Takara Bio USA, Inc.

Guide-it™ SNP Screening Kit User Manual

Cat. Nos. 632652, 632653
(091718)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: techUS@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

I. Introduction 4
A. Summary 4
B. Protocol Overview 6
II. List of Components 7
III. Additional Materials Required 7
IV. Oligos Required for the Protocol 8
V. Protocol: Design and Test Displacement Oligo and Flap-Probe Oligo 8
A. Design Wild-type and SNP Control Oligos, Displacement Oligo, and Flap-Probe Oligo 8
B. Test Displacement Oligo and Flap-Probe Oligo 13
VI. Protocol: Extract Genomic DNA From Your Edited Clonal Cell Lines 16
A. Duplicate Your 96-Well Plate 16
B. Lyse Cells with MightyPrep Reagent and Extract Genomic DNA 16
VII. Protocol: Amplify the Target Site by PCR 17
A. Design Primers 17
B. Amplify the Target Site 18
VIII. Protocol: Perform the Enzymatic Assay 19
A. Anneal PCR Products with Displacement and Flap-Probe Oligos 19
B. Perform Enzymatic Reaction 19
Appendix A. Troubleshooting Guide 21
Appendix B. Glossary of Terms 22

Table of Figures

Figure 1. Tripartite structures formed by the annealing of PCR product and user generated displacement and flap-probe oligos. 4
Figure 2. Schematic of the Guide-it SNP Screening Kit workflow. 5
Figure 3. Workflow of the Guide-it SNP Screening Kit. 6
Figure 4. An example showing the design of wild-type and SNP control oligos used to test the performance of displacement and flap-probe oligos. 9
Figure 5. An example showing the design of the flap-probe oligo. 10
Figure 6. An example showing the design of the displacement oligo 11
Figure 7. Alternative displacement and flap-probe oligo designs. 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 the flap-probe oligos designed to detect SNPs at various genomic loci 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 SNP Screening Kit. 18

Table of Tables

Table 1. Oligos needed to perform SNP screening using this protocol 8
Table 2. Conditions to be applied in the Tm calculation of the displacement oligo and flap-probe oligo. 10
Table 3. 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 4. Annealing master mix preparation guidelines..... 14
Table 5. Enzymatic reaction master mix preparation guidelines. 14
Table 6. PCR master mix preparation guidelines..... 18
Table 7. Annealing master mix preparation guidelines..... 19
Table 8. Enzymatic reaction master mix preparation guidelines 19
Table 9. Troubleshooting Guide for the Guide-it SNP Screening Kit. 21
Table 10. Glossary of terms 22

I. Introduction

A. Summary

One of the most powerful applications of genome editing is the introduction of nucleotide substitutions at specific genomic sites to mimic single nucleotide polymorphisms (SNPs) related to human diseases or to generate stop codons that yield precise gene knockouts. However, screening thousands of clones for a single edited nucleotide remains a challenge, especially in the absence of a corresponding phenotype. The Guide-it SNP Screening Kit provides the ability to quickly identify edited clones from 96-well plates, employing a simple and rapid workflow that comprises PCR amplification of the genomic target site followed by an enzymatic assay using Guide-it Flapase (structure-specific endonuclease) and Guide-it Flap Detector that generates a fluorescent readout.

The kit protocol involves PCR amplification of the genomic region surrounding the site of the desired nucleotide substitution (SNP) 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):

- The displacement oligo hybridizes 3' relative to the interrogated base and has an extra noncomplementary base at its 3' end (indicated by the "n" in Figure 1).
- The flap-probe oligo is designed to encode the SNP and hybridizes to the target sequence 5' relative to the interrogated base and has a noncomplementary and specific fixed sequence at its 5' end.

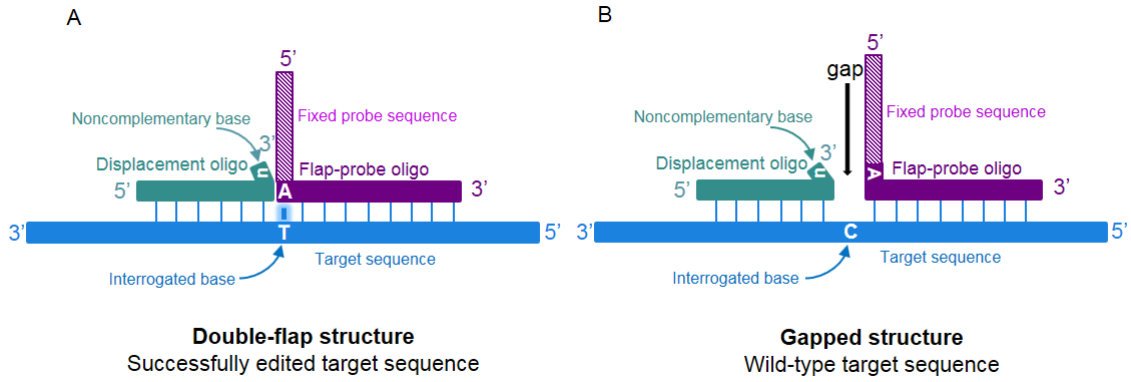


Figure 1. Tripartite structures formed by the annealing of the genomic target site and user-generated displacement and flap-probe oligos. Annealing of the displacement oligo (green) and the flap-probe oligo (purple) with the PCR product (blue) containing the genomic target sequence is shown. **Panel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

