Takara Bio USA, Inc.

Guide-it™ Knockin Screening Kit User Manual

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Table of Contents

I. Introduction 4
A. Summary 4
B. Protocol Overview 7
II. List of Components 8
III. Additional Materials Required 8
IV. Protocol for Detecting Single Base Change or Heterozygosity 9
A. Design Wild-Type and SNP Control Oligos Based on the Target Sequence (Design 1) 9
B. Design Flap-Probe Oligos (Design 1) 10
C. Design Displacement Oligo (Design 1) 11
D. Alternative Design (Design 2) 12
E. Test Displacement and Flap-Probe Oligos 13
F. Protocol: Extract Genomic DNA From Your Edited Clonal Cell Lines 16
G. Protocol: Amplify the Target Site by PCR 17
H. Protocol: Perform the Enzymatic Assay 19
V. Protocol for Detecting Longer Insertions 22
A. Design Control, Flap-probe, and Displacement Oligos for the Detection of the 5' Terminus of the Insert (Design 1) 23
B. Design Control, Flap-probe, and Displacement Oligos to Detect the 3' Terminus of the Insert (Design 1) 25
C. Alternative Probe Design for the Detection of KI Inserts (Design 2) 28
D. Test the Two Sets of Displacement and Flap-Probe Oligos 29
E. Protocol: Extract Genomic DNA From Your Edited Clonal Cell Lines 32
F. Protocol: Amplify the Target Site by PCR 33
G. Protocol: Perform the Enzymatic Assay 35
VI. References 36
Appendix A. Troubleshooting Guide 37
Appendix B. Glossary of Terms 38

Table of Figures

Figure 1. Tripartite structures formed by the annealing of the genomic target site and user-generated displacement and flap-probe oligos for the detection of a single base mutation. 5
Figure 2. Schematic of the Guide-it Knockin Screening Kit workflow for detecting SNPs or clonal heterozygosity. 6
Figure 3. Workflow of the Guide-it Knockin Screening Kit 7
Figure 4. Sample design of wild-type and SNP control oligos used to test the performance of displacement and flap-probe oligos. 10
Figure 5. An example showing the design of the flap-probe oligo. 11

Figure 6. An example showing the design of the displacement oligo. 12
Figure 7. Alternative design (Design 2). 12
Figure 8. An example of an alternative design of the control, displacement, and flap-probe oligos. 13
Figure 9. Results obtained from testing the functionality of displacement and flap-probe oligos designed to detect clone heterozygosity using control oligos as samples. 16
Figure 10. Optimal range of cell densities for genomic DNA extraction using MightyPrep Reagent. 17
Figure 11. Examples of PCR-amplified target DNA used as samples with the Guide-it Knockin Screening Kit. 19
Figure 12. Design of the displacement and flap-probe oligos for the detection of an insert at its 5' and 3' termini. 22
Figure 13. The Guide-it Knockin Screening Kit provides a method for detecting full-length knockin insertions. 23
Figure 14. An example showing the design of wild-type and KI control oligos for the detection of the 5' terminus of the insert. 24
Figure 15. An example showing the design of the flap-probe oligo for the detection of the 5' terminus of the insert. 25
Figure 16. An example showing the design of the displacement oligo for the detection of the 5' terminus of the insert. 25
Figure 17. An example showing the design of wild-type and KI control oligos used to test the performance of displacement and flap-probe oligos for the detection of the 3' junction of the insert. 26
Figure 18. An example showing the design of the flap-probe oligo for the detection of the 3' terminus of the insert. 27
Figure 19. An example showing the design of the displacement oligo for the detection of the 3' terminus of the insert. 27
Figure 20. Alternative probe design for the detection of KI inserts (Design 2). 28
Figure 21. An example of alternative design (Design 2) for the control, displacement, and flap-probe oligos. 29
Figure 22. Testing the functionality of displacement and the flap-probe oligos designed to detect the insertion of a myc tag in *UGT1A9* gene. 32
Figure 23. Scheme showing the design of primers to amplify the target site. 33

Table of Tables

Table 1. Conditions for calculating Tm of the displacement and flap-probe oligos. 10
Table 2. Noncomplementary nucleotide to be added at the 3'end of the displacement oligo based on the nucleotide substitution being screened for (wild type>SNP). 11
Table 3. Annealing master mix preparation guidelines. 14
Table 4. Enzymatic reaction master mix preparation guidelines. 15
Table 5. PCR master mix preparation guidelines. 18
Table 6. Annealing master mix preparation guidelines. 19
Table 7. Enzymatic reaction master mix preparation guidelines 20
Table 8. Annealing master mix preparation guidelines. 30
Table 9. Enzymatic reaction master mix preparation guidelines. 31
Table 10. PCR master mix preparation guidelines if one pair of primers is used. 34
Table 11. PCR master mix preparation guidelines if two pairs of primers are used. 34
Table 12. Annealing master mix preparation guidelines. 35
Table 13. Enzymatic reaction master mix preparation guidelines 35
Table 14. Troubleshooting guide for the Guide-it Knockin Screening Kit. 37
Table 15. Glossary of terms 38

I. Introduction

A. Summary

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

During the workflow of any knockin (KI) experiment 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 the Guide-it Knockin Screening Kit, which employs 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 the genomic locus targeted for editing. The assay involves PCR amplification of the target site, followed by an enzymatic assay (using Guide-it Flapase, a structure-specific endonuclease) and a fluorescence-based readout generated by the Guide-it Green/Red Flap detectors. A positive fluorescent signal from the assay is highly correlated with the presence of a specific sequence at the edited site. The assay can also be tailored depending on the length of the edit. For single base substitutions, it allows the detection of clonal heterozygosity with green or red fluorescence corresponding to the presence of SNP or wild-type alleles, respectively. For longer KI insertions, the dual-color detection capability allows for the simultaneous detection of seamless insertions at both 5’ and 3’ ends of the recombinant sequence.

The assay is based on the capacity of Guide-it Flapase to recognize and cleave a specific tripartite structure formed by the target site and two user-defined oligo probes (Figure 1). The kit protocol involves PCR amplification of the targeted genomic region followed by hybridization between the PCR product and two oligo probes (referred to as the “displacement oligo” and the “flap-probe oligo” [Figure 1, see Appendix B for a glossary of terms]). If you are detecting a single base change, the displacement oligo hybridizes the PCR product 3’ relative to the interrogated base and has an extra noncomplementary base at its 3’ end (indicated by “n” in Figure 1).

The flap-probe oligo is designed to encode the sequence you want to detect and one of two fixed, noncomplementary sequences at its 5’ end that triggers the generation of either green or red fluorescence by the Guide-it Flap Detector. This flap-probe oligo hybridizes the PCR product 5’ relative to the interrogated base.

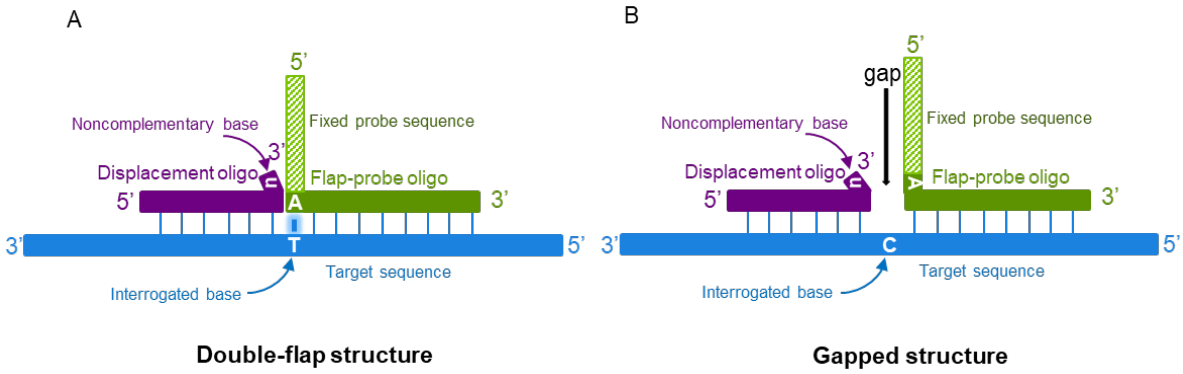


Figure 1. Tripartite structures formed by the annealing of the genomic target site and user-generated displacement and flap-probe oligos for the detection of a single-base mutation. Annealing of the displacement oligo (purple) and the flap-probe oligo (green) with the PCR product (blue) containing the genomic target sequence is shown. **Panel A**. In a scenario where the interrogated base encodes the desired edit, the flap-probe oligo forms a complete base pairing at the target site (double-flap structure). **Panel B**. If there isn't a complete base pairing at the target site with the flap-probe oligo, a gap is formed (gapped structure).

Hybridization of the displacement and flap-probe oligos with the PCR product can yield either of two different tripartite structures depending on whether both probes have full complementarity with the PCR product (Figure 1):

1. A double-flap structure is formed where there is full complementarity between the flap-probe oligo, the displacement oligo, and the PCR product. In the enzymatic assay following the formation of the tripartite structure, Guide-it Flapase specifically recognizes the double-flap structure and subsequently cleaves the flap. The release of this fixed-sequence portion of the flap-probe oligo is detected downstream by the Guide-it Flap Detector, generating either a green or red fluorescent signal (depending on the fixed sequence) that can be measured using a plate reader (or a qPCR machine).

2. If there is not full complementarity, the flap-probe oligo remains unpaired and causes a gap in the structure. In this case, Guide-it Flapase does not cleave the flap, and no fluorescent signal is generated.

The detection of a positive fluorescent signal in this assay indicates presence of the desired sequence encoded in the PCR product, and this signal can be used to screen hundreds of clones or bulk-edited populations for successful outcomes in knockin experiments involving genome-editing tools (e.g., CRISPR/Cas9 system).

NOTE: For detecting successful HR events in heterogenous bulk-edited cell populations, small adjustments must be made to the kit protocol which are described in detail at relevant sections of the user manual (Sections IV.F–H; Sections V. E–G).

This kit includes two different Guide-it Flap detectors, each of which generates a specific fluorescent signal (green or red). Each Guide-it Flap detector detects a specific DNA sequence, allowing the simultaneous use of two different flap-probe oligos to detect the presence of two different sequences at the target site (e.g., SNP and wild-type) (Figure 2).

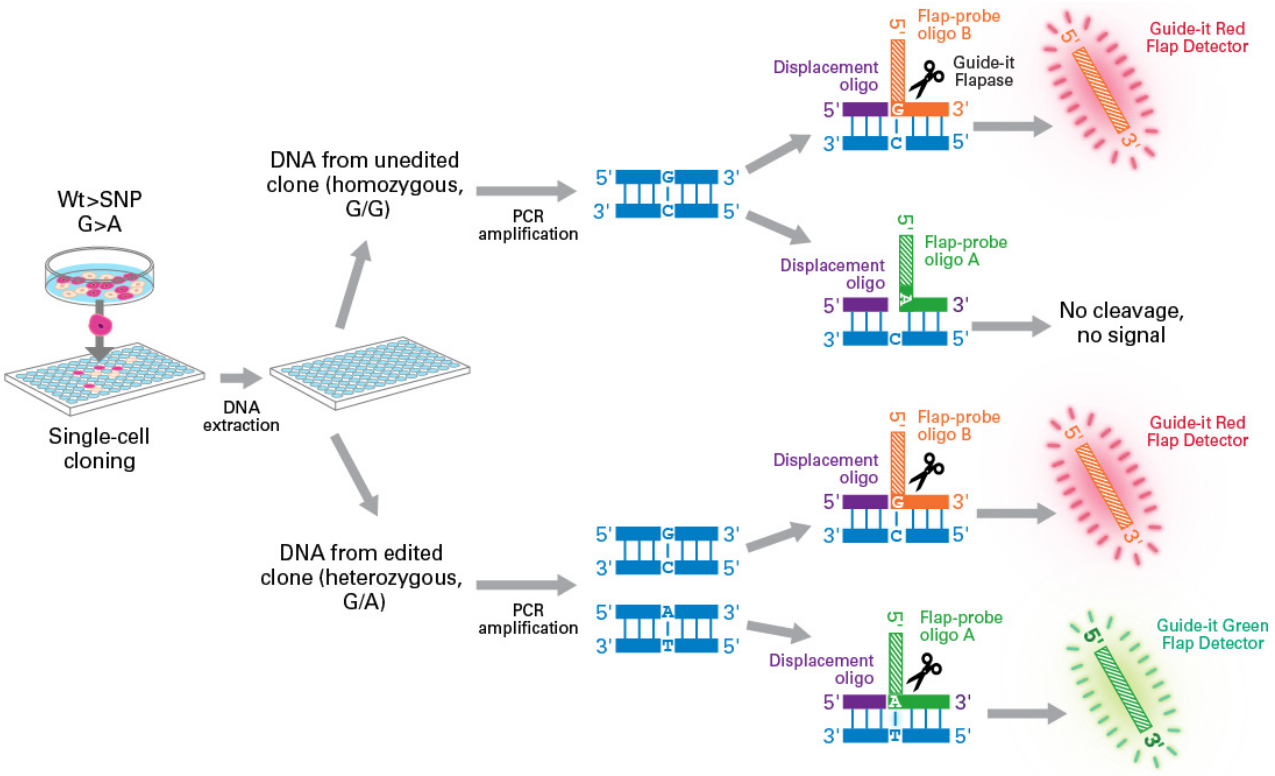
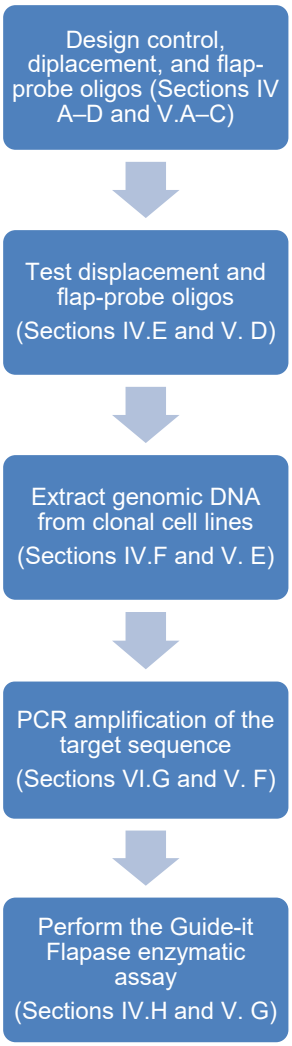


