Streamlined genome engineering: identifying successful homologous recombination events using a fluorescence-based screening method

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Abstract

One of the most powerful applications of genome editing is the ability to introduce precise changes at genomic loci of interest by pairing site-specific nuclease activity with repair processes mediated by homologous recombination (HR). Common objectives of this approach include the insertion of long sequences encoding fusion tags or expression cassettes, the introduction of single base substitutions that mimic single nucleotide polymorphisms (SNPs) associated with human disease, or the creation of stop codons to generate precise gene knockouts.

In the context of most genome editing workflows involving knockin (KI) of insertions or substitutions in cultured cells, there are two different stages when the detection of successful HR events is critically important. The first stage involves optimization of experimental conditions to achieve the highest percentage of error-free recombination events in an edited population before moving forward with the isolation of single-cell clones. The second stage involves identification of cell lines carrying the edit of interest after single-cell isolation and expansion in 96-well plates. To address this need, we developed a simple fluorescence-based method that enables detection of successful HR events independent of their length (from single nucleotide substitutions to longer insertions) or surrounding targeted genomic loci in edited populations as well as in clones from 96-well plates. 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 is highly correlated with the correct introduction of the desired edit. For scenarios involving KI of longer sequences, the assay allows for the simultaneous detection of seamless insertions at both 5' and 3' ends of the recombinant sequence.

For engineering SNPs, the assay enables detection of single nucleotide substitutions with high sensitivity in both mixed and clonal populations and can be used to positively identify heterozygous clones carrying one copy each of the edited (SNP) and unedited (WT) alleles.

We employed this method in a variety of experimental contexts, including the creation of isogenic cell lines carrying SNPs in the *FAH* gene related to tyrosinemia, and the introduction of a myc tag fused with the gene *UGT1A9* (related to drug metabolism). In all editing experiments, we performed the assay at both stages identified above: first to detect which sgRNAs generated higher KI efficiencies in the edited cell population, and second to screen for successfully edited clonal cell lines following isolation and expansion of single-cell clones in 96-well plates. During the screening process for successful fusion of the myc tag with *UGT1A9*, we could discern clonal cell lines with either partial or complete insertions due to the assay's ability to interrogate both 5' and 3' ends of the insert. Using previously characterized genomic DNA samples obtained from the Coriell Institute, we also demonstrated the capability of the assay to simultaneously detect WT and SNP alleles in the same sample, a common objective for research projects that require engineering cell lines that are heterozygous for a given SNP of interest.

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

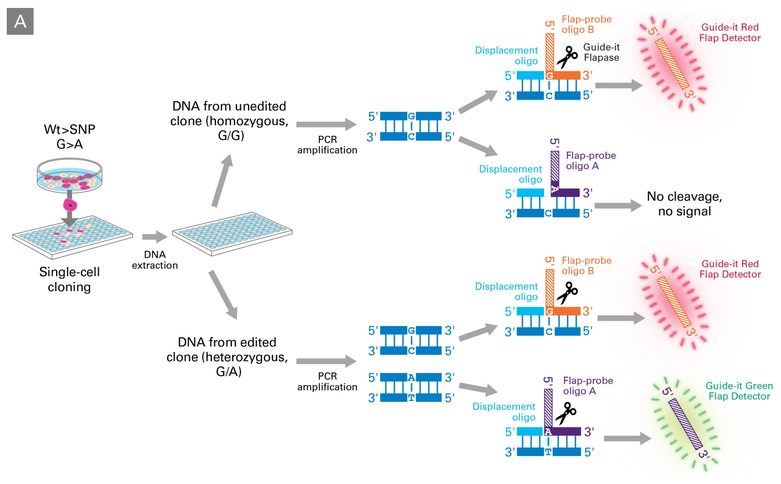

Figure 1. Workflow for the detection of single base substitutions or longer knockin insertions. Panel A. This example workflow demonstrates analysis of a G>A substitution, where G is the wild-type base edited to an A. After genome editing, single cells are isolated via FACS or limiting dilution then 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 DNA extraction and subsequent PCR amplification of the target site, the PCR product is annealed with two different combinations of oligo probes in parallel (in the same well): the Displacement oligo in combination with either Flap-probe oligo A or Flap-probe oligo B. In the example above, the Displacement oligo and Flap-probe oligo B fully anneal to PCR products corresponding to the WT allele (G), while the Displacement oligo and Flap-probe oligo A fully anneal to PCR products corresponding to the edited allele (A). 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'. Each flap-probe oligo has a specific fixed and distinct flap sequence that allows for the generation of a green or a red fluorescence signal. If the HR event has been successful and seamless, the full hybridization of the probes at both ends (5' and 3') will generate 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) would indicate an insertion truncated on either the 5' or 3' end, respectively.

