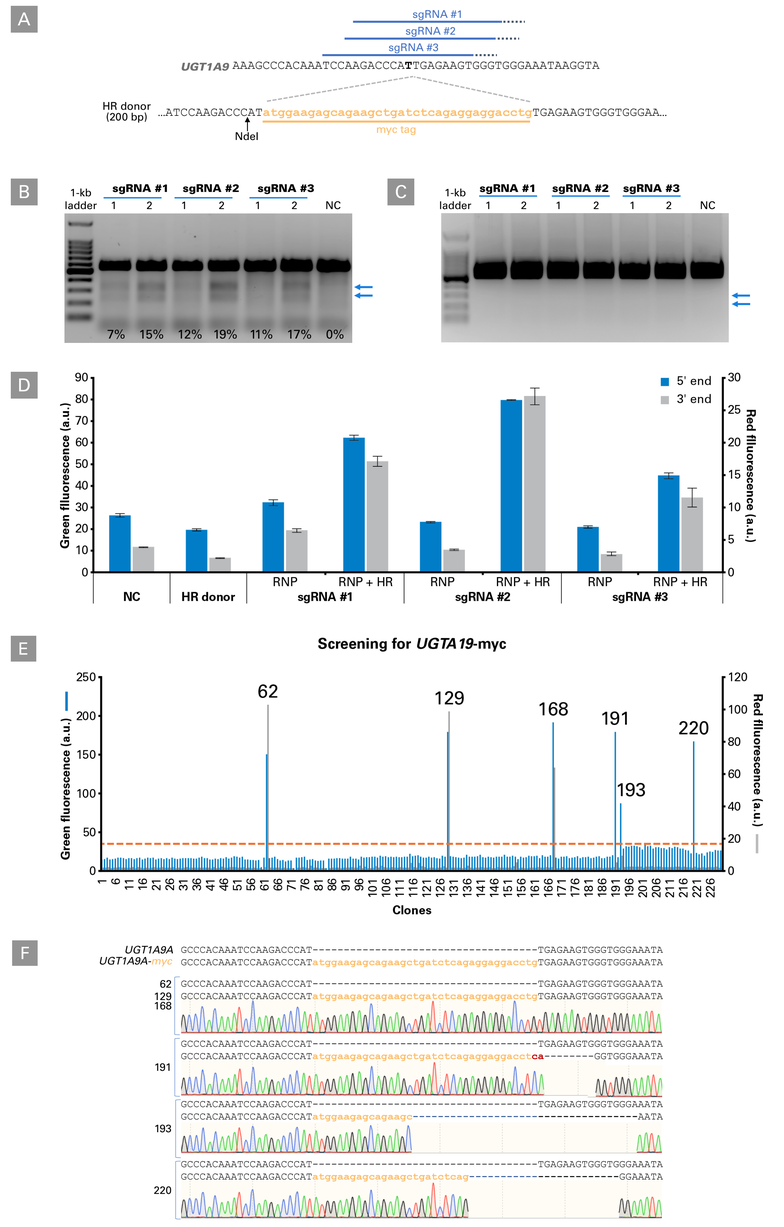

Figure 4. Detection of the tagging of the *UGT1A9* gene with a myc tag in hiPSCs using the Guide-it Knockin Screening Kit. Panel A. ChiPSC18 cells were electroporated with Cas9-RNP complex together with an oligo encoding a myc tag flanked by homology arms allowing for integration at the 3′ end of the *UGT1A9* gene. Three different sgRNAs targeting an area around the insertion site were tested. **Panel B**. The Guide-it Mutation Detection Kit (Cat. # 631443) was used to detect editing events at the target site: (1) RNP; (2) RNP + donor; (NC) negative control. The bands of interest are marked with blue arrows. **Panel C.** No successful HR events could be detected in any case when RFLP assay was used to detect the KI in the pool of edited cells (the blue arrows point to the theoretical positions of the bands). **Panel D.** The Guide-it Knockin Screening Kit was used to detect the successful, full-length HR events in the pool of edited cells. After extraction of the genomic DNA and amplification of the target region by PCR, the DNA samples were hybridized with two sets of probes designed to detect the presence of correct insertion at the 5′ and 3′ ends. The highest signal was obtained from the population electroporated with an RNP complex containing sgRNA #2. This population was chosen for the subsequent single-cell clone isolation. **Panel E.** Single cells were isolated by flow cytometry and expanded. Thirty days after seeding, clonal cell lines were interrogated for myc-tag insertion using the Guide-it Knockin Screening Kit. Out of 230 clones, only three were positive for a correct insertion at both ends (green and red fluorescence could be detected in clones 62, 129, and 168); whereas three other clones only gave a positive signal related to a correct 5′ insertion (only a green signal could be detected in clones 191, 193, and 220). **Panel F**. The results from the Guide-it assay were corroborated by Sanger sequencing. The three positive clones (62, 129, and 168) were heterozygous for the insertion (in blue) with one allele encoding for the wild-type sequence. No homozygous clone for the insertion could be obtained. The other three clones (191, 193, and 220) were also heterozygous with an allele encoding for the wild-type and the other one for a truncated myc tag (extra bases are depicted in red).

Conclusions

- The Guide-it Knockin Screening Kit provides a fast, simple method for detecting precise edits—including single nucleotide substitutions and insertion of longer sequences—both in mixed and clonal cell populations.
- The kit workflow can be completed in less than four hours and involves PCR amplification of the genomic target region followed by an enzymatic assay with a fluorescent readout. No special equipment is required except for a fluorescence plate reader with green and red filter sets.
- The kit assay can be applied towards the detection of any possible substitution or insertion at any genomic locus.
- The application of dual-color chemistry allows for the confirmation of precise gene knockins at the 5′ and 3′ junctions of inserted sequences or the simultaneous detection of edited (SNP) and unedited (WT) alleles in parallel from the same sample, enabling rapid identification of heterozygous clones.

Takara Bio USA, Inc.
United States/Canada: +1.800.662.2566 • Asia Pacific: +1.650.919.7300 • Europe: +33.(0)1.3904.6880 • Japan: +81.(0)77.565.6999

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