Screening successful homologous recombination events using a fluorescence-based method

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

Takara Bio USA, Inc., Mountain View, CA 94043, USA *Corresponding Author

Abstract

One of the most powerful applications of genome editing is the ability to introduce precise changes in specific sites by homologous recombination (HR). These HR editing events range from the insertion of long sequences encoding fusion tags or expression cassettes to single base changes that mimic single-nucleotide polymorphisms (SNPs) related to human diseases, to the introduction of stop codons to generate precise gene knockouts.

During the workflow of any knockin (KI) experiment, it is critical to detect successful and accurate HR events at two different stages. Stage one involves optimization of the experimental conditions to achieve the highest percentage of recombination before moving forward with single-cell clone isolation of the edited population. The second stage involves identification of the cell line 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 allows the 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 target site, followed by an enzymatic assay, and a fluorescence-based read out using a standard plate reader. A positive fluorescent signal from the assay is highly correlated with the correct introduction of the desired edit. In case of KI of longer sequences, the assay allows for the simultaneous detection of seamless insertions at both 5' and 3' ends of the recombinant sequence due to dual-color detection capability.

As a proof of concept, we applied this method in different KI experiments in hiPSCs with the creation of isogenic cell lines carrying SNPs in the *FAH* gene related to tyrosinemia and the introduction of myc tag in the gene *UGT1A9* (related to drug metabolism). In all editing experiments, we performed the assay at both stages: first to detect which sgRNAs generated a higher percentage of KI, and second to screen for the edited clonal cell lines after isolation and expansion of single cells in 96-well plates. In case of tagging of the *UGT1A9* gene, during the screening process, we could discern clonal cell lines with partial or complete insertions due to the detection of both 5' and 3' ends. The engineered cell lines carrying the desired edit could be further differentiated to hepatocytes, thus generating a valuable tool for disease and metabolism studies.

Conclusions: The Guide-it™ SNP and KI Screening kits provide a quick, high-throughput method to detect HR events following genome editing experiments in the edited population as well as in clonal cell lines.

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

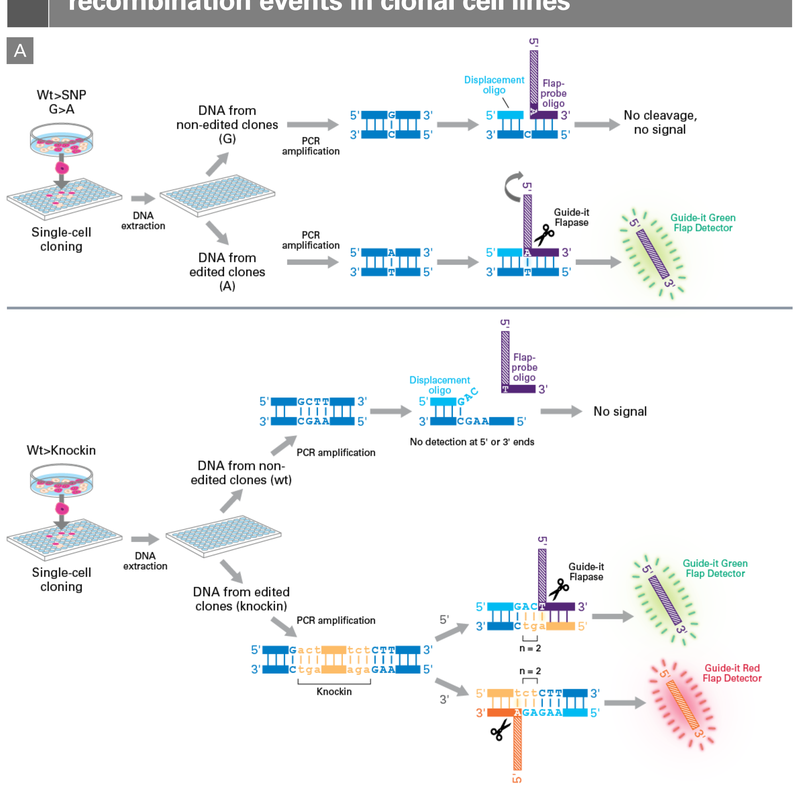

Figure 1. Workflow for the detection of single-base substitutions or longer knockin insertions. Panel A. This example workflow shows the detection of a G>A transition (where G is the wild-type base edited to an A). After the genome editing event, single cells are isolated via FACS or limiting dilution and expanded to clonal cell lines that can be either wild-type or successfully edited. After DNA extraction and subsequent PCR amplification of the target site, the PCR product is annealed with two different complementary oligo probes—the displacement oligo and the flap-probe oligo that are designed to hybridize with the target site in the region adjacent to the interrogated base (defined as the actual SNP base for which you are screening). After the annealing of the two DNA oligos with the PCR product, Guide-it Flapase recognizes a complete base-pairing between the flap-probe oligo and the PCR product in the resulting three-dimensional structure and subsequently cleaves the flap. This cleavage event causes the release of the flap oligo, which is detected downstream by the Guide-it Flap Detector, generating a fluorescence signal that can be measured using a plate reader. **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 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) would indicate an insertion truncated on either the 5' or 3' end, respectively.