2 Screening for a tyrosinemia-related SNP in the *FAH* gene in hiPSCs

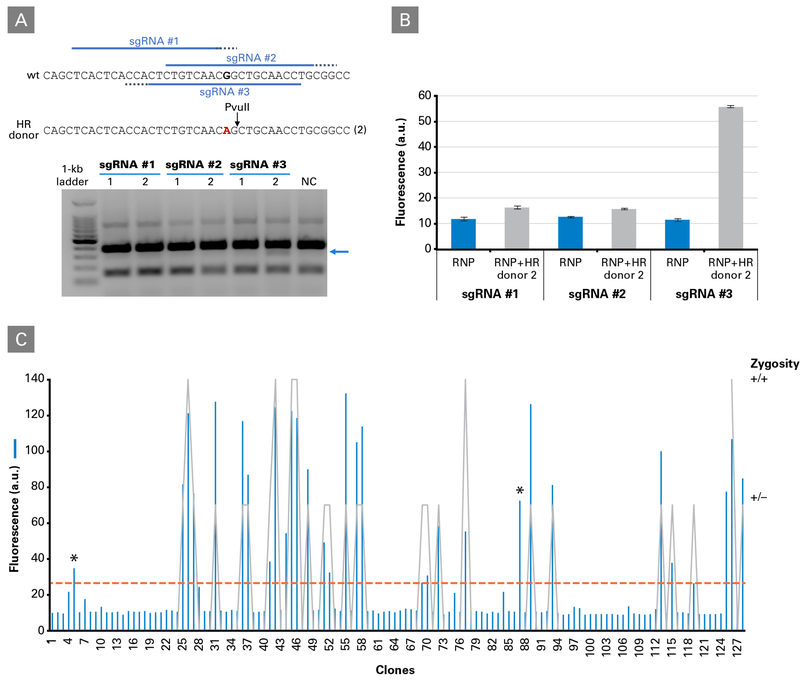

Figure 2. Detection of a tyrosinemia-related SNP in hiPSCs using the Guide-it SNP Screening Kit. Panel A. A SNP in the *FAH* locus related to tyrosinemia (NM_000137.2(FAH):c.1009G>A (p.Gly337Ser)) was generated in cells from the Cellartis® Human iPS Cell Line 18 (ChiPSC18) Kit (Cat. # Y00305) by electroporation of Cas9-RNP complexes together with a short oligo encoding the SNP acting as the HR donor (using the Cellartis iPSC rCas9 Electroporation and Single-Cell Cloning System; Cat. # 632643). Different sgRNAs (indicated by #1, #2, and #3) were tested, and an RFLP assay was used to detect HR in the pool of edited cells. Successful substitution of the edited base (shown in red) introduced a new restriction site for the PvuII enzyme. In this case, HR could be detected for only one of the sgRNAs used (as seen in the gel image—the band of interest is marked with a blue arrow). Legend: (NC) negative control; (1) cells electroporated with RNP with no HR donor; (2) RNP + HR donor 2. **Panel B.** The Guide-it SNP Screening Kit was also used to detect successful HR in the pool of edited cells before single-cell isolation. After extraction of the genomic DNA with NucleoSpin Tissue Columns (Cat. # 740952.250S) and amplification of the target region by PCR, the DNA sample was hybridized with a displacement oligo and a flap-probe oligo designed to detect the SNP. A fluorescent signal was only detected for sgRNA #3 which correlates with the result obtained by the RFLP assay. **Panel C.** Single cells were isolated by limiting dilution and expanded following the protocol established by the Cellartis iPSC Single-Cell Cloning DEF-CS™ Culture Media Kit (Cat. # Y30021). Forty-five days after seeding, clonal cell lines were interrogated for the SNP using the Guide-it SNP Screening Kit and approximately 24% of the clonal cell lines generated a positive fluorescent signal. Correlation between production of a fluorescent signal above a specific threshold (orange dotted line) and presence of the intended SNP at the site of interest was confirmed by Sanger sequencing (gray peaks indicate zygosity of the SNP allele for each clone). Samples that were determined to be nonclonal are marked with an asterisk.

3 Screening for WT and SNP alleles in the same sample

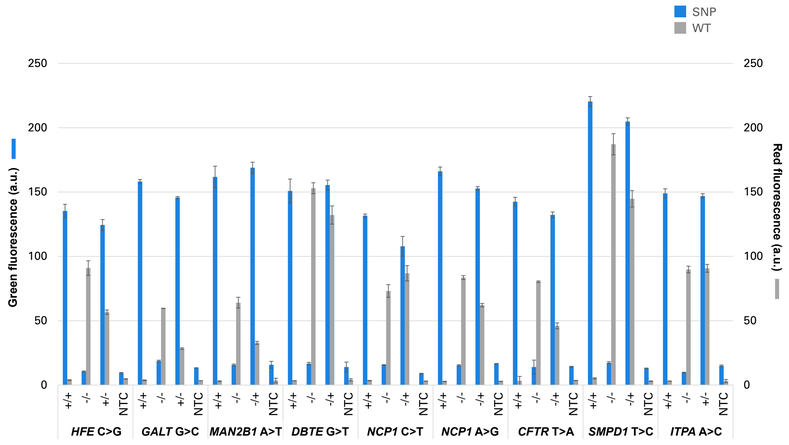

Figure 3. Simultaneous detection of WT and SNP alleles in previously characterized genomic DNA samples. Genomic DNA samples carrying WT and SNP alleles in homozygous or heterozygous configurations at a variety of different loci were obtained from the Coriell Institute and analyzed using the dual-color version of the Guide-it SNP 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 samples heterozygous 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.