Based on their design, hybridization of the displacement and flap-probe oligos with the PCR product yields one of two different tripartite structures depending on whether the desired nucleotide substitution has occurred (Figure 1 and 2):

- If the editing is successful, a double-flap structure is formed where the edited nucleotide is paired with the complementary nucleotide included in the flap-probe oligo. 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 a fluorescence signal that can be measured using a plate reader.

– If the editing is not successful, the wild-type nucleotide is not complementary to the corresponding nucleotide in the flap-probe oligo and remains unpaired generating a gap in the structure. In this case, Guide-it Flapase does not cleave the flap, and no fluorescent signal is generated.

In this manner, detection of a positive fluorescent signal in this assay indicates successful introduction of the desired SNP. This correlation between fluorescence and the presence of a specific nucleotide in a defined position can be used to screen hundreds of clones for successful outcomes in single-nucleotide substitution experiments involving genome-editing tools such as the CRISPR/Cas9 system.

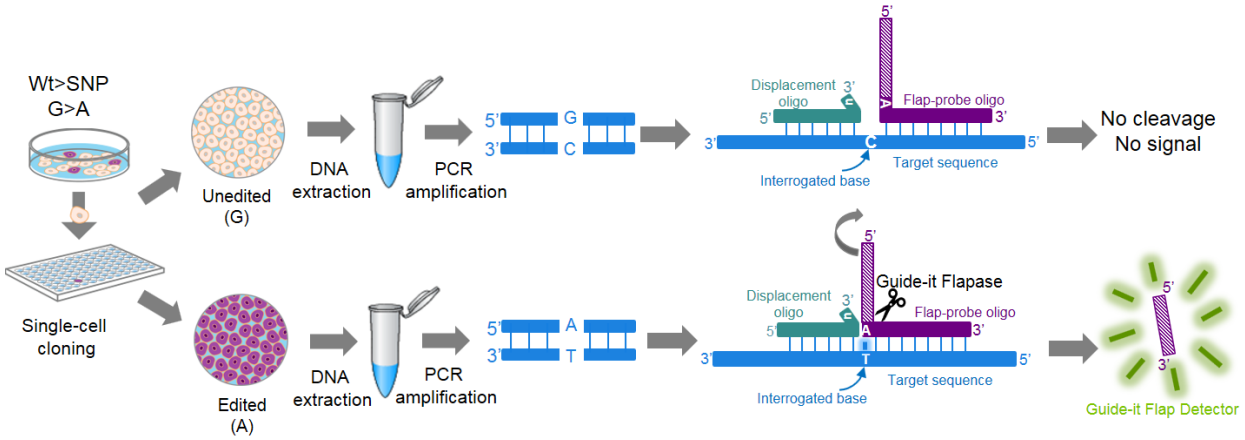
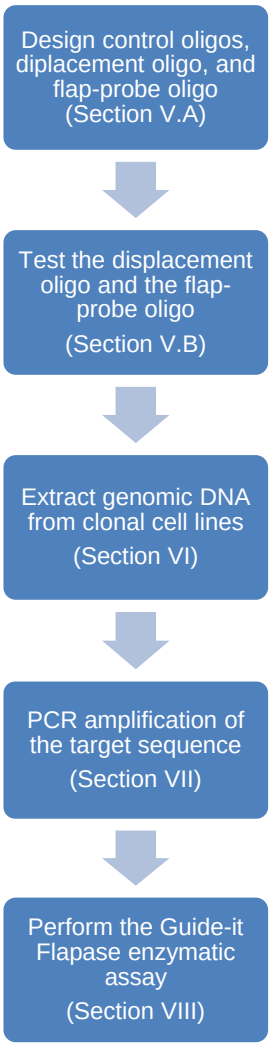


Figure 2. Schematic of the Guide-it SNP Screening Kit workflow. This example workflow depicts the detection of a G>A substitution (editing of a wild-type guanine to adenine). Following genome editing, single cells are isolated via FACS or limiting dilution and expanded to clonal cell lines that carry either wild-type (G, top) or successfully edited nucleotides (A, bottom) 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 two different complementary oligos—a displacement oligo (green) and a flap-probe oligo (dark/light purple) that has a fixed, noncomplementary sequence (light purple) that forms a flap. Hybridization of the oligos with the PCR product yields one of two structures depending on the outcome of the editing. When editing has occurred successfully, the flap-probe oligo forms a complete base pairing at the target site yielding a double-flap structure (bottom). Guide-it Flapase is a specific nuclease that only cleaves and releases the flap from the double-flap structure which is detected by the Guide-it Flap Detector generating a fluorescent signal. When editing has not occurred, the flap-probe oligo does not form a complete pairing at the target site generating a gapped structure (top) and cannot be cleaved by Guide-it Flapase. In this manner, detection of a fluorescent signal using the Guide-it SNP Screening Kit is indicative of whether the desired nucleotide substitution has occurred.