2 Screening for SNPs in *FAH*-related tyrosinemia in hiPSCs

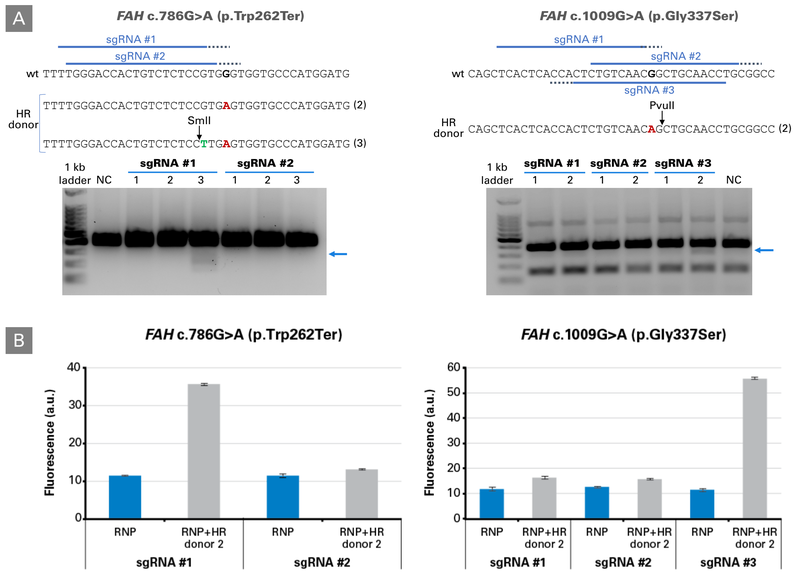

Figure 2. Detection of tyrosinemia-related SNPs in hiPSCs using the Guide-it SNP Screening Kit. Panel A. Two SNPs in the *FAH* locus related to tyrosinemia (p.Gly337Ser and p.Trp262Ter) were generated in cells from the Cellartis® Human iPS Cell Line 18 (ChiPSC18) Kit (Cat. # Y00305) by electroporation of the Cas9 RNP complex 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 the HR in the pool of edited cells. In the case of *FAH* Gly337Ser, the edited base (shown in red) introduced a new restriction site for the PvuII enzyme; in the case of Trp262Ter, a second template (HR donor 3) with another base mutation (shown in green) was used to generate a new restriction site for the enzyme SmlI. In each case, the HR event could be detected in only one of the sgRNAs used (as seen in the gel images—the band of interest is marked with a blue arrow); (NC) negative control; (1) cells electroporated with RNP with no HR donor; (2) RNP + HR donor 2; (3) RNP + HR donor 3 with an extra base change encoding a restriction site. **Panel B.** The Guide-it SNP Screening Kit was also used to detect the successful HR event 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. In both cases, the fluorescent signal correlated 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 respective SNPs using the Guide-it SNP Screening Kit. In each case, approximately 19–24% of the clonal cell lines generated a positive fluorescent signal. The correlation between the fluorescence above a specific detection signal (orange dotted line) and the existence of the SNPs in the interrogated base was confirmed by Sanger sequencing in all the tested clonal cell lines to be homo or heterozygous. Non-clonal samples are marked with an asterisk.

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

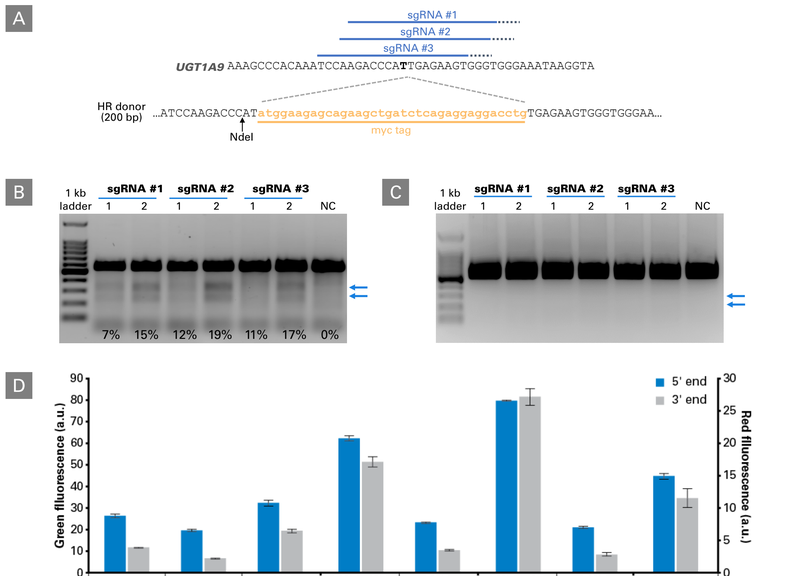

Figure 3. Detection of the tagging of the *UGT1A9* gene with a myc tag in hiPSCs using the Guide-it KI Screening Kit. Panel A. ChiPSC18 cells were electroporated with Cas9 RNP complex together with an oligo encoding for a myc tag flanked by homology arms allowing for integration at the 3'-end of the *UGT1A9* gene. Three different sgRNAs targeting an area around the insertion site were tested. **Panel B**. 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 position 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).

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