Figure 2. Schematic of the Guide-it Knockin Screening Kit workflow for detecting a single base change. This example workflow depicts the detection of a G>A substitution. Following genome editing, single cells are isolated via FACS or limiting dilution and expanded to clonal cell lines that may carry wild-type (G, top) or successfully edited nucleotides (A, bottom), or indels at the target site. After DNA extraction and subsequent PCR amplification of the region surrounding the target site, the resulting PCR products (blue) are hybridized with different complementary oligos—a displacement oligo (purple) and either of two flap-probe oligos (green [A] or orange [B]) that each have a fixed, noncomplementary sequence (shaded green or orange) that forms a flap. When there is a full hybridization between the probes and the amplified sequence, the flap-probe oligo forms a complete base pairing at the target site yielding a double-flap structure. Guide-it Flapase specifically cleaves and releases the flap from the double-flap structure which then binds to the Guide-it Flap Detector and generates a fluorescent signal (green or red). When there is not full complementarity (e.g., when indels are formed), the flap-probe oligo generates a gapped structure that cannot be cleaved by Guide-it Flapase and no signal is generated. Thus, detection of a specific fluorescent signal (green or red) using the Guide-it Knockin Screening Kit is indicative of whether the desired nucleotide encoded in the corresponding flap-probe oligo is present in the analyzed clone.

B. Protocol Overview



Design the displacement, flap-probe, and control oligos (60–90 bp in length) based on the target sequence encoding the wild-type and edited sequence. The design of oligos for detecting SNPs or longer knockins are different and are discussed separately in Sections IV and V, respectively.

Test the performance of the displacement and flap-probe oligos in the enzymatic assay using wild-type and SNP/KI control oligos as control samples.

Extract genomic DNA from clonal cell lines using MightyPrep Reagent.

Amplify the target sequence with Terra™ PCR Direct Polymerase.

Anneal the PCR products with the displacement and flap-probe oligos, perform the enzymatic assay with Guide-it Flapase, and measure fluorescence on a standard fluorescence plate reader (or qPCR machine).

Figure 3. Workflow of the Guide-it Knockin Screening Kit.

II. List of Components

The Guide-it Knockin Screening Kit consists of the Guide-it Flap Reagents (not sold separately), the Guide-it Knockin Control Set (not sold separately), and MightyPrep Reagent for DNA. **These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions.** The substitution of reagents in the kit and/or modification of the protocol may lead to unexpected results.

Storage Conditions

- Store MightyPrep Reagent for DNA at 4°C.
- Store all other reagents at –20°C.

Guide-it Knockin Screening Kit	**632659 (100 rxns)**	**632660 (400 rxns)**
MightyPrep Reagent for DNA (Store at 4°C)	**9182A**	**9182**
MightyPrep Reagent for DNA	5 ml	20 ml
Guide-it Knockin Control Set (Store at –20°C)	**632661**	**632661**
Guide-it Knockin Positive Control Mix	300 µl	300 µl
Guide-it Knockin Negative Control Mix	300 µl	300 µl
Guide-it Flap Reagents (Store at –20°C)	**632662 (100 rxns)**	**632663 (400 rxns)**
Terra PCR Direct Polymerase Mix	50 µl	200 µl
2X Terra PCR Direct Buffer (with Mg^{2+}, dNTP)	750 µl x 2	1 ml x 5
Dilution Buffer	8 ml	32 ml
RNase-free Water	20 ml	35 ml x 2
Annealing Buffer	200 µl	800 µl
Flapase Buffer	300 µl	1.2 ml
Guide-it Flapase	100 µl	400 µl
Guide-it Green Flap Detector (40X)	50 µl	200 µl
Guide-it Red Flap Detector (40X)	50 µl	200 µl

III. Additional Materials Required but not Supplied

- PBS -/- (Dulbecco's Phosphate Buffered Saline without Ca^{2+} & Mg^{2+} [Sigma-Aldrich, Cat. No. 8537 or equivalent])
- Hot plate
- Thermal cycler
- 96-well plates compatible with PCR thermal cycler
- Cap strips or optical film for 96-well plate compatible with PCR thermal cycler
- Twelve-channel pipettes (recommended): 20 µl and 200 µl
- Low-speed benchtop centrifuge for 96-well plates
- Low-binding DNA Eppendorf tubes (e.g.; Eppendorf, Cat. No. 022431005)
- 96-well black plate with clear bottom (e.g., Corning, Cat. No. 3631)
- NucleoSpin Tissue (Cat. # 740952.250) for screening bulk-edited populations

- Plate reader or qPCR machine that allows detection of green fluorescence with 485 nm excitation/535 nm emission and red fluorescence with 590 nm excitation/620 nm emission

IV. Protocol for Detecting single-nucleotide substitutions

NOTES:

– Please read this **entire section** to understand the design of the necessary oligos prior to designing and ordering them.

– Please use our online tool to streamline the oligo design process: takarabio.com/oligo-tool-snp

– If you are screening bulk populations before clonal cell expansion, small adjustments must be made to the protocol; follow corresponding instructions provided in Sections IV.F–H (for genomic DNA isolation and incubation time for the enzymatic reaction, respectively).

This assay is highly sensitive to any mutation(s) in the sequences hybridizing with the displacement and flap-probe oligos. If the PAM site is to be mutated, or there are any other changes in the original sequence near the edited site that are encoded in the HR template, the design of the probes will need to be based on the final edited sequence. This also applies if you will use a mixture of oligos encoding for the SNP and the wild-type with PAM mutations in order to obtain a higher rate of heterozygous clones (Paquet et al. 2016). We have described the rationale behind probe designs in Sections A–D below.

NOTE: For oligo design, we strongly recommend using our software, available at takarabio.com/oligo-tool-snp, to design the wild-type and SNP control oligos as well as the displacement and flap-probe oligos. Our software designs the flap-probe oligos so that red and green fluorescent signals correlate with wild-type and SNP sequences, respectively. For detecting single-nucleotide substitutions, only one displacement oligo is needed (Figure 2). Once you have your oligos, you can start with Section IV.E; Test Displacement and Flap-probe Oligos.

A. Design Wild-Type and SNP Control Oligos Based on the Target Sequence (Design 1)

Design the wild-type and SNP control oligos that will be used as samples to test the performance of the flap-probe and displacement oligos. These control oligos encode your target sequence and can be used as positive controls in the actual screening experiment.

The wild-type and SNP control oligos are around 60–90 nucleotides in length, with the interrogated base positioned around the middle of each oligo (Figure 4).

NOTE: Since the control oligos must hybridize the full length of the flap-probe and displacement oligos, they could be longer than 90 bases depending on the length (and GC content) of the designed oligos.

While the sequences of these oligos are typically derived from the antisense strand, this is not a requirement, and the sense strand may be used instead. In fact, in certain instances it may be necessary to use the sense strand [see Note below and Section IV.D]. The sequence of the SNP control oligo should be the same as the wild-type control oligo, except it includes the desired SNP at the target site (Figure 4).

NOTE: If the target sequence immediately 5’ relative to the SNP is either of the following: 5’-GGAGX-3’, 5’-GGAGNX-3’, 5’-GACGX-3’, or 5’-GACGNX3’ (where N is any nucleotide, and X is the interrogated base), the opposite strand **must** be used to design the wild-type and SNP oligos. Inclusion of these sequences causes the Guide-It Flap Detector to recognize the displacement oligo as the cleaved fragment and generates background fluorescence. An example demonstrating the design of displacement and flap-probe oligos for such a scenario is provided in Figures 7 and 8.

Figure 4. Sample design of wild-type and SNP control oligos used to test the performance of displacement and flap-probe oligos. Wild-type and SNP control oligos used to screen for a g.1488G>T substitution (nucleotides in bold) in the *RECQL4* gene are shown. In this example, the wild-type and SNP control oligo sequences (black and blue, respectively) are antisense to the coding sequence.

B. Design Flap-Probe Oligos (Design 1)

This assay requires two different flap-probe oligos, one each for detecting the SNP and wild-type sequences. The flap-probe oligos each consist of a complementary region (complementary to the target sequence that includes the interrogated base [wild type or SNP] and its adjacent 5' region) and a specific, fixed, 12-nucleotide sequence at their 5' ends (Figure 5).

The flap-probe oligo sequence is determined by the target sequence and must have specific melting temperatures (Tm) to anneal properly. These values should be calculated using an oligo design tool (e.g., https://idtdna.com/calc/analyzer) with the conditions specified in Table 1.

Table 1. Conditions for calculating Tm of the displacement and flap-probe oligos.

	Concentration
Oligo	0.25 µM
Na^+	50 mM
Mg^{2+}	7.5 mM
dNTPs	0

To design the flap-probe oligos:

1. Starting with and including the interrogated base, select a continuous sequence that is complementary to the SNP or wild-type control oligo, runs towards its 5' end, and has a calculated Tm of 60–63°C.
2. Add the following 12-nucleotide flap sequence at the 5'end of the sequence selected in the previous step:
   5'-ACG GAC GCG GAG-3' – for the SNP (green fluorescence)
   5' AGG CCA CGG ACG – for the wild type (red fluorescence).
3. Include the blocking modification hexanediol (/3C6/) at the 3' end of the oligo (Figure 5). This modification is standard in oligo synthesis. For the purpose of this kit, standard desalting is enough, and extra purification steps like PAGE or HPLC are not required.

IMPORTANT: Do not modify the fixed 12-nucleotide sequences at the 5' ends of the flap-probe oligos (specified in Step 2, above) since they are responsible for generating the fluorescent signals. Each flap-probe oligo must also include the desired base to be detected in order to establish a complete, specific pairing and to generate a positive fluorescent signal.

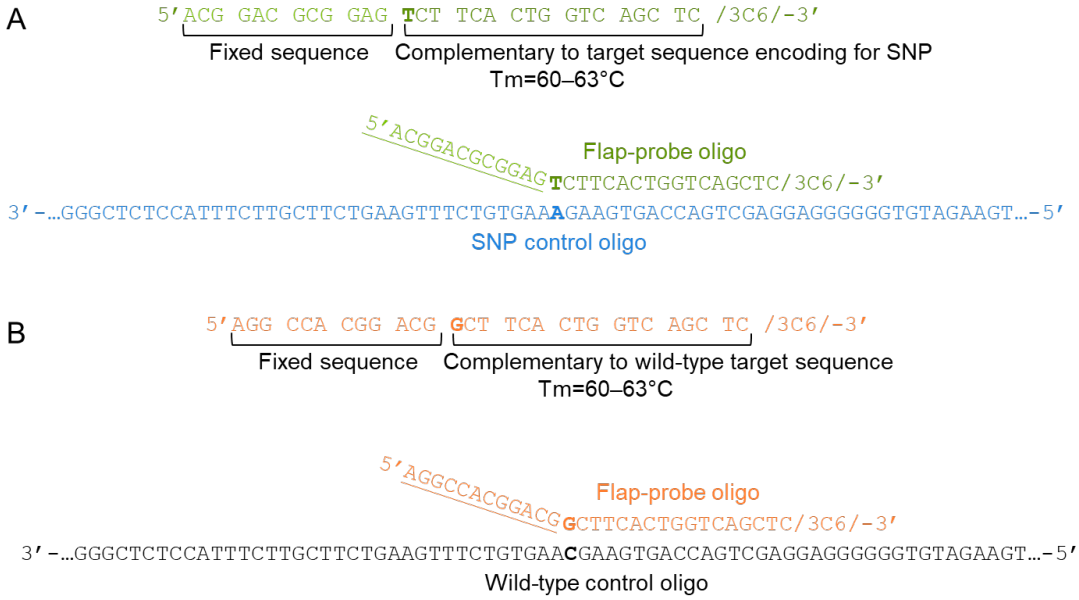


Figure 5. An example showing the design of the flap-probe oligos. **Panel A.** A flap-probe oligo designed to detect a g.1488G>T substitution in the *RECQL4* gene is shown. The flap-probe oligo (green) contains a noncomplementary, fixed 12-nt sequence at its 5' end (underlined) while its 3' end is complementary to the SNP control oligo. **Panel B.** Shows design of a flap-probe oligo for detecting the WT genomic target sequence with a fixed 12-nt sequence (underlined). The respective oligos include SNP or WT nucleotides at the position of the interrogated base (shown in bold).

C. Design Displacement Oligo (Design 1)

The displacement oligo is designed to be complementary to the portion of the target sequence that is immediately adjacent and 3' relative to the interrogated base (Figure 6). The displacement oligo also must include a noncomplementary nucleotide at its 3' end that is determined by the substitution being screened for (Table 2).