B. Protocol Overview



Design control oligos (~90 bp in length) based on the genomic sequence encoding the wild-type and SNP sequence. Design a displacement oligo and a flap-probe oligo following the guidelines provided in this protocol. See Table 1 for a complete list of oligos and primers required for the protocol.

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

Once displacement and flap-probe oligos have been successfully tested, extract genomic DNA from clonal cell lines using MightyPrep Reagent.

Amplify the target sequence by Terra™ PCR Direct Polymerase.

Anneal the PCR products with the displacement oligo and flap-probe oligo and perform the enzymatic assay with Guide-it Flapase. After the reaction is completed, measure fluorescence on a standard fluorescence plate reader.

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

II. List of Components

The Guide-it SNP Screening Kit consists of the Guide-it Flap Reagents (not sold separately), the Guide-it 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 a 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 SNP Screening Kit	**632652 (100 rxns)**	**632653 (400 rxns)**
MightyPrep Reagent for DNA (Store at 4°C)	**9182A**	**9182**
MightyPrep Reagent for DNA	5 ml	20 ml
Guide-it SNP Control Set (Store at –20°C)	**632654**	**632654**
Guide-it SNP Positive Control Mix	300 µl	300 µl
Guide-it SNP Negative Control Mix	300 µl	300 µl
Guide-it Flap Reagents (Store at –20°C)	**632655 (100 rxns)**	**632656 (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	10 ml	20 ml x 2
Annealing Buffer	350 µl	1.4 ml
Flapase Buffer	350 µl	1.4 ml
Guide-it Flapase	100 µl	400 µl
Guide-it Flap Detector (40X)	50 µl	200 µl

III. Additional Materials Required

The following materials are required but not supplied:

- 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)
- Plate reader that allows detection of green fluorescence with 485 nm excitation/535 nm emission
- 96-well black plate with clear bottom (e.g.; Corning, Cat. No. 3631)

IV. Oligos Required for the Protocol

For the detection of a particular SNP, a set of user-generated oligos are needed that are specific to the genomic target sequence and the SNP.

Table 1. Oligos needed to perform SNP screening using this protocol

Control oligos (Section V.A.1 or V.A.4)	
Wild-type control oligo	Derived from the wild-type sequence and contains the interrogated base in the middle (~85–90 bp)
SNP control oligo	Derived from the wild-type sequence and contains the SNP in the middle (~85–90 bp)
Oligos for the enzymatic reaction (Section V.A.2 and V.A.3)	
Flap-probe oligo	Derived from the SNP control oligo with a noncomplementary, fixed 12-nt sequence at its 5' end
Displacement oligo	Derived from the SNP control oligo and anneals to the target sequence immediately 3' to the SNP
PCR amplification of the target site (Section VII.A)	
PCR primers	Forward and reverse primers for amplifying ~200–700 bp of genomic target sequence surrounding the interrogated base

V. Protocol: Design and Test Displacement Oligo and Flap-Probe Oligo

A. Design Wild-type and SNP Control Oligos, Displacement Oligo, and Flap-Probe Oligo

First, generate wild-type and SNP control oligos that correspond to the genomic target sequence and act as the negative and positive controls for the enzymatic assay. Then, using the SNP control oligo as a template, design and test the displacement and flap-probe oligos that are specific and complementary to the SNP control oligo. Please read the entire section to understand the design of the oligos before ordering them.

NOTES:

– If the target sequence immediately 5' relative to the SNP is the following: 5'-GGAGX-3' or 5'-GGAGNX-3' (where N is any nucleotide and X is the SNP, as shown below) please refer to Section V.A.4.

```
5′   ATGGTGGAGTATTCC  3′              5′   ATGGTGGAGCTATTCC  3′
3′   TACCACCTCATAAGG  5′              3′   TACCACCTCGATAAGG  5′
                        X=T                                  N=C, X=T
```

– If the target sequence immediately 3' relative to the interrogated base has repeats, this may affect the hybridization of the flap-probe oligo. Please follow instructions for alternative design (Section V.A.4).

1. Generate wild-type and SNP control oligos based on the target sequence

Design the wild-type and SNP control oligos that will be used as control samples to test the performance of the flap-probe and displacement oligos. The wild-type and SNP control oligos encode the target sequence including either the wild-type or the SNP and can also be used as negative and positive controls, respectively, in the actual screening experiments.