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

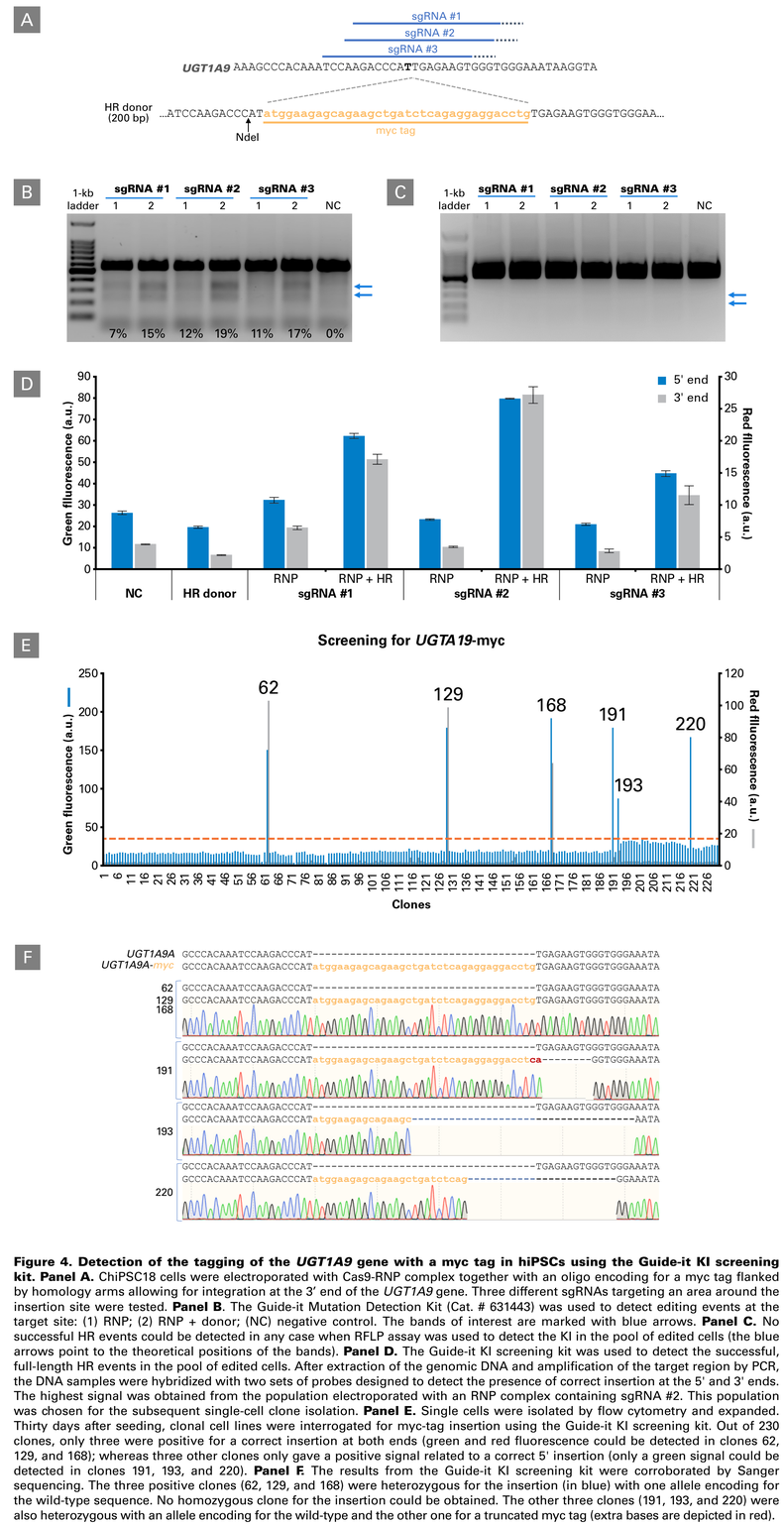

Figure 4. Detection of the tagging of the *UGT1A9* gene with a myc tag in hiPSCs using the Guide-it KI screening kit. Panel A. ChiPSC18 cells were electroporated with Cas9-RNP complex together with an oligo encoding for a myc tag flanked by homology arms allowing for integration at the 3′ end of the *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 KI 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 KI 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 KI screening kit 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 SNP 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 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 confirmation of precise gene knockins at 5′ and 3′ junctions of inserted sequences or simultaneous detection of edited (SNP) and unedited (WT) alleles in parallel from the same sample, enabling rapid identification of heterozygous clones.

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