To design the displacement oligo:

1. Starting with the position immediately adjacent and 3' to the interrogated base, select a continuous sequence that is complementary to the SNP or wild-type control oligo, runs towards its 3' end, and has a calculated Tm of 70–72.5°C. **This sequence should not include the interrogated base**.
2. Add an extra nucleotide to the 3' end of the sequence generated in the previous step. This nucleotide must be specific to the substitution being screened for as specified in Table 2:

Table 2. Noncomplementary nucleotide to be added at the 3'end of the displacement oligo based on the nucleotide substitution being screened for (wild type>SNP).

Substitution	Noncomplementary nucleotide
C>G or G>C	A
T>A or A>T	C
A>G or G>A	C
A>C or C>A	G
T>C or C>T	A
G>T or T>G	C

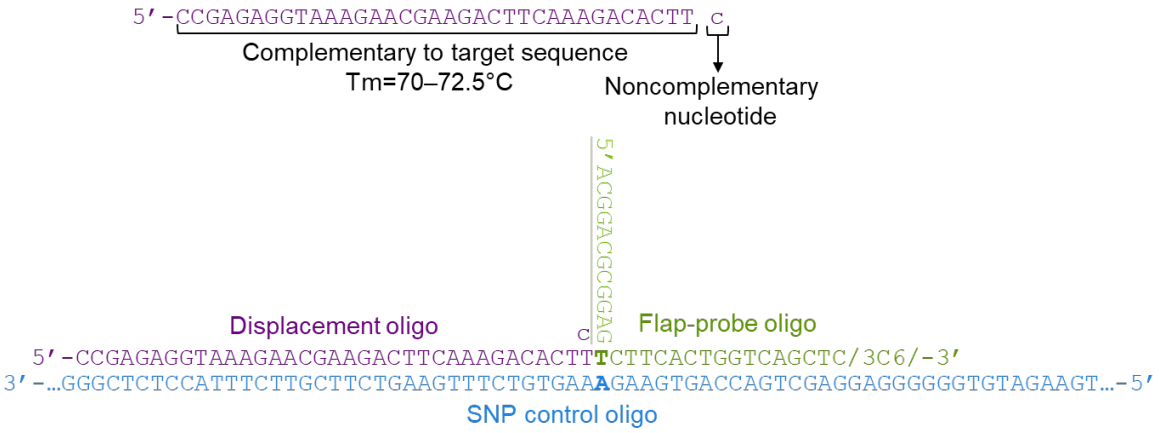


Figure 6. An example showing the design of the displacement oligo. A displacement oligo design for the detection of a g.1488G>T substitution (nucleotides in bold) in the *RECQL4* gene is shown. The displacement oligo (purple) is complementary to the SNP control oligo 3' of the SNP, with a noncomplementary and substitution-specific nucleotide at its 3' end (in lowercase). In this case, since the substitution is G>T, the noncomplementary base should be an C (as specified in Table 2). The sequence of the SNP control oligo is shown in blue and the flap-probe oligo in green.

D. Alternative Design (Design 2)

If the sequence immediately 5' to the interrogated base is either 5'-GGAGX-3', 5'-GGAGNX-3', 5'-GACGX-3' or 5'-GACGNX3' (where N is any nucleotide and X is the interrogated base; Figure 7), the control, displacement, and flap-probe oligos need to be designed taking the opposite strand of the one used in Section IV.A as reference (e.g., for a SNP detection assay involving a coding sequence, if the control oligos cannot be derived from the antisense strand due to the above condition, they should instead be derived from the sense strand [Figure 7]).

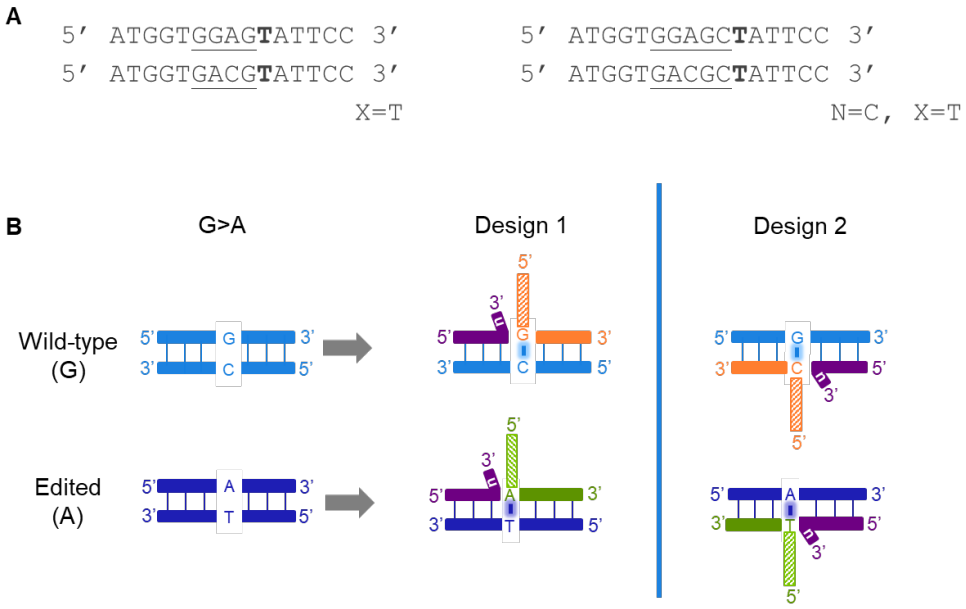


Figure 7. Alternative design (Design 2). **Panel A.** Sequences 5'of the interrogated base (underlined) that requires the use of Design 2 for generation of the displacement and flap-probe oligos, where X is the interrogated base (in bold) and N can be any nucleotide. **Panel B.** Displacement and flap-probe oligos (purple and green/orange, respectively) can be designed to detect a G>A substitution using the sequence of either DNA strand as a target sequence. In Design 1 (explained in Section IV.A.), the SNP control oligo is derived from the DNA strand depicted in the 3'→5' orientation; whereas for Design 2 (alternative design), the SNP control oligo is derived from the DNA strand in the 5'→3' orientation. For either approach, the desired base is detected when there is a complete pairing between the oligos and Guide-it Flapase recognizes the resulting double-flap structure.

Inclusion of the sequences (5'-GGAGX-3', 5'-GGAGNX-3', 5'-GACGX-3' or 5'-GACGNX3') causes the Guide-It Flap Detector to recognize the displacement oligo as the cleaved fragment and generates background fluorescence.

Regardless of which strand the target sequence is derived from, the displacement and flap-probe oligo design parameters (e.g., Tm values, inclusion of noncomplementary nucleotides, etc.) described in Sections IV.B and IV.C remain unchanged. An example demonstrating the design of displacement and flap-probe oligos for a scenario in which the complement of a candidate target sequence contains 5'-GGAGNX-3' (where N=A and X=C) is provided in Figure 8.

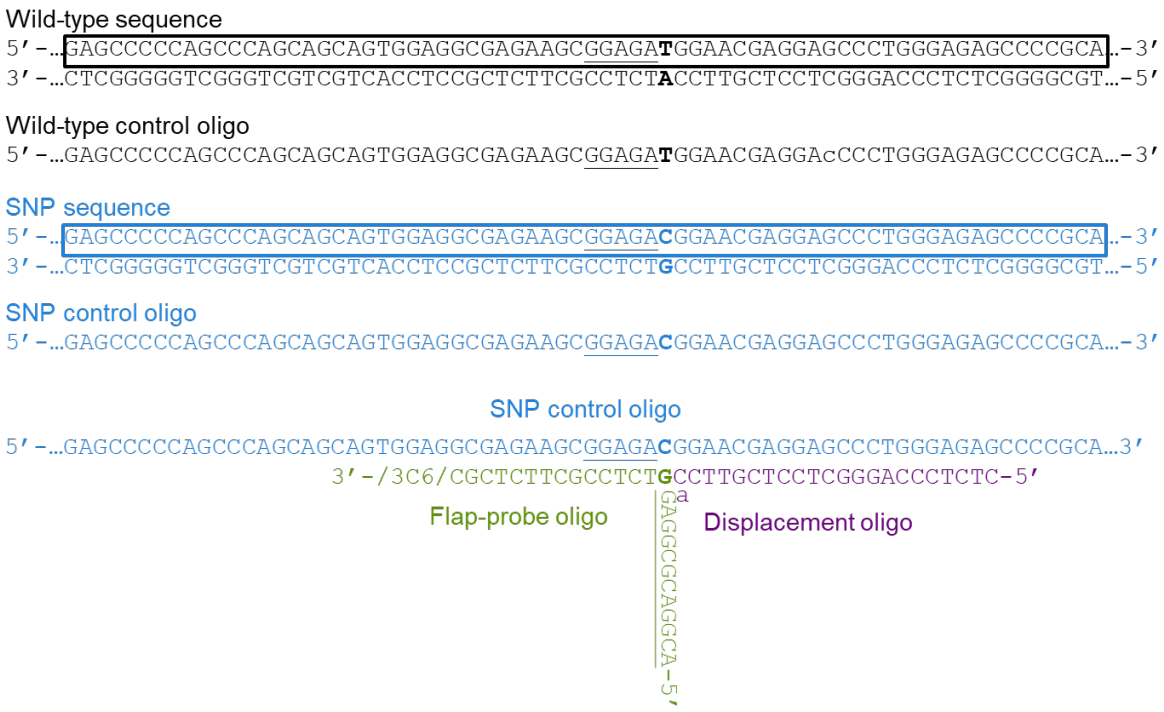


Figure 8. An example of an alternative design (Design 2) of the control, displacement, and flap-probe oligos. Control, flap-probe, and displacement oligos designed to detect the mutation g.1540T>C in the gene *RECQL4* are shown. The existence of the underlined sequence 5'-GGAGA-3' at the 5' of the interrogated base (C) requires that the wild-type and SNP control oligos (black and blue, respectively) are generated from the sense strand. The displacement oligo (purple) anneals to the region 3' relative to the interrogated base, with a noncomplementary nucleotide at its 3' end (shown in lowercase). In this case, for the T>C substitution, an A has been chosen as the noncomplementary base (Table 2). The flap-probe oligo (green, containing a noncomplementary, fixed 12-nt sequence at its 5' end, underlined) and including the SNP (in bold) anneals to the region 5' relative to the interrogated base. The same design (Design 2) should be used to generate the flap-probe oligo targeting the wild-type nucleotide.

E. Test Displacement and Flap-Probe Oligos

The designed displacement oligo and the flap-probe oligos must first be tested to verify that they are suitable for the enzymatic assay. We also advise that you use the Guide-it Knockin Control Set to confirm that the settings of your fluorescent plate reader (or qPCR machine) allow for detection of the signals generated by the Guide-it Green and Red Flap Detectors. The Guide-it Knockin Positive Control Mix mimics a heterozygous sample for the g.1298G>T substitution in the *MTHFR* gene premixed with the corresponding displacement and flap-probe oligos in Annealing Buffer. It will generate green and red fluorescent signals due to the presence of SNP and wild-type oligos, respectively. The Guide-it Knockin Negative Control Mix contains an oligo with a different base at the target site (not encoded for by either of the flap-probe oligos) and therefore will not generate any fluorescent signal.

1. Anneal Wild-Type and SNP Control Oligos with Displacement and Flap-Probe Oligos

1. Thaw the Guide-it Knockin Positive Control Mix and the Guide-it Knockin Negative Control Mix on ice. They will be used as positive and negative controls for the reaction.
2. Resuspend the wild-type and SNP control oligos (designed using the oligo design tool or as explained in Section IV.A or D) completely in RNase-free Water such that the final concentration of each oligo is 1 nM.

NOTE: We recommend using low-binding plastic tubes due to the low concentration at which the control oligos are used.

3. Prepare a heterozygous positive control solution by mixing wild-type and SNP control oligos in equal volume so the final concentration of each oligo is 0.5 nM.
4. In a 96-well PCR plate (Plate 1), pipette 10 µl of the wild-type control oligo, SNP control oligo, heterozygous positive control solution, or water (non-template control, NTC) per well. We recommend performing every reaction in triplicate.
5. Resuspend the displacement oligo completely in RNase-free Water such that the final concentration is 1 µM.
6. Resuspend each flap-probe oligo completely in RNase-free Water such that the final concentration is 20 µM.
7. Prepare the annealing master mix in a 200-µl PCR tube as follows:

Table 3. Annealing master mix preparation guidelines.

Number of wells (96-well plate)	Annealing Buffer (µl)	Flap-probe oligo A [20 µM] (µl)	Flap-probe oligo B [20 µM] (µl)	Displacement oligo [1 µM] (µl)	Rnase-free Water (µl)
12	24	6	6	12	12
N*	2.0 x N	0.5 x N	0.5 x N	1 x N	1 x N

***The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

8. Add 5 µl of the annealing master mix prepared in Step 7 to each well containing wild-type control oligo, SNP control oligo, heterozygous positive control (mix of wild-type/SNP control oligos), or water (non-template control (NTC).
9. Add 15 µl of Guide-it Knockin Positive Control Mix or Guide-it Knockin Negative Control Mix (provided with the kit) to other wells. We recommend performing every reaction in triplicate.
10. Cover the plate with optical film or cap strips.
11. Centrifuge the plate at 700*g* for 1 min.
12. Place the plate on a thermal cycler.
13. Program and run your thermal cycler with the following conditions:

95°C	5 min
Step down from	95°C to 63°C at 0.1°C/sec
63°C	10 min
63°C	Hold

2. Perform Enzymatic Reaction

1. While the oligos are annealing, prepare the enzymatic reaction master mix as detailed in Table 4. If the annealing reactions were performed in triplicates, there will be 18 total

reactions: 12 reactions for testing the design of the flap-probes and displacement oligos, and 6 reactions for confirming the plate reader (or qPCR machine) functionality using Guide-it Knockin Control Mix.