The wild-type and SNP control oligos must be between 85–90 nucleotides in length, with the interrogated base positioned around the middle of each oligo. For editing experiments involving coding sequences, the sequence of the wild-type control oligo is typically derived from the antisense strand (i.e., the wild-type control oligo is the reverse-complement of the sense strand), but there is no actual requirement for this, and in certain instances it may be necessary to use the sequence of the sense strand (Section V.A.4). The sequence of the SNP control oligo should be the same as the wild-type, except that it includes the desired SNP at the target site (Figure 4).

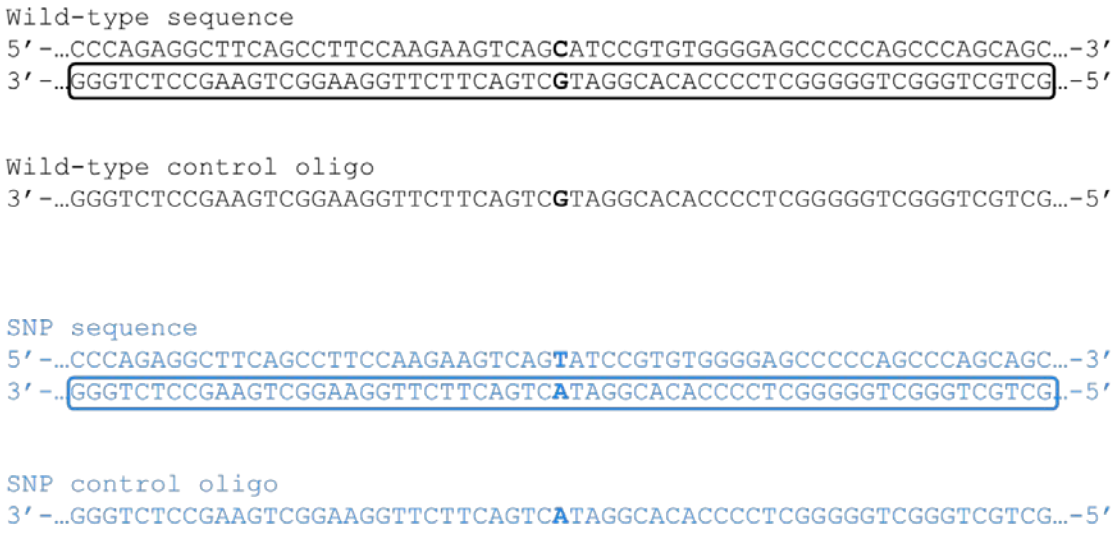


Figure 4. An example showing the design of wild-type and SNP control oligos used to test the performance of displacement and flap-probe oligos. Wild-type and SNP control oligos used to screen for g.1488C>T substitution (nucleotides in bold) in the *REQL4* gene are shown. In this example, the wild-type and SNP control oligo sequences (black and blue, respectively) are the reverse-complement strands of the coding sequence.

2. Design flap-probe oligo

The sequences of the displacement and flap-probe oligos are determined by the target sequence, and to anneal successfully they need to have specific melting-temperature (Tm) values that should be calculated using an oligo design tool (idtdna.com/calc/analyzer) with the conditions specified in Table 2.

Table 2. Conditions to be applied in the Tm calculation of the displacement oligo and flap-probe oligo.

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

As described in Section I.A, the flap-probe oligo is complementary to the target sequence that includes the interrogated base and its adjacent 5' region and has a specific and fixed 12-nucleotide sequence at its 5' end (Figure 5). To design the flap-probe oligo:

1. Starting with and including the interrogated base, select a continuous sequence with a Tm equal to 60–63°C (calculated using an oligo design tool such as idtdna.com/calc/analyzer with the conditions specified in Table 2) that is complementary to the SNP control oligo and runs towards its 5' end.
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'
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 sufficient, and extra purification steps like PAGE or HPLC are not required.

NOTES:

– Do not modify the fixed 12-nucleotide sequence at the 5' end of the flap-probe oligo (specified in Step 2, above) since it is responsible for generating the fluorescent signal.

– The flap-probe oligo must include the interrogated base to establish a complete and specific pairing with the SNP control oligo or SNP-containing PCR product to generate a positive fluorescent signal.