Table 4. Enzymatic reaction master mix preparation guidelines.

Number of wells (96-well plate)	Flapase Buffer (µl)	Guide-it Green Flap Detector (µl)	Guide-it Red Flap Detector (µl)	Flapase (µl)
18	54	9	9	18
N*	3.0 x N	0.5 x N	0.5 x N	1 x N

***The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

2. Mix by pipetting to ensure proper mixing.
3. In a new 96-well PCR plate (Plate 2), pipette 5 µl of the enzymatic reaction master mix into the same wells as the annealing reactions from Plate 1 (e.g., if well A1 on Plate 1 contains an annealing reaction, pipette enzymatic master mix into A1 on Plate 2).
4. Check the reaction placed in the thermal cycler in Section IV.E.1, Step 13. If the incubation has completed and is on hold at 63°C, stop the reaction. If the incubation is still in process, keep Plate 2 covered at 4°C until the program reaches the hold step.
5. Transfer the contents from each well of Plate 1 to the corresponding well of Plate 2 (e.g., transfer the contents of Plate 1, well A1 to Plate 2, well A1, and so on).
6. Cover the plate with optical film or cap strips.
7. Centrifuge the plate at 700*g* for 30 sec.
8. Place the plate on a thermal cycler and run the following program:

63°C	75 min
4°C	Hold

NOTE: If you are planning to use a qPCR machine for the fluorescence reading, and have used compatible 96-well plates, you can skip Steps 9–11 and directly read the fluorescence.

9. After the reaction is complete (on hold at 4°C), take a black, 96-well, clear-bottom plate (Plate 3) and add RNAse-free Water to each well.

NOTE: The specific volume of water will depend on your plate reader. Confirm the minimum final volume required to obtain an accurate reading with your plate reader and adjust the volume of RNase-free Water added to each well accordingly, accounting for reaction volume (20 µl) that will be added to each well from Plate 2.

10. Once the reaction in Step 8 has reached the hold step at 4°C, transfer the contents from each well of Plate 2 (96-well PCR plate) to the corresponding well of Plate 3 (black 96-well plate) (e.g., transfer the contents of Plate 2, well A1 to Plate 3, well A1, and so on).
11. Centrifuge the plate at 700*g* for 1 min to eliminate any air bubbles.
12. Proceed to read green fluorescence on a plate reader with a filter set that allows for the detection of a green dye with an excitation maximum of 485 nm and an emission maximum of 535 nm.
13. Proceed to read red fluorescence on a plate reader with a filter set that allows for the detection of a red dye with an excitation maximum of 590 nm and an emission maximum of 620 nm.

NOTE: If a plate reader has different filter sets, use wells containing Guide-it Knockin control mixes to determine which filter set combination gives the maximum ratio between the positive and negative samples.

14. Calculate the median and standard deviation for each sample for each wavelength (red and green).

    The outcome of this assay indicates whether the design of your flap-probe and displacement oligos allow the accurate and specific detection of desired bases in your target loci, as shown in Figure 9:

    - Wild-type (-/-): red fluorescence
    - Homozygous SNP (+/+): green fluorescence
    - Heterozygous SNP/wild-type (+/-): red and green fluorescence

    The ratio between the Guide-it Knockin Positive and Negative Control Mix wells should be ≥3, while only background fluorescence should be detected in the NTC wells.

NOTE: If the probes do not reach the indicated ratio, please reach out to our technical service for an alternative probe design at technical_support@takarabio.com.

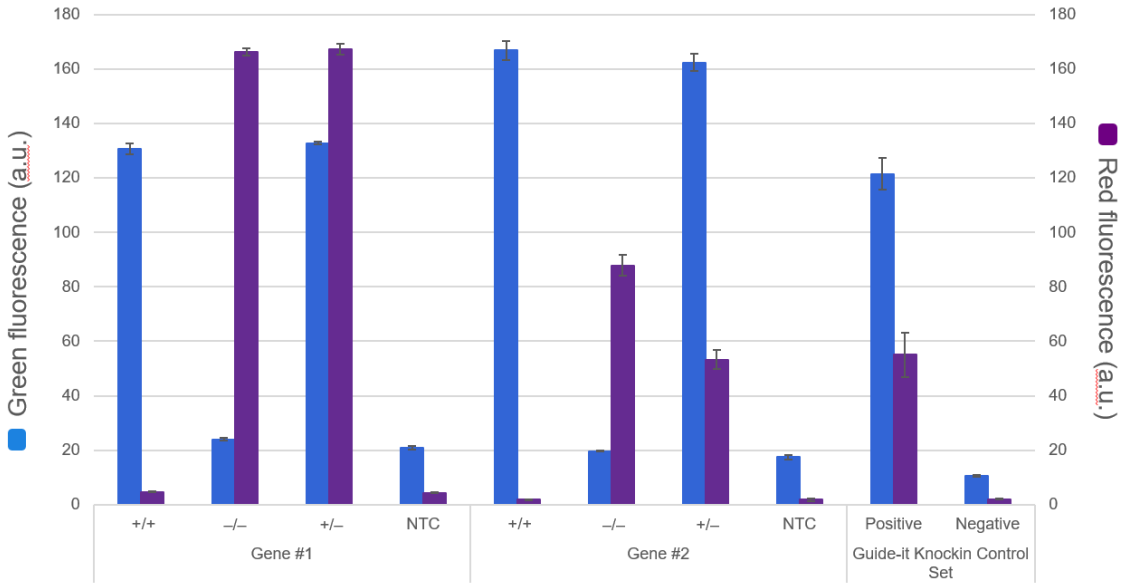


Figure 9. Results obtained from testing the functionality of displacement and flap-probe oligos designed to detect clone heterozygosity using control oligos as samples. All the flap-probe and displacement oligo combinations tested enabled accurate detection of the desired nucleotide substitutions, as indicated by the strong fluorescent signals for the positive samples (in both wavelengths, red and green) relative to the corresponding negative samples (NTC). Note that fluorescence values are arbitrary and will depend on the plate reader.

F. Protocol: Extract Genomic DNA From Your Edited Clonal Cell Lines

NOTE: If you are screening bulk populations before clonal cell expansion, **do not use** the MightyPrep Reagent for the isolation of genomic DNA. Instead, extract the genomic DNA using NucleoSpin Tissue (Cat. # 740952.250) and quantify the genomic DNA via nanodrop.

Once you have confirmed that your displacement and flap-probe oligos function properly, proceed to screen your edited clonal cell lines for the presence of the desired base at the target site.

1. Duplicate Your 96-Well Plate

If your clonal cell lines are growing in a 96-well plate, duplicate the plate so you will have a master plate as a reference to recover the successfully edited clonal cell lines later. Master plates may be frozen or maintained in the incubator.

2. Lyse Cells with MightyPrep Reagent and Extract Genomic DNA

Since the clonal cell lines in a 96-well plate can have different growth rates, the protocol has been optimized for cell densities ranging from 2 x 10^4 to 2 x 10^5 cells per well (Figure 10).

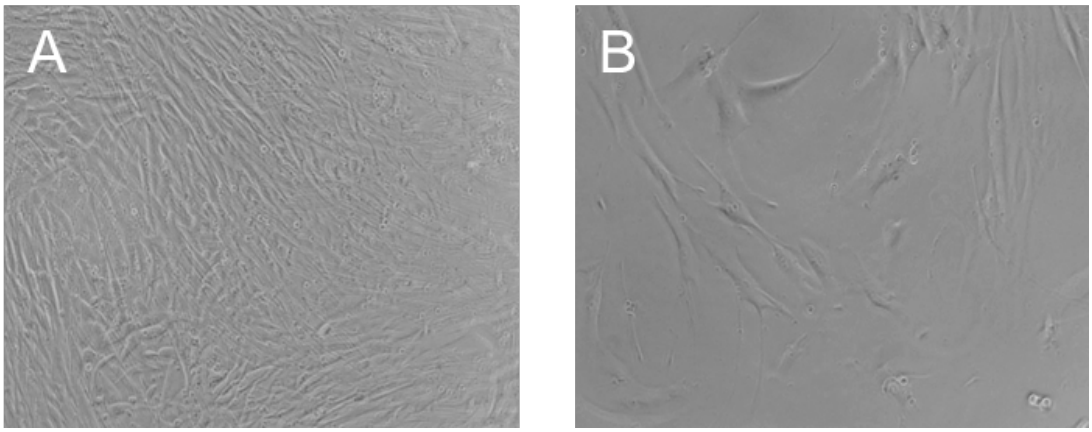


Figure 10. Optimal range of cell densities for genomic DNA extraction using MightyPrep Reagent. Primary fibroblasts were seeded at different cell densities—from 2 x 10^5 (**Panel A**) to 2 x 10^4 (**Panel B**) cells per well—and were tested successfully for the presence of the SNP (*DMP1* c.274C>G).

1. Heat the hot plate to 95°C.
2. Aspirate and discard the media from the 96-well plate.
3. Wash the cells twice with 100 µl of PBS -/-.
4. Aspirate and discard the PBS and add 50 µl of MightyPrep Reagent to each well.
5. Cover the 96-well plate with optical film or cap strips, then place on top of the hot plate for 10 min.
6. Centrifuge the plate at 1,200*g* for 10 min.

   NOTE: Cell lysis can be confirmed by microscopy at this point.
7. Carefully remove 45 µl of supernatant (containing genomic DNA) from each well of the plate and transfer it to a new 96-well plate. This plate can be stored at –20°C until further use.

G. Protocol: Amplify the Target Site by PCR

NOTE: If you are screening bulk populations before clonal cell expansion, quantify the genomic DNA isolated with NucleoSpin Tissue kit via nanodrop and use 150 ng of genomic DNA per 25 µl of PCR reaction.

1. Design Primers

Design gene-specific primers to amplify the targeted region around the interrogated base using the following guidelines:

- The optimal amplicon size should be 200–700 bp, with the targeted base located at least 100 bp from either end (5’ or 3’).
- The gene-specific primers should have a Tm ≥60°C.

- Avoid primer combinations likely to form dimers since primer dimerization can decrease the sensitivity of the assay.
- Maximize specificity of the primers using primer design tools (e.g., ncbi.gov/tools/primer-blast/).

NOTES:
– We recommend testing the primer pairs on sample genomic DNA extracted with MightyPrep Reagent or NucleoSpin Tissue using Terra Direct PCR Polymerase with the suggested PCR conditions (Section IV.G.2) to ensure correct amplification and absence of primer dimers.
– Note that the PCR reaction does not need to produce a single band (Figure 11).
– It is important to maximize the specificity of the primers using tools for primer design such as ncbi.gov/tools/primer-blast/.

2. Amplify the Target Site

NOTE: If you are screening bulk populations before clonal cell expansion, use 150 ng of genomic DNA per 25 µl of PCR reaction as sample in the PCR reaction. Modify the added volume of water accordingly to obtain a final reaction volume of 25 µl (Table 5).

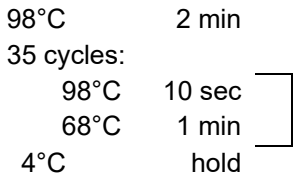
1. Prepare a master mix of the PCR reaction mixture depending on the number of samples to be analyzed as shown in Table 5 below:

Table 5. PCR master mix preparation guidelines

Components	1X	96X*
2X Reaction Buffer	12.5 µl	1200 µl
RNase-free Water	8.5 µl	816 µl
Forward Primer (10 µM)	0.75 µl	72 µl
Reverse Primer (10 µM)	0.75 µl	72 µl
Polymerase	0.5 µl	48 µl
Total Volume	23 µl	2208 µl

*Prepare 10–15% extra to account for pipetting errors.

2. Add 23 µl of the PCR master mix prepared in Step 1 to each well of a 96-well plate for PCR.
3. Take 2 µl of genomic DNA extracted in Section IV.F. from each well using a multichannel pipette and transfer to the PCR plate containing the PCR master mix. The total volume of the PCR reaction will be 25 µl.
4. Cover the PCR plate with optical film or cap-strips.
5. Centrifuge the PCR plate briefly at 700*g*.
6. Place the place on a thermal cycler and perform PCR with the following conditions:



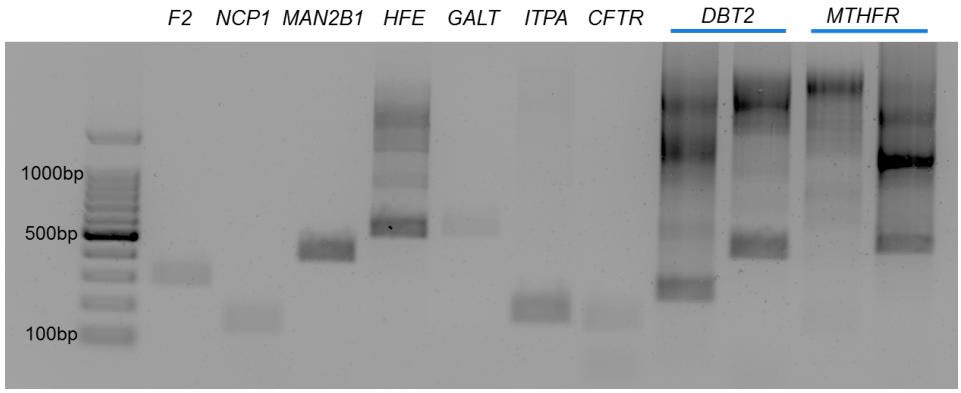


Figure 11. Examples of PCR-amplified target DNA used as samples with the Guide-it Knockin Screening Kit. Please note that the production of multiple amplicons during the PCR step does not negatively affect the enzymatic reaction. The size of the amplified target can range between 200 bp and 700 bp. For the detection of a single base substitution involving the genes *DBT2* and *MTHFR*, two different sized amplicons (ranging in size between 150 bp—400 bp) were successfully used for the enzymatic assay.

H. Protocol: Perform the Enzymatic Assay

NOTE: If you are screening bulk populations, the incubation time for the enzymatic reaction must be increased (see Section H.2 Step 8).

1. Anneal PCR Products with Displacement and Flap-Probe Oligos

1. Take the PCR products generated in Section IV.G and dilute them 1/40 with Dilution Buffer (i.e., 2 µl of each PCR reaction in 78 µl of Dilution Buffer).
2. If positive and negative controls are desired, thaw the wild-type and SNP control oligos. The concentration of each oligo should be 1 nM in water (the same concentration that was used for testing in Section IV.E). Prepare a solution in water by mixing wild-type and SNP control oligos in equal volume to 0.5 nM for each oligo. This would be the positive control.
3. In a 96-well PCR plate (Plate 1), pipette 10 µl of the mixture of wild-type and SNP control oligos (positive sample), or water (non-template control, NTC) in two separate wells.
4. To the remaining empty wells of Plate 1, pipette 10 µl of the diluted PCR product per well.
5. Prepare the annealing master mix in a 1.5-ml PCR tube as described in Table 6.

Table 6. Annealing master mix preparation guidelines

Number of wells (96-well plate)	Annealing Buffer (µl)	Flap-probe oligo SNP [20µM] (µl)	Flap-probe oligo WT [20µM] (µl)	Displacement oligo [1µM] (µl)	RNAse-free Water (µl)
96	192	48	48	96	96
N*	2 x N	0.5 x N	0.5 x N	1 x N	1 x N

*The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

6. Add 5 µl of the annealing master mix prepared in Step 5 to each sample-containing well (including the controls).
7. Cover the plate with optical film or cap-strips.
8. Centrifuge the plate at 700*g* for 1 min.

9. Place the plate on a thermal cycler and run the following program:

95°C	5 min
Step down from 95°C to 63°C at 0.1°C/sec	
63°C	10 min
63°C	hold

2. Perform Enzymatic Reaction

1. While the oligos are being annealed, prepare the enzymatic reaction master mix described in Table 7:

Table 7. Enzymatic reaction master mix preparation guidelines

Number of wells (96-well plate)	Flapase Buffer (µl)	Guide-it Green Flap Detector (µl)	Guide-it Red Flap Detector (µl)	Guide-it Flapase (µl)
96	288	48	48	96
N*	3.0 x N	0.5 x N	0.5 x N	1 x N

***The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

2. Mix by pipetting to ensure proper mixing.
3. In a new 96-well PCR plate (Plate 2), pipette 5 µl of the enzymatic reaction master mix per well. This mix should be added to the same wells to which the annealing reactions were added in Plate 1.
4. Check the reaction placed in the thermal cycler in Section IV.H.1., Step 9. If the incubation has completed and is on hold at 63°C, stop the reaction. If the incubation is still in process, keep Plate 2 covered at 4°C until the program reaches the hold step
5. Once the program has reached the hold step at 63°C, transfer the contents from each well of Plate 1 to the corresponding well of Plate 2.
6. Cover the PCR plate with optical film or cap-strips.
7. Centrifuge the plate briefly at 700*g* for 30 sec.
8. Place the plate in a thermal cycler and run the following program:

NOTE: If screening a bulk population, increase the incubation time at 63°C to 5 hours.

63°C	75 min
4°C	hold

NOTE: If you are planning to use a qPCR machine for the fluorescence reading and have used compatible 96-well plates, you can skip Steps 9–11 and directly read the fluorescence.

9. After the reaction is complete (on hold at 4°C), take a black, 96-well, clear-bottomed plate (Plate 3) and add RNase-free Water to each well.

NOTE: The specific volume will depend on your plate reader. Confirm the minimum volume required to obtain an accurate reading with your plate reader and adjust the volume of RNase-free Water added to each well accordingly, accounting for the reaction volume (20 µl) that will be added to each well from Plate 2.

10. Once the reaction in Step 8 has reached the hold step at 4°C, transfer the contents from each well of Plate 2 (96-well PCR plate) to the corresponding well of Plate 3 (black 96-well plate).
11. Centrifuge the plate at 700*g* for 1 min to eliminate any air bubbles.

12. Proceed to read green fluorescence on a plate reader with a filter set that allows for the detection of a green dye with an excitation maximum of 485 nm and an emission maximum of 535 nm.
13. Read red fluorescence in a plate reader with a filter set that allows for the detection of a red dye with an excitation maximum of 590 nm and an emission maximum of 620 nm.
14. A positive fluorescent signal corresponds to the existence of the encoded base in the flap-probe oligo in the specific clonal cell sample.
    - Wild-type: red fluorescence
    - Homozygous SNP: green fluorescence
    - Heterozygous SNP/wild-type: red and green fluorescence
15. Pick out cells from your master cell plate corresponding to those that generated a positive fluorescent signal and confirm the results using Sanger sequencing (if desired).

V. Protocol for Detecting Longer Insertions

NOTES:

- Please read this **entire section** to understand the design of the necessary oligos prior to ordering them.
- If you are screening bulk populations before clonal cell expansion, small adjustments must be made to the protocol; follow corresponding instructions provided in Sections V.E–G.

Due to the high sensitivity of the Guide-it Flapase to any change in the target sequence, it can also be used to detect seamless insertions of sequences longer than one base. In this application, the fluorescence readout is correlated to the correct and seamless insertion of the HR template at its 5' and 3' termini (green and red, respectively). Two sets of displacement and flap-oligo probes are needed, one for each junction (5' or 3') (Figures 12 and 13):

- The displacement oligos encode the wild-type sequence and two bases of the insertion at their junction and have one extra noncomplementary base at their 3' end (indicated by "n" in Figure 12).
- The flap-probe oligos are designed to encode the inserted sequence starting two bases downstream of the insertion site and have noncomplementary and specific fixed 12-nt sequences at their 5' end.

Also, wild-type and knockin control oligos must be generated for each junction in order to test the performance of both sets of probes. These control oligos encode your wild-type target sequence (wild-type control oligo) and the final edited sequence (knockin [KI] control oligo) and can be used as controls in the actual screening experiment.

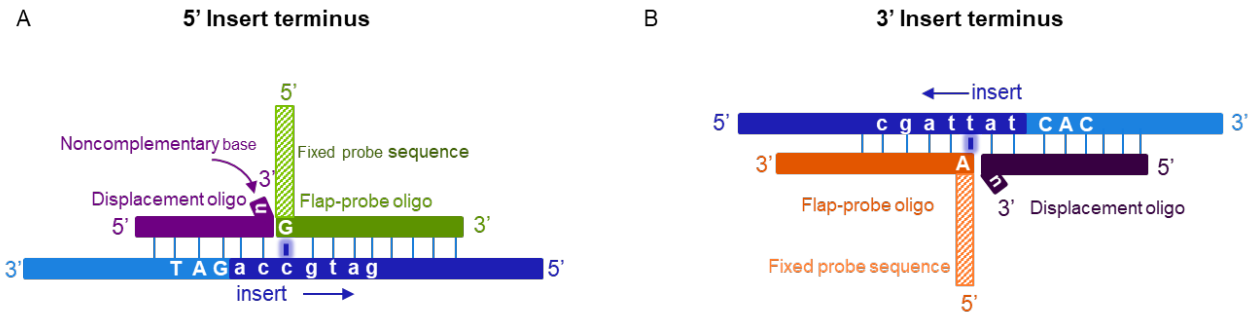


Figure 12. Design of the displacement and flap-probe oligos for the detection of an insert at its 5' or 3' terminus. The displacement oligos (in dark and light purple) hybridize with the wild-type sequence (light blue uppercase) and two bases of the insert (dark blue lowercase) while the flap-probe oligos (in green and orange) fully hybridize with the insert.

NOTE: This assay is highly sensitive to any mutation(s) in the target sequences hybridizing with the displacement and flap-probe oligos. If the PAM site is to be mutated, or there are any other changes in the original sequence near the edited site that are encoded in the HR template, the design of the probes will need to be based on the final edited sequence.

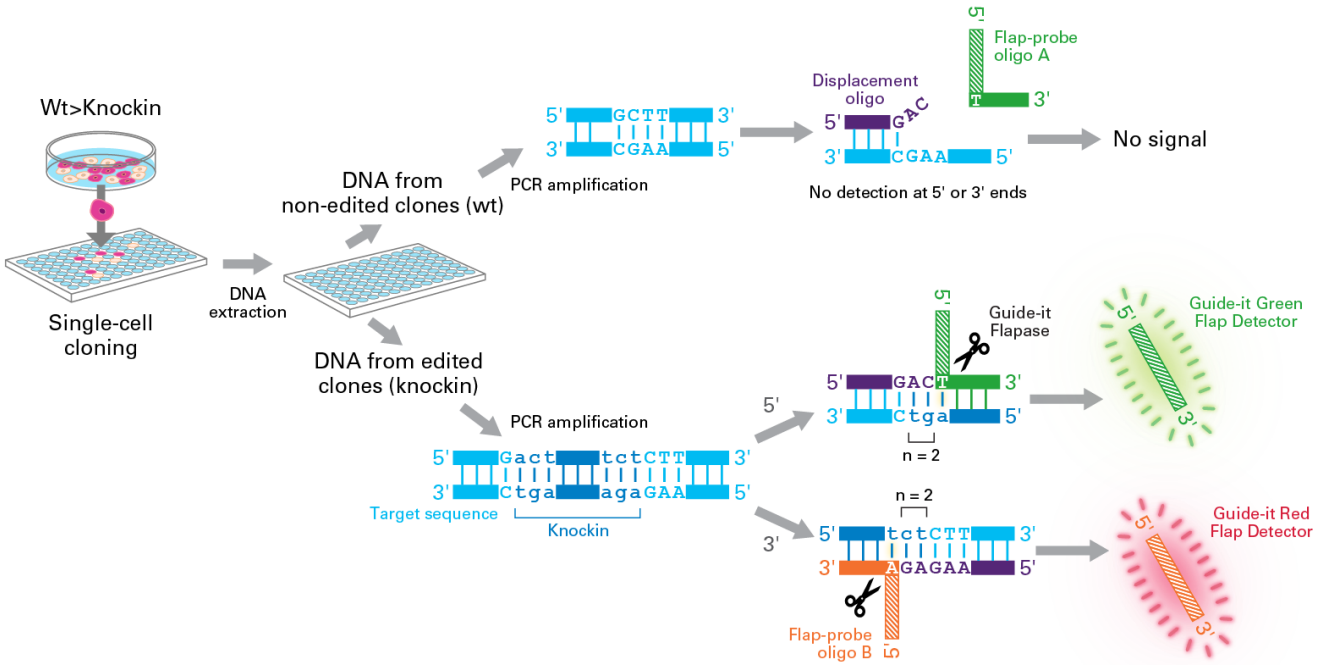


Figure 13. The Guide-it Knockin Screening Kit provides a method for detecting full-length knockin insertions. After the genome editing event, single cells are isolated via FACS or limiting dilution and expanded to clonal cell lines that may carry wild-type, indel, or successfully edited alleles. After DNA extraction from the clonal cells and subsequent PCR amplification of the target site, the PCR product is annealed with two different sets of displacement and flap probes: one that hybridizes with the 5' end of the insert (Flap-probe oligo A; green), and the other with the 3' end (Flap-probe oligo B; orange). If the full-length HR event has been successful and seamless, the full hybridization of the probes at both termini will generate both green and red fluorescent signals after the cleavage of the respective flap probes 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.

NOTE: If the sequence at the junction between the wild-type (uppercase) and insert (lowercase) is any of the following:

```
5′- GGag -3′
5′- GGAgn -3′
5′- GAcg -3′
5′- GACgn -3′
```

Where n is any nucleotide and the insert sequence indicated with lowercase, the opposite strand **must** be used to design the control, displacement, and flap-probe oligos (following Design 2, Section V.C). The inclusion of these sequences causes the Guide-It Flap Detector to recognize the displacement oligo as the cleaved fragment and generates background fluorescence.

A. Design Control, Flap-probe, and Displacement Oligos to Detect the 5' Terminus of the Insert (Design 1)

1. Design Wild-Type and KI Control Oligos (for 5' Terminus)

Design one wild-type and one KI control oligo for the 5' terminus of the insert. These control oligos will be used as samples to test the performance of the flap-probe and displacement oligos designed for the 5' insert junction. The wild-type and KI control oligos are between 60–90 nucleotides in length, with the insertion site positioned around the middle of each oligo (Figure 14). The sequence of the KI control oligo must be generated from the final sequence of your edited site.

NOTE: Since the control oligos must hybridize the full length of the flap-probe and displacement oligos, they could be longer than 90 bases depending on the length (and the GC content) of the designed oligo probes.

For editing experiments involving coding sequences in the 5' terminus, the sequence of the wild-type control oligo is typically derived from the antisense strand, but this is not a requirement. In certain instances, it may be necessary to use the sequence of the sense strand (Section V.C). When designing the probes for the 3' terminus, the other strand must be used in order to avoid steric hindrance.

Figure 14. An example showing the design of wild-type and KI control oligos for the detection of the 5' terminus of the insert. Oligos encoding for the wild-type (black) and knockin final sequence (blue) of a myc tag insertion (dark blue, lowercase) in the *UGT1A9* gene. Specifically, these oligos are used to test the performance of displacement and flap-probe oligos to detect the 5' junction of the insertion.