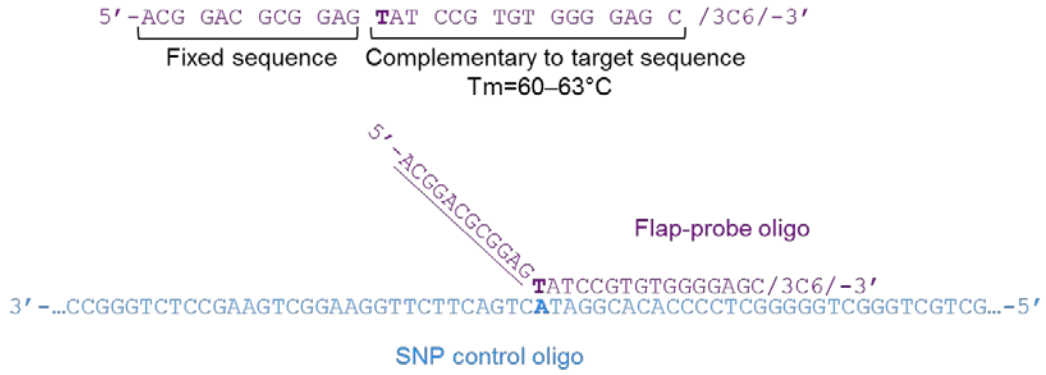


Figure 5. An example showing the design of the flap-probe oligo. A flap-probe oligo designed to screen for a g.1488C>T substitution in the *REQCL4* gene is shown. The flap-probe oligo (purple) contains a noncomplementary, fixed 12-nt sequence at its 5' end (underlined) while its 3' end is complementary to the SNP control oligo (or the genomic target sequence) and includes the edited base (T in bold). The SNP control oligo sequence is displayed in blue.

3. Design displacement oligo

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) and includes a noncomplementary nucleotide at its 3' end that is determined specifically by the substitution being screened for (Table 3). To design the displacement oligo:

1. Starting with the position in the SNP control oligo that is immediately 3' relative to the interrogated base, 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 2) that is complementary to the SNP control oligo, and runs towards its 3' end. **It should not include the interrogated base**.
2. To the 3' end of the sequence generated in the previous step, add an extra nucleotide that is specific to the substitution being screened for, as specified in Table 3:

Table 3. 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	T
G>C	A
T>A or A>T	C
A>G	T
G>A	C
A>C or C>A	G
T>C or C>T	A
G>T or T>G	C

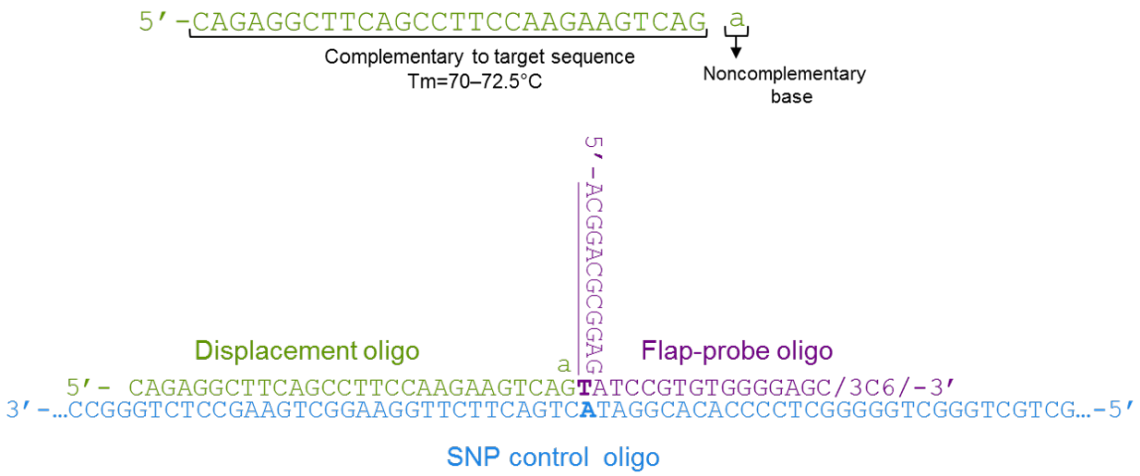


Figure 6. An example showing the design of the displacement oligo. Example of displacement oligo design for detection of g.1488C>T substitution (nucleotides in bold) in the *REQL4* gene. The displacement oligo (in green) is complementary to the SNP control oligo 3' of the SNP, with a noncomplementary and substitution-specific nucleotide at its 3' end (in lowercase). In this case, since the substitution is C>T, the noncomplementary base should be an A (as specified in Table 3). The sequence of the SNP control oligo is shown in blue and the flap-probe oligo in purple.

4. Alternative design

If the sequence immediately 5’ to the interrogated base is either 5’-GGAGX-3’ or 5’-GGAGNX-3’ (where N is any nucleotide and X is the SNP, Figure 7), the control, displacement and flap-probe oligos need to be designed taking the opposite strand as the one used in Section V.A.1 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).