2. Design Flap-probe Oligo (for 5' Terminus)

As the sequences of the displacement and flap-probe oligos are determined by the final edited sequence, these oligos should be designed using the 5' KI control oligo (or the sequence of your HR template) as a reference. These oligos must have specific melting temperatures to anneal properly. These values should be calculated using an oligo design tool (www.idtdna.com/calc/analyzer) with the conditions specified in Table 1.

To design the flap-probe oligo for the 5' terminus use the 5' KI control oligo (or the sequence of your HR template) as a reference (Figure 15):

1. Starting three bases downstream of the 5' insertion site, select a continuous sequence that is complementary to the insert, runs towards its 5' end, and has a calculated Tm of 60–63°C.
2. Add the following 12-nucleotide flap sequence to the 5' end of the sequence selected in the previous step:
   5'-ACG GAC GCG GAG-3' – for the 5' terminus (green fluorescence)
3. Include the blocking modification hexanediol (/3C6/) at the 3' end of the oligo. This modification is standard in oligo synthesis. For the purpose of this kit, standard desalting is sufficient, and extra purification steps like PAGE or HPLC are not required.

IMPORTANT: Do not modify the fixed 12-nucleotide sequence at the 5' end of the flap-probe oligo (specified in Step 3, above) since it is responsible for generating the fluorescent signal.
The flap-probe oligo must also fully hybridize with the final edited sequence to generate a positive fluorescent signal.

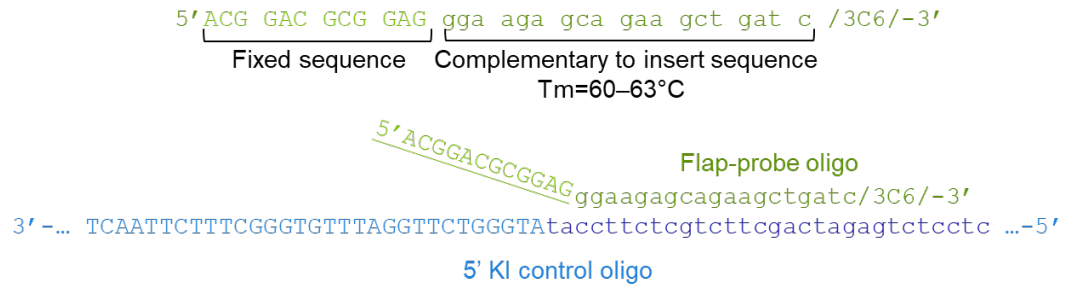


Figure 15. An example showing the design of the flap-probe oligo for the detection of the 5' terminus of the insert. A flap-probe oligo designed to detect the 5' insertion of a myc tag (lowercase) is shown. The flap-probe oligo (green) contains a noncomplementary, fixed 12-nt sequence at its 5' end (underlined uppercase) that will generate a green fluorescent signal while its 3' end is complementary to the insert.

3. Design Displacement Oligo (for 5' Terminus)

The displacement oligo is designed to be complementary to the junction sequence (Figure 16) and must include a noncomplementary nucleotide at its 3' end that breaks hybridization with the insert sequence. To design the displacement oligo:

1. Starting with the first two bases of the insert, select a continuous sequence that is complementary to the wild-type sequence, runs towards its 3' end, and has a calculated Tm of 70–72.5°C (calculated using an oligo design tool such as idtdna.com/calc/analyzer with the conditions specified in Table 1). The final displacement oligo will contain two bases of the insert followed by wild-type sequence.
2. To the 3' end of the sequence generated in the previous step, add an extra nucleotide to break the full hybridization with the insert sequence (e.g., any base that is not complementary to the third base of the insert).

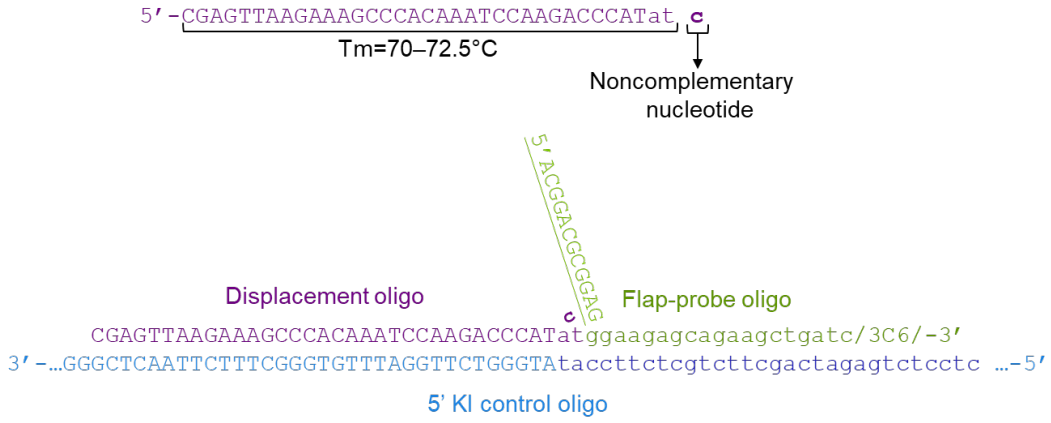


Figure 16. An example showing the design of the displacement oligo for the detection of the 5' terminus of the insert. Example of displacement oligo designed to detect the 5' terminus of a myc-tag insertion. The displacement oligo (in purple) hybridizes with the wild-type sequence and includes the first two bases of the insert, with a noncomplementary nucleotide at its 3' end (in bold). In other words, this extra nucleotide (c) does not hybridize with the corresponding base in the 5' KI control oligo (c). The sequence of the KI control oligo is shown in blue and the flap-probe oligo in green.

B. Design Control, Flap-probe, and Displacement Oligos to Detect the 3' Terminus of the Insert (Design 1)

Design wild-type and KI control oligos that encode for the 3' terminus of the insertion. They are between 60–90 nucleotides in length, with the insertion site positioned around the middle of each oligo.

NOTE: Since the control oligos must hybridize with the full length of the flap-probe and displacement oligos, they could be longer than 90 bases depending on the length (and the GC content) of the designed oligo probes.

For editing experiments involving coding sequences in the 3' terminus, the sequence of the 3' wild-type control oligo is typically derived from the sense strand (the opposite strand as the one used in the 5' terminus design). This is especially important when the insert encodes a short sequence like a myc tag to avoid steric hindrances.

As mentioned previously, the sequence of the 3' KI control oligo should be determined by the final edited sequence or in other words, the final sequence of your HDR template (Figure 17).

Figure 17. An example showing the design of wild-type and KI control oligos used to test the performance of displacement and flap-probe oligos for the detection of the 3' terminus of the insert. Oligos encoding for the wild-type (black) and knockin final sequence (blue) of a myc tag insertion (dark blue lowercase) in the *UGT1A9* gene. Specifically, these oligos are used to test the performance of displacement and flap-probe oligos to detect the 3' junction of the insertion.

1. Design Flap-probe Oligo (for 3' Terminus)

To design the flap-probe oligo, take the 3' KI control oligo (or the sequence of your HDR template) as reference (Figure 18):

1. Starting three bases upstream of the 3' insertion site, select a continuous sequence that is complementary to the insert, runs towards its 5' end, and has a calculated Tm of 60–63°C (calculated using an oligo design tool such as idtdna.com/calc/analyzer with the conditions specified in Table 1).

2. Add the following 12-nucleotide flap sequence to the 5' end of the sequence selected in the previous step:
   5'- AGG CCA CGG ACG- 3' –triggers red fluorescence.

3. Include the blocking modification hexanediol (/3C6/) at the 3' end of the oligo. This modification is standard in oligo synthesis. For the purpose of this kit, standard desalting is sufficient, and extra purification steps like PAGE or HPLC are not required.

IMPORTANT: Do not modify the fixed 12-nucleotide sequence at the 5' end of the flap-probe oligo (specified in Step 3, above) since it is responsible for generating the fluorescent signal.
The flap-probe oligo must also fully hybridize with the final edited sequence to generate a positive fluorescent signal.

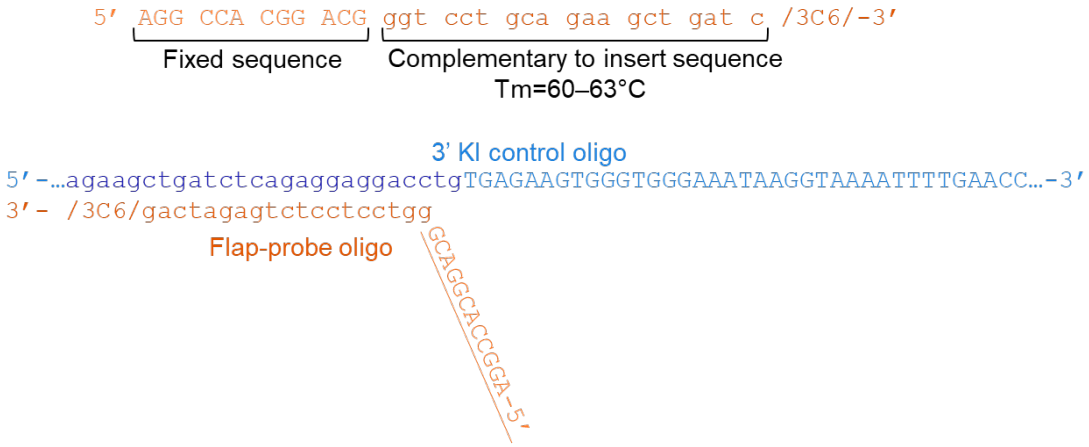


Figure 18. An example showing the design of the flap-probe oligo for the detection of the 3' terminus of the insert. A flap-probe oligo designed to detect the 3' junction of a myc-tag insertion is shown. The flap-probe oligo (orange) contains a noncomplementary, fixed 12-nt sequence at its 5' end (underlined uppercase) that will generate a red fluorescent signal while its 3' end is complementary to the insert.

2. Design the Displacement Oligo (for 3' Terminus)

The displacement oligo is designed to be complementary to the junction sequence (Figure 19) and includes a noncomplementary nucleotide at its 3' end that is determined specifically by the edited sequence. To design the displacement oligo:

1. Starting with the last two bases of the insert, select a continuous sequence with a Tm equal to 70–72.5°C (calculated using an oligo design tool such as idtdna.com/calc/analyzer with the conditions specified in Table 1) that is complementary to the wild type sequence, and runs towards its 3' end. The final displacement oligo will contain the last two bases of the insert on its 3' terminus followed by wild-type sequence.
2. To the 3' end of the sequence generated in the previous step, add an extra nucleotide to break the full hybridization with the insert sequence; in other words, add any base that it is not complementary to the third last base of the insert in its 3' terminus.

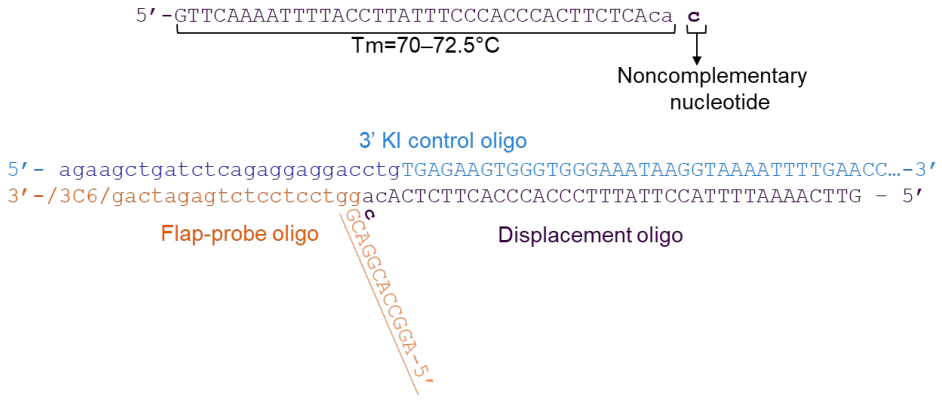


Figure 19. An example showing the design of the displacement oligo for the detection of the 3' terminus of the insert. An example of a displacement oligo designed to detect the 3' terminus of a myc-tag insert (dark blue, lowercase) is shown. The displacement oligo (in dark purple) includes the last two bases of the insert followed by a noncomplementary nucleotide at its 3' end (c in bold). The sequence of the 3' KI control oligo is shown in light blue and the flap-probe oligo in orange.

C. Alternative Probe Design for the Detection of Longer Insertions (Design 2)

The only limitation in the oligo design is the existence of the following sequences (in the sense or antisense strand) in the junction between the wild-type and the insertion at its 5' or 3' terminus (where n is any nucleotide and lowercase encode for the insert).

```
5′- GGag -3′
5′- GGAgn -3′
5′- GAcg -3′
5′- GACgn -3′
```

In other words, if the sequence in the 3' of the displacement oligo (before adding the extra noncomplimentary nucleotide at its 3' end) is 5'-GGAG-3', 5'-GGAGN-3', 5'-GACG-3', or 5'-GACGN-3' (where N is any nucleotide), the control, displacement and flap-probe oligos need to be designed taking the opposite strand as the one used in Section V.A and B as reference (Figure 20). This implies that, in Design 2, the displacement oligo will be hybridizing completely with the insert sequence and the flap-probe oligo will contain two bases of the insertion and the wild-type sequence (encoding the junction).