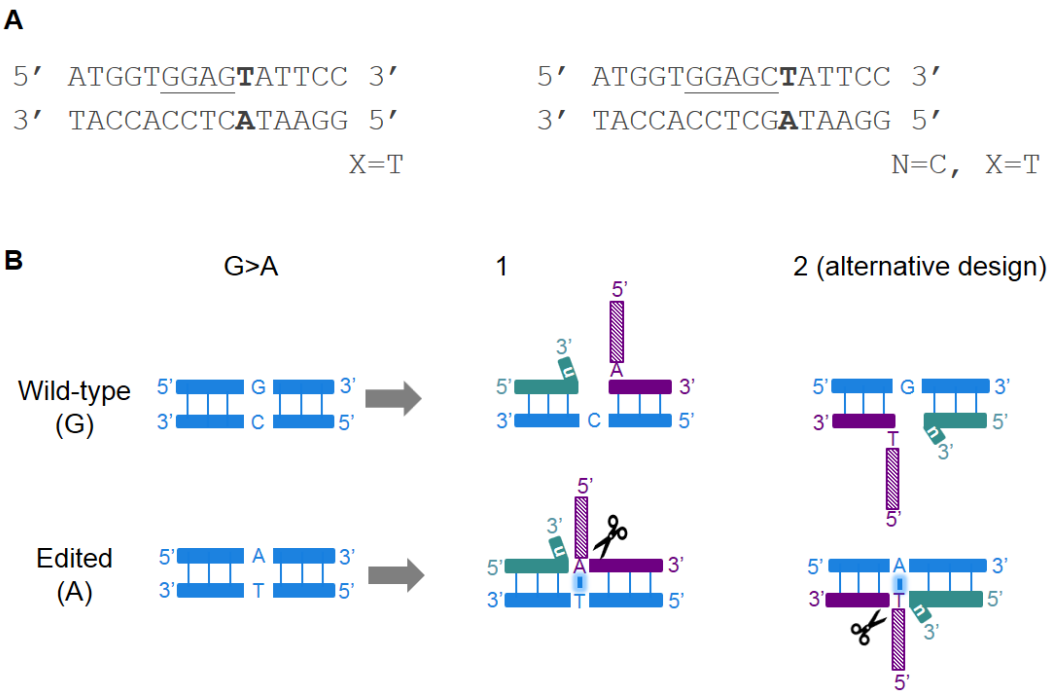


Figure 7. Alternative displacement and flap-probe oligo design. Panel A. Sequences 5’of the interrogated base (in bold) that requires the use of an alternative design (**Panel B2**) for generation of the displacement and flap-probe oligos, where X is the interrogated base and N can be any nucleotide. **Panel B.** Displacement and flap-probe oligos (green and purple, respectively) can be designed to detect a G>A substitution using the sequence of either DNA strand as a target sequence. In option 1 (explained in Section V.A.1), the SNP control oligo is derived from the DNA strand depicted in the 3’→5’ orientation; whereas for option 2 (alternative design), the SNP control oligo is derived from the DNA strand in the 5’→3’ orientation. For either approach, the desired SNP is detected when there is a complete pairing between the oligos and Guide-it Flapase recognizes the resulting double-flap structure.

The reason for this limitation is that inclusion of the sequences 5’-GGAGX-3’ or 5’-GGAGNX-3’ at the 3’ end of the displacement oligo would cause the Guide-it Flap Detector to recognize the displacement oligo as the cleaved flap, generating background fluorescent signal.

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 V.A.2 and V.A.3 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’-GGAGX-3’ or 5’-GGAGNX-3’ is provided in Figure 8.

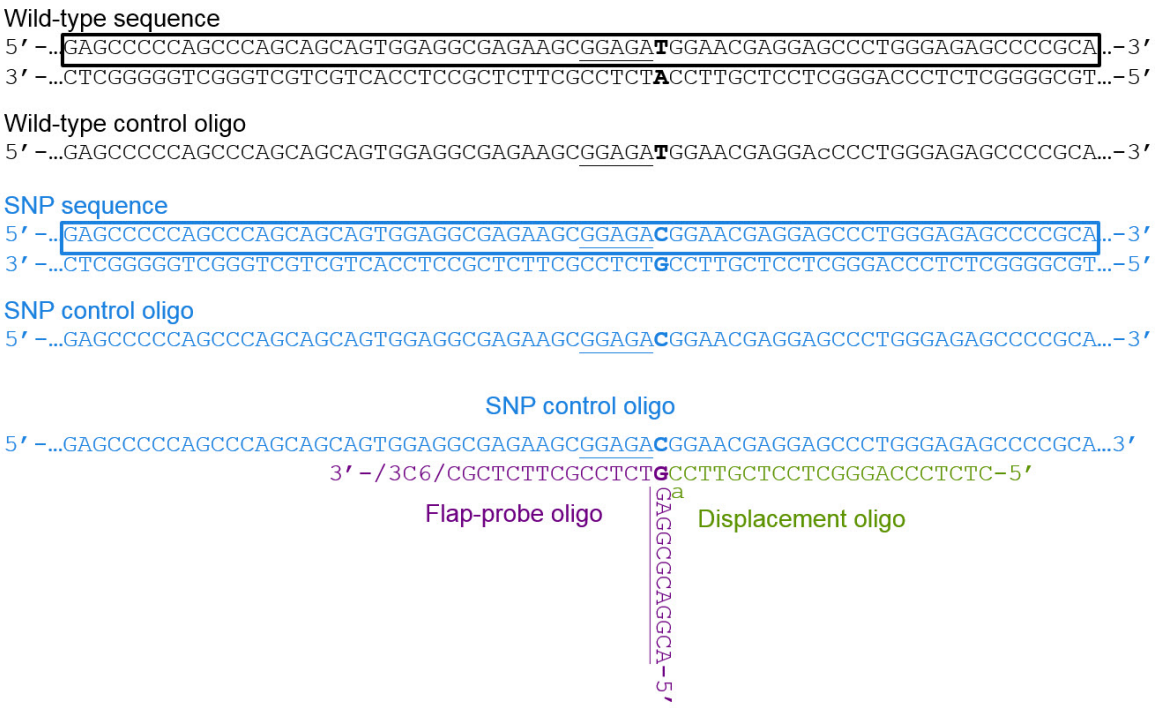


Figure 8. An example of an alternative design of the control, displacement, and flap-probe oligos. Control, flap-probe, and displacement oligos designed to screen for g.1540T>C in the gene *RECQL4* are shown. The existence of the underlined sequence 5'-GGAGA-3' at the 5' of the SNP (C) requires that the wild-type and SNP control oligos (black and blue, respectively) are generated from the sense strand. The displacement oligo (green) 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 3). The flap-probe oligo (purple, 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.

B. Test Displacement Oligo and Flap-Probe Oligo

The displacement oligo and the flap-probe oligo designed against the target sequence must first be tested to verify that they are suitable for the enzymatic assay. We also advise that you use the Guide-it SNP Control Set to confirm that the settings of your fluorescent plate reader allow for detection of the signal generated by the Guide-it Flap Detector. The Guide-it SNP Control Set includes wild-type and SNP control oligos encoding target sequences for the g.1298G>T substitution in the *MTHFR* gene premixed with the corresponding displacement and flap-probe oligos in Annealing Buffer.

1. Anneal wild-type and SNP control oligos with displacement and flap-probe oligos

1. Thaw the Guide-it SNP Positive Control Mix and the Guide-it SNP 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 in Section V.A.1 or V.A.4) 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. In a 96-well PCR plate, (Plate #1), pipette 10 µl of the wild-type control oligo (negative sample), SNP control oligo (positive sample), or water (non-template control, NTC) per well. We recommend performing every reaction in triplicate.

4. Resuspend the displacement oligo completely in RNase-free Water such that the final concentration is 1 µM.
5. Resuspend the flap-probe oligo completely in RNase-free Water such that the final concentration is 20 µM.
6. Prepare the annealing master mix in a 200-µl PCR tube as follows (the volumes provided below are for the exact number of wells as your samples, please prepare 5–10% extra to account for pipetting errors):

Table 4. Annealing master mix preparation guidelines.

Number of wells (96-well plate)	Annealing Buffer (µl)	Flap-probe oligo [20 µM] (µl)	Displacement oligo [1 µM] (µl)
9	31.5	4.5	9
n*	3.5 x n	0.5 x n	1 x n

*Where n is the number of wells containing samples (prepare 5–10% extra master mix to account for pipetting errors). If performing fewer or more than nine reactions, calculate the required volumes as indicated.

7. Add 5 µl of the annealing master mix prepared in Step 6 to each well of Plate #1 containing template: wild-type control oligo (negative sample), SNP control oligo (positive sample) or water (non-template control, NTC).
8. Add 15 µl of Guide-it SNP Positive Control Mix or Guide-it SNP Negative Control Mix (provided with the kit) to other wells of Plate #1. We recommend performing every reaction in triplicate.
9. Cover the plate with optical film or cap-strips.
10. Centrifuge the plate at 700*g* for 1 min.
11. Place the plate on a thermal cycler.
12. 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. Since the reactions specified in the previous section were performed in triplicates, the number of total reactions at this point will be fifteen: 9 reactions for testing the design of the flap-probe and displacement oligos, and 6 reactions for confirming assay functionality using Guide-it SNP Control Mix (the volumes provided below are for the exact number of wells as your samples, prepare 5–10% extra to account for pipetting errors).

1. Thaw the Flapase buffer, Guide-it Flap Detector and Guide-it Flapase on ice.
2. Prepare the enzymatic reaction master mix as detailed in Table 5.

Table 5. Enzymatic reaction master mix preparation guidelines.

Number of wells (96-well plate)	Flapase Buffer (µl)	Guide-it Flap Detector (µl)	Flapase (µl)
15	52.5	7.5	15
n*	3.5 x n	0.5 x n	1 x n

*Where n is the number of wells containing samples (prepare 5–10% extra master mix to account for pipetting errors).