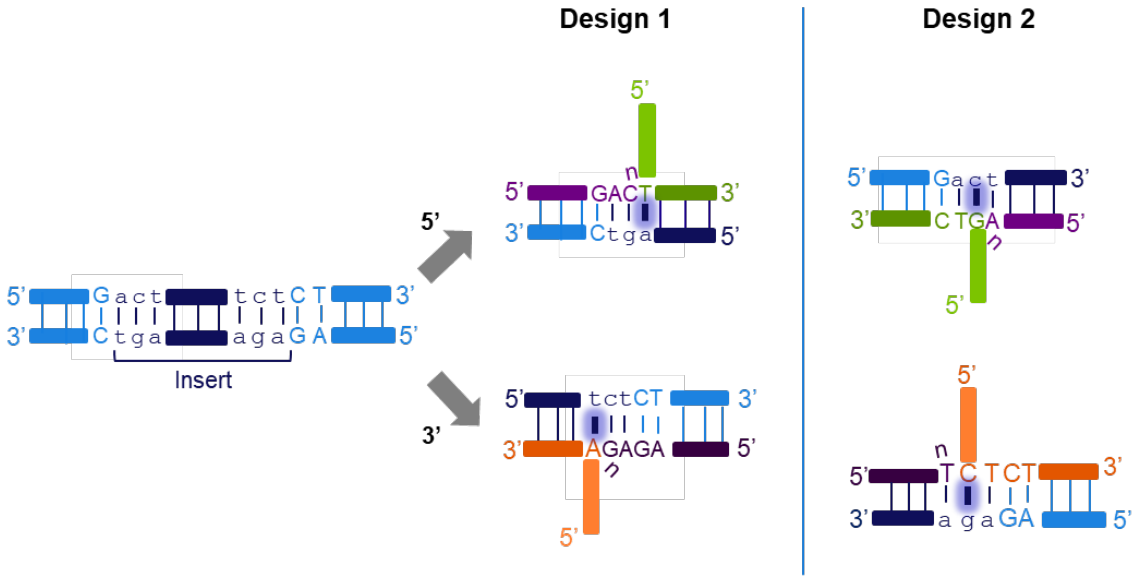


Figure 20. Alternative probe design for the detection of KI inserts (Design 2). Displacement and flap-probe oligos can be designed to detect the 5' and 3' junction of the insert using the sequence of either DNA strand as a target sequence. In Design 1 (explained in Section V.A–B), the 5' KI control oligo is derived from the DNA strand depicted in the 3'→5' orientation; whereas for Design 2, the 5' KI control oligo is derived from the DNA strand in the 5'→3' orientation.

The reason for this limitation is that inclusion of these sequences would cause the Guide-it Flap Detector (Green or Red) to recognize the displacement oligo as the cleaved flap, generating background fluorescent signal.

Regardless of the strand the oligos are derived from, the displacement and flap-probe oligo design parameters (e.g., Tm values, inclusion of noncomplementary nucleotides, etc.) described in Sections V.A and B remain unchanged. An example demonstrating the design of displacement and flap-probe oligos for a scenario in which the complement of a candidate target sequence contains either 5'-GACGN-3' (where N is an A) is provided in Figure 21.

Figure 21. An example of alternative design (Design 2) for the control, displacement, and flap-probe oligos for the 3' junction. When Design 1 (**Panel A**) is applied to create the displacement probe (dark purple) in the shown test case, the generated oligo contains the sequence -GACGA- at its 3' end (underlined). This sequence would trigger a false fluorescence signal due to its equivalence to the 3' end of the fixed sequence added at the flap-probe oligo. Therefore, Design 2 (**Panel B**) is applied to generate the control oligos and the displacement and the flap-probe oligos. In this case, the flap-probe oligo (orange) contains two bases of the insertion (lowercsae) followed by the wild-type sequence at its 5'end. The displacement oligo encodes for the insert (starting 3 bases upstream of the insertion site).

D. Test the Two Sets of Displacement and Flap-Probe Oligos

Both pairs of your designed displacement and flap-probe oligos must first be tested to verify that they are suitable for the enzymatic assay. We also advise that you use the Guide-it Knockin Control Set to confirm that the settings of your fluorescent plate reader (or qPCR machine) allow for the detection of the signal generated by the Guide-it Green and Guide-it Red Flap detectors. The Guide-it Knockin Positive Control Mix will generate both green and red fluorescence in a manner similar to a sample that has undergone successful HR. The Guide-it Knockin Negative Control mix will not generate any fluorescent signal (Section IV.E).

1. Anneal Wild-Type and KI Control Oligos with Displacement and Flap-Probe Oligos

1. Thaw the Guide-it Knockin Positive Control Mix and the Guide-it Knockin Negative Control Mix on ice. They will be used as positive and negative controls for the reaction, respectively.
2. Resuspend the 5' and 3' wild-type and 5' and 3' KI control oligos (designed in Sections V.A and B, respectively) completely in RNase-free Water such that the final concentration of each oligo is 2 nM.

NOTE: We recommend using low-binding plastic tubes due to the low concentration at which the control oligos are used.

3. Prepare a wild-type control oligo mixture by mixing 5' and 3' wild-type control oligos in equal volume so the final concentration of each oligo is 1 nM. This is your negative control.
4. Prepare a KI control oligo mixture by adding 5' and 3' KI control oligos in equal volume so the final concentration of each oligo is 1 nM. This is your positive control for a complete insertion (5'+3').

5. Dilute the 5' KI control oligo with RNAse-free Water such that the final concentration is 1 nM. This is your positive control for the 5' insertion.
6. Dilute the 3' KI control oligo with RNAse-free Water such that the final concentration is 1 nM. This is your positive control for the 3' insertion.
7. In a 96-well PCR plate (Plate 1), pipette 10 µl of the wild-type control oligo mixture (step 3), (5'+3') KI control oligo (step 4), 5' KI control oligo (step 5), 3' KI control oligo (step 6), or water (non-template control, NTC) per well. We recommend performing every reaction in triplicate.
8. Resuspend each displacement oligo completely in RNase-free Water such that the final concentration is 1 µM.
9. Resuspend each flap-probe oligo completely in RNase-free Water such that the final concentration is 20 µM.
10. Prepare the annealing master mix for N samples in a 200-µl PCR tube as follows:

Table 8. Annealing master mix preparation guidelines.

Number of wells (96-well plate)	Annealing Buffer (µl)	Flap-probe oligo 5' [20 µM] (µl)	Flap-probe oligo 3' [20 µM] (µl)	Displacement oligo 5' [1 µM] (µl)	Displacement oligo 3' [1 µM] (µl)
15	30	7.5	7.5	15	15
N*	2.0 x N	0.5 x N	0.5 x N	1 x N	1 x N

*The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

11. Add 5 µl of the annealing master mix prepared in Step 10 to each well containing wild-type oligos, (5'+3') KI oligos, 5' KI oligo, 3' KI oligo or water (non-template control (NTC).
12. Add 15 µl of Guide-it Knockin Positive Control Mix or Guide-it Knockin Negative Control Mix (provided with the kit) to other wells. We recommend performing every reaction in triplicate.
13. Cover the plate with optical film or cap-strips.
14. Centrifuge the plate at 700*g* for 1 min.
15. Place the plate on a thermal cycler.
16. Program and run your thermal cycler with the following conditions:

95°C 5 min
Step down from 95°C to 63°C at 0.1°C/sec
63°C 10 min
63°C hold

2. Perform Enzymatic Reaction

While the oligos are annealing, prepare the enzymatic reaction master mix as detailed in Table 9. If the annealing reactions in the previous section were performed in triplicates, there will be 21 total reactions: 15 reactions for testing the design of the flap-probes and displacement oligos, and 6 reactions for confirming the plate reader (or qPCR machine) functionality using Guide-it Knockin Control Mix:

Table 9. Enzymatic reaction master mix preparation guidelines.

Number of wells (96-well plate)	Flapase Buffer (µl)	Guide-it Green Flap Detector (µl)	Guide-it Red Flap Detector (µl)	Flapase (µl)
21	63	10.5	10.5	21
N*	3.0 x N	0.5 x N	0.5 x N	1 x N

***The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

1. Mix by pipetting to ensure proper mixing.
2. In a new 96-well PCR plate (Plate 2), pipette 5 µl of the enzymatic reaction master mix into the same wells as the annealing reactions from Plate 1 (e.g., if well A1 on Plate 1 contains an annealing reaction, pipette enzymatic master mix into well A1 on Plate 2).
3. Check the reaction placed in the thermal cycler in Section V.D.1., Step 16. If the incubation has completed and is on hold at 63°C, stop the reaction. If the incubation is still in process, keep Plate 2 covered at 4°C until the program reaches the hold step.
4. Transfer the contents from each well of Plate 1 to the corresponding well of Plate 2.
5. Cover the plate with optical film or cap strips.
6. Centrifuge the plate at 700*g* for 30 sec.
7. Place the plate on a thermal cycler and run the following program:

63°C	75 min
4°C	Hold

   NOTE: If you are planning to use a qPCR machine for the fluorescence reading, and have used compatible 96-well plates, you can skip steps 8–10 and directly read the fluorescence.

8. After the reaction is complete (on hold at 4°C), take a black, 96-well, clear-bottom plate (Plate 3) and add RNAse-free Water to each well.

   NOTE: The specific volume of water will depend on your plate reader. Confirm the minimum final volume required to obtain an accurate reading with your plate reader and adjust the volume of RNase-free Water added to each well accordingly, accounting for reaction volume (20 µl) that will be added to each well from Plate 2.

9. Once the reaction in Step 7 has reached the hold step at 4°C, transfer the contents from each well of Plate 2 (96-well PCR plate) to the corresponding well of Plate 3 (black 96-well plate) (e.g., transfer the contents of Plate 2, well A1 to Plate 3, well A1, and so on).
10. Centrifuge the plate at 700*g* for 1 min to eliminate any air bubbles.
11. Proceed to read green fluorescence on a plate reader with a filter set that allows for the detection of a green dye with an excitation maximum of 485 nm and an emission maximum of 535 nm.
12. Proceed to read red fluorescence on a plate reader with a filter set that allows for the detection of a red dye with an excitation maximum of 590 nm and an emission maximum of 620 nm.

    NOTE: If your plate reader has different filter sets, use wells containing Guide-it Knockin control mixes to determine which filter set combination gives the maximum ratio between the positive and negative samples.

13. Calculate the median and standard deviation for each sample for each wavelength (red and green).

The outcome of this assay will allow you to determine whether the design of your two sets of flap-probe and displacement oligos allow the accurate and specific detection of successful and seamless HR in your target loci. As shown in Figure 22, the samples should generate the following:

- Wild-type: no fluorescence
- (5'+3') KI: green and red fluorescence
- 5' KI: green fluorescence
- 3' KI: red fluorescence.

The ratio between the positive and negative samples should be ≥3, while only sample background should be detected in the NTC wells.

NOTE: If the probes do not reach the desired ratio, please reach to our technical support for an alternative probe design at technical_support@takarabio.com.

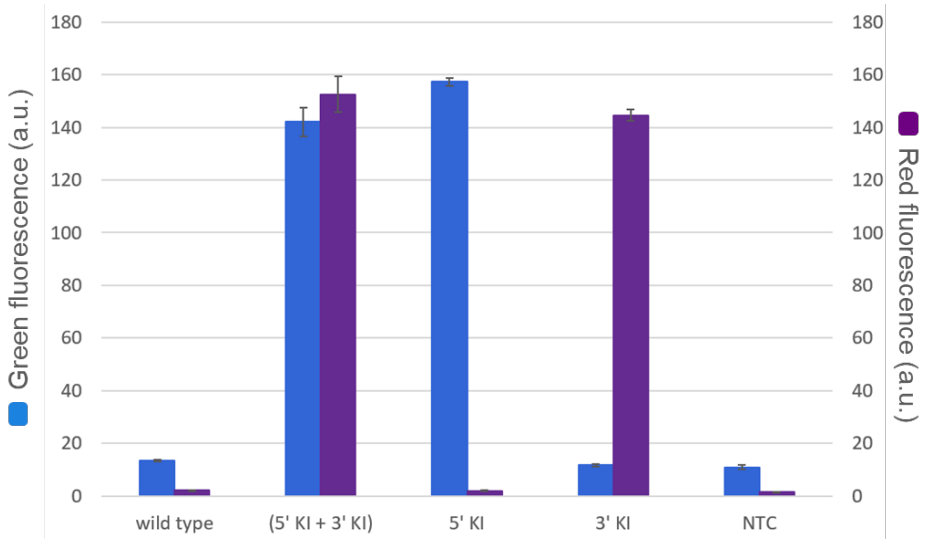


Figure 22. Testing the functionality of displacement and the flap-probe oligos designed to detect the insertion of a myc tag in the *UGT1A9* gene. All the flap-probe and displacement oligo combinations tested enabled accurate detection of the 5' and 3' junction of the insertion, as indicated by the strong fluorescent signals for the positive samples (in both wavelengths, red and green) relative to the corresponding negative samples (NTC and wild-type).

E. Protocol: Extract Genomic DNA From Your Edited Clonal Cell Lines

NOTE: If you are screening bulk populations before clonal cell expansion, **do not use** the MightyPrep Reagent for the isolation of genomic DNA. Instead, extract the genomic DNA using NucleoSpin Tissue (Cat. # 740952.250) and quantify the genomic DNA via nanodrop.

Once you have confirmed that your displacement and flap-probe oligos function properly for the detection of both junctions, proceed to screen your edited clonal cell lines for the presence of the desired insert.

1. Duplicate Your 96-Well Plate

If your clonal cell lines are growing in a 96-well plate, duplicate the plate so you will have a master plate as a reference to recover the successfully edited clonal cell lines later (you can keep the master plate frozen or maintain it in the incubator).

2. Lyse Cells with MightyPrep Reagent and Extract Genomic DNA

Since the clonal cell lines in a 96-well plate can have different growth rates, the protocol has been optimized for a wide range of cell densities: from 2 x 10^5 to 2 x 10^4 cells per well (Figure 10).

1. Turn on the hot plate to 95°C.
2. Aspirate and discard the media from the 96-well plate.
3. Wash the cells with 100 µl of PBS -/- twice.
4. Aspirate and discard the PBS and add 50 µl of MightyPrep Reagent to each well.
5. Place the 96-well plate covered with optical film or cap strips on top of the hot plate (at 95°C) for 10 min.
6. Centrifuge the plate at 1,200*g* for 10 min.

NOTE: Cell lysis can be confirmed by microscopy at this point.

7. Carefully remove 45 µl of supernatant (containing genomic DNA) from each well of the plate and transfer it to a new 96-well plate, which can be stored at –20°C until further use.

F. Protocol: Amplify the Target Site by PCR

1. Design Primers

Design gene-specific primers to amplify the edited region around the insertion using the following guidelines:

- Design primers that hybridize outside the homology arms.
- The optimal amplicon size should be 200–1,700 bp. If the total length of the insert and the homology arms is higher than 1,700 bp, two sets of primers may be used simultaneously (Figure 23).
- The specific primers should have a Tm ≥60°C.
- Avoid primer combinations likely to form dimers since primer dimerization can decrease the sensitivity of the assay.
- Maximize specificity of the primers using primer design tools (e.g., www.ncbi.nlm.nih.gov/tools/primer-blast/).

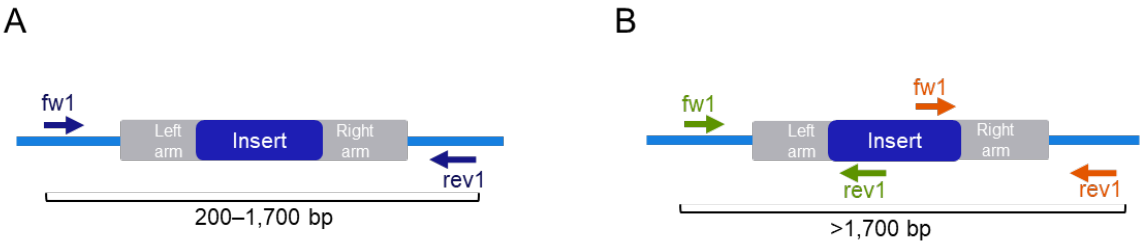


Figure 23. Scheme showing the design of primers to amplify the target site. Panel A. If the total amplicon is between 200–1,700 bp, the primer pair hybridizes outside the homology arms. **Panel B**. If the total amplicon is longer than 1,700 bp, two primer sets (green and orange) will be used: one of the primers in each pair anneals outside the region spanned by the homology arm, and the other one inside the insert.

2. Amplify the Target Site

NOTE: If you are screening bulk populations before clonal cell expansion, use 150 ng of genomic DNA (isolated using the NucleoSpin Tissue kit) as template per 25 µl of PCR reaction. Modify the added volume of water accordingly to obtain a final reaction volume of 25 µl (Table 10 or 11).

1. Prepare a master mix of the PCR reaction mixture depending on the number of samples to be analyzed as follows:

Table 10. PCR master mix preparation guidelines if one pair of primers is used (Figure 23. Panel A)

Components	1X	96X*
2X Reaction Buffer	12.5 µl	1200 µl
RNase-free Water	8.5 µl	816 µl
Forward Primer (10 µM)	0.75 µl	72 µl
Reverse Primer (10 µM)	0.75 µl	72 µl
Polymerase	0.5 µl	48 µl
Total Volume	23 µl	2208 µl

Table 11. PCR master mix preparation guidelines if two pairs of primers are used (Figure 23. Panel B)

Components	1X	96X*
2X Reaction Buffer	12.5 µl	1200 µl
RNase-free Water	7 µl	672 µl
5' Forward Primer (10 µM)	0.75 µl	72 µl
5' Reverse Primer (10 µM)	0.75 µl	72 µl
3' Forward Primer (10 µM)	0.75 µl	72 µl
3' Reverse Primer (10 µM)	0.75 µl	72 µl
Polymerase	0.5 µl	48 µl
Total Volume	23 µl	2208 µl

*The volumes provided in the two tables above are for 1 and 96 wells exactly; please prepare 10–15% extra to account for pipetting errors.

2. Add 23 µl of the PCR master mix prepared in Step 1 to each well of a 96-well plate for PCR.
3. Take 2 µl of genomic DNA extracted in Section V.E. from each well using a multichannel pipette and transfer to the PCR plate containing the PCR master mix. The total volume of the PCR reaction will be 25 µl.

NOTE: If you are screening bulk populations before clonal cell expansion, use 150 ng of genomic DNA extracted using the NucleoSpin Tissue kit per 25 µl of PCR reaction. In this case, modify the added volume of water accordingly to a final reaction volume of 25 µl.

4. Cover the PCR plate with optical film or cap-strips.
5. Centrifuge the PCR plate briefly at 700*g*.
6. Place the plate on a thermal cycler and perform PCR with the following conditions:

98°C	2 min	
35 cycles:		
98°C	10 sec	┐
68°C	1 min/kb	┘
4°C	hold	

G. Protocol: Perform the Enzymatic Assay

NOTE: If you are screening bulk populations, the incubation time for the enzymatic reaction must be increased (see Section G.2 Step 8).

1. Anneal PCR Products with Displacement and Flap-Probe Oligos

1. Take the PCR products generated in Section V.F and dilute them 1/40 with Dilution Buffer (i.e., 2 µl of each PCR reaction in 78 µl of Dilution Buffer).
2. If negative/positive controls are desired, thaw the wild-type control oligo mixture and the KI control oligo mixture prepared in Section V.D.1 Steps 3–4.
3. In a 96-well PCR plate (Plate 1), pipette 10 µl of the wild-type control oligo mixture (negative control) and KI control oligo mixture (positive control) in two separate wells.
4. In the remaining wells of Plate 1, pipette 10 µl of the diluted PCR product per well.
5. Prepare the annealing master mix in a 1.5-ml PCR tube as described in Table 12 below:

Table 12. Annealing master mix preparation guidelines

Number of wells (96-well plate)	Annealing Buffer (µl)	Flap-probe oligo 5' [20 µM] (µl)	Flap-probe oligo 3' [20 µM] (µl)	Displacement oligo 5' [1 µM] (µl)	Displacement oligo 3' [1 µM] (µl)
96	192	48	48	96	96
N*	2 x N	0.5 x N	0.5 x N	1 x N	1 x N

*The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

6. Add 5 µl of the annealing master mix prepared in Step 5 to each sample-containing well (including the controls).
7. Cover the plate with optical film or cap-strips.
8. Centrifuge the plate at 700*g* for 1 min.
9. Place the plate on a thermal cycler and run the following program:

95°C 5 min
Step down from 95°C to 63°C at 0.1°C/sec
63°C 10 min
63°C hold

2. Perform Enzymatic Reaction

1. While the oligos are being annealed, prepare the enzymatic reaction master mix described in Table 13:

Table 13. Enzymatic reaction master mix preparation guidelines

Number of wells (96-well plate)	Flapase Buffer (µl)	Guide-it Green Flap Detector (µl)	Guide-it Red Flap Detector (µl)	Guide-it Flapase (µl)
96	288	48	48	96
N	3.0 x N	0.5 x N	0.5 x N	1 x N

*The volumes provided are for N wells exactly; please prepare 10–15% extra to account for pipetting errors.

2. Mix by pipetting to ensure proper mixing.

3. In a new 96-well PCR plate (Plate 2), pipette 5 µl of the enzymatic reaction master mix per well. This mix should be added to the same wells to which the annealing reactions were added in Plate 1.
4. Check the reaction placed in the thermal cycler in Section V.G.1, Step 9. If the incubation has completed and the cycler is on hold at 63°C, stop the reaction. If the incubation is still in process, keep Plate 2 covered at 4°C until the program reaches the hold step.
5. Once the program has reached the hold step at 63°C, transfer the contents from each well of Plate 1 to the corresponding well of Plate 2.
6. Cover the PCR plate with optical film or cap-strips.
7. Centrifuge the plate briefly at 700*g* for 30 sec.
8. Place the plate in a thermal cycler and run the following program:

NOTE: If screening bulk populations, increase incubation time at 63°C to 5 hours.

63°C	75 min
4°C	hold

NOTE: If you are planning to use a qPCR machine for the fluorescence reading and have used compatible 96-well plates, you can skip Steps 9–11 and directly read the fluorescence Step 12.

9. After the reaction is complete (on hold at 4°C), take a black, 96-well, clear-bottomed plate (Plate 3) and add RNase-free Water to each well.

NOTE: The specific volume of water will depend on your plate reader. Confirm the minimum final volume required to obtain an accurate reading with your plate reader and adjust the volume of RNase-free Water added to each well accordingly, accounting for the reaction volume (20 µl) that will be added to each well from Plate 2.

10. Once the reaction in Step 8 has reached the hold step at 4°C, transfer the contents from each well of Plate 2 (96-well PCR plate) to the corresponding well of Plate 3 (black 96-well plate).
11. Centrifuge the plate at 700*g* for 1 min to eliminate any air bubbles.
12. Proceed to read green fluorescence on a plate reader with a filter set that allows for the detection of a green dye with an excitation maximum of 485 nm and an emission maximum of 535 nm.
13. Proceed to read red fluorescence in a plate reader with a filter set that allows for the detection of a red dye with an excitation maximum of 590 nm and an emission maximum of 620 nm.
14. A positive red and green fluorescent signal correspond to the existence of the complete knockin in the clonal cell sample.
    - Wild-type/indels: no fluorescence
    - Complete KI: green and red fluorescence
    - KI with a truncation in the 3’ terminus: green fluorescence
    - KI with a truncation in the 5’ terminus: red fluorescence
15. Pick out cells from your master cell plate corresponding to those that generated a positive fluorescent signal and confirm the results using Sanger sequencing (if desired).

VI. References

Paquet, D., Kwart, D., Chen, A., Sproul, A., Jacob, S., Teo, S., … Tessier-Lavigne, M. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature*, *533*(7601), 125–129 (2016).

Appendix A. Troubleshooting Guide

Table 14. Troubleshooting guide for the Guide-it Knockin Screening Kit.

Problem	Possible Explanation	Solution
Displacement oligo and flap-probe oligo design		
No fluorescence detected	Control Oligos wrongly diluted in Dilution Buffer	• Control Oligos should be diluted in RNase-free Water and not in Dilution Buffer
	Configuration of the plate reader or qPCR machine	• Use Guide-it Knockin Control Set to check for the proper filter set in the plate reader or qPCR thermal cycler
	Not enough volume in the well for a correct readout	• Check the minimum volume required for the plate reader to get an accurate measurement or, if using qPCR, whether the Eppendorf tubes/plates used are compatible with the qPCR machine
	Error in the oligo design	• Check and correct any errors in the oligo design (if designed manually) • Check if the 12-nt fixed sequence is present at the 5' end of the flap-probe oligo and its sequence is correct • Addition of the noncomplementary extra nucleotide at the 3' end of the displacement oligo
Fluorescence values of the designed oligos are much lower than the Guide-it Control Mix **or** the ratio of positive vs negative signal is <3	Need to optimize design	• Increase the Tm of the flap-probe oligo (you can use an RT-PCR machine to detect the kinetics of the reaction) • Use the opposite strand than the one you originally used (e.g., sense or anti-sense) to design oligos
Negative control has the same fluorescence as positive control and NTC (e.g., only background signal is detected)	Oligos diluted in Dilution Buffer	• Control Oligos should be diluted in RNase-free Water and not in Dilution Buffer
PCR amplification of the target DNA		
No amplification	Oligo design is not optimal	• Redesign the primers using suggested online tools
Failed enzymatic assay		
No fluorescent signal in the positive SNP/KI control oligo	Oligos diluted in Dilution Buffer	• Oligos should be diluted in RNase-free water
	Problems with the plate reader	• Check that your plate reader or qPCR thermal cycler is functioning properly
	Not enough volume in the well for a correct readout	• Check the minimum volume required for the plate reader to get an accurate measurement or, if using a qPCR machine, whether the Eppendorf tubes/plates used are compatible with the qPCR machine

Appendix B. Glossary of Terms

Table 15. Glossary of terms

Interrogated base/sequence	Nucleotide base or sequence that is being screened for (can be wild-type or edited)
Wild-type control oligo	User-generated oligo encoding the wild-type target sequence
SNP control oligo	User-generated oligo encoding the target sequence with the desired nucleotide substitution
KI control oligo	User-generated oligo encoding the target sequence with the desired edited sequence (if a long insert is being detected)
Displacement oligo	User-generated oligo that anneals to the target sequence and forms a tripartite structure that is the substrate for the Guide-it Flapase
Flap-probe oligo	User-generated oligo that anneals fully to target sequence and contains a fixed 12-nt sequence at its 3' end that forms a flap structure
Guide-it Flapase	A structure-specific endonuclease that cleaves and releases the fixed flap sequence of the flap-probe oligo from double-flap structures. It does not recognize gapped structures
Guide-it Green/Red Flap Detector	Recognizes the fixed, 12-nt sequence of the flap-probe oligo once it is cleaved by the Guide-it Flapase and generates a fluorescent signal (green or red)

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fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
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