3. Mix by pipetting to ensure proper mixing.
4. 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.
5. Check the reaction placed in the thermal cycler in Section V.B.1., Step 11. If the 10-min incubation at 63°C has completed and the machine is on hold at 63°C, stop the reaction. If the 10-min incubation at 63°C is still in process, keep Plate #2 covered at 4°C until the program reaches the hold step.
6. 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 (e.g., transfer the contents of Plate #1, well A1 to Plate #2 well A1; transfer the contents of Plate #1, well A2 to Plate #2, well A2; and so on).
7. Cover the plate with optical film or cap strips.
8. Centrifuge the plate briefly at 700*g* for 30 sec.
9. Place the plate on a thermal cycler and run the following program:

63°C	75 min
4°C	hold

10. After the reaction is complete (on hold at 4°C), take a 96-well black/clear-bottom plate (Plate #3) and add 35 µl of RNase-free Water to each well (to achieve the minimum final volume required for the plate reader to perform an accurate reading upon addition of the reactions from Plate #2).

    NOTE: 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 volume of reaction that will be added to each well from Plate #2 (20 µl).

11. Once the reaction in Step 9 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 A1; transfer the contents of Plate #2, well A2 to Plate #3, well A2; and so on).
12. Centrifuge the plate briefly to eliminate any air bubbles (700*g* for 1 min).
13. Proceed to read the fluorescence in a plate reader with a filter set that allows for the detection of a dye with an excitation maximum of 485 nm and an emission maximum of 535 nm.

    NOTE: If a plate reader has different filter sets, use the Guide-it SNP control wells 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: negative (wild type), positive (SNP), and non-template control (NTC), as well as the Guide-it SNP Control Positive Mix and Guide-it SNP Control Negative Mix (see Figure 9 for an example).

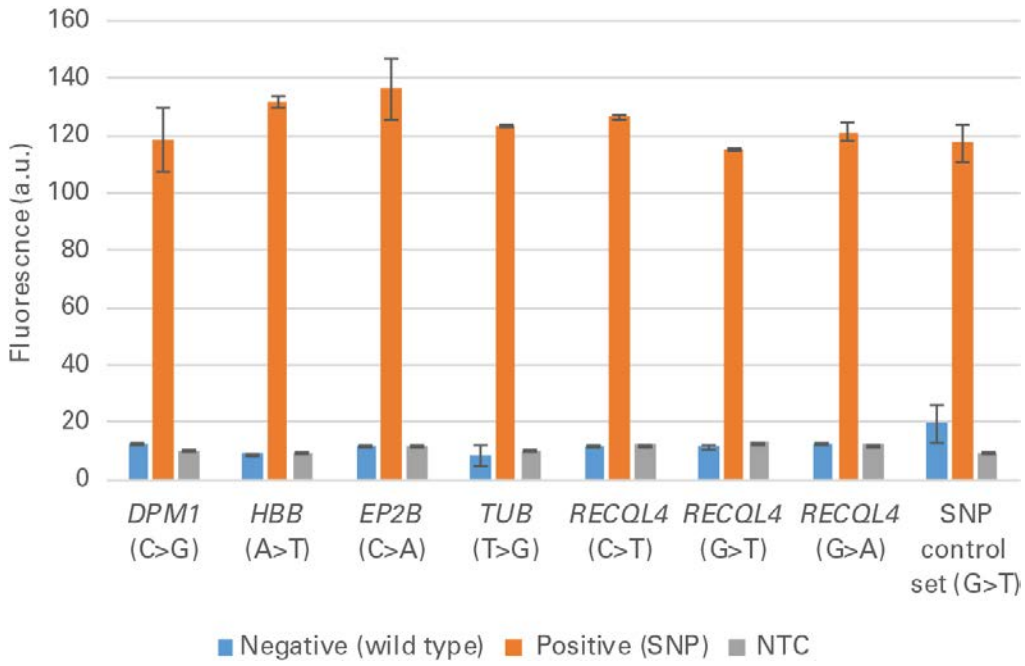


Figure 9. Results obtained from testing the functionality of displacement and the flap-probe oligos designed to detect SNPs at various genomic loci using control oligos as samples. Displacement and flap-probe oligos were designed to detect SNPs in the indicated human genes. All the flap-probe and displacement oligo combinations tested enabled accurate detection of the desired nucleotide substitution, as indicated by the strong fluorescent signals for the positive samples (orange) relative to the corresponding negative samples (blue) and NTCs (gray). Fluorescence values obtained with the provided Guide-it SNP Control Set are also shown (right). Please take into account that the fluorescence values are arbitrary and depend on the plate reader.

The outcome of this assay will allow you to determine whether the design of your flap-probe oligo and displacement oligo will enable accurate detection of SNPs in edited cells. The ratio between signals obtained from positive and negative samples should be higher than 6. If you do not obtain a positive fluorescent signal with your SNP control oligo, first check your oligo design, and then order and test new oligos if necessary.

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

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 SNP.

A. 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).

B. 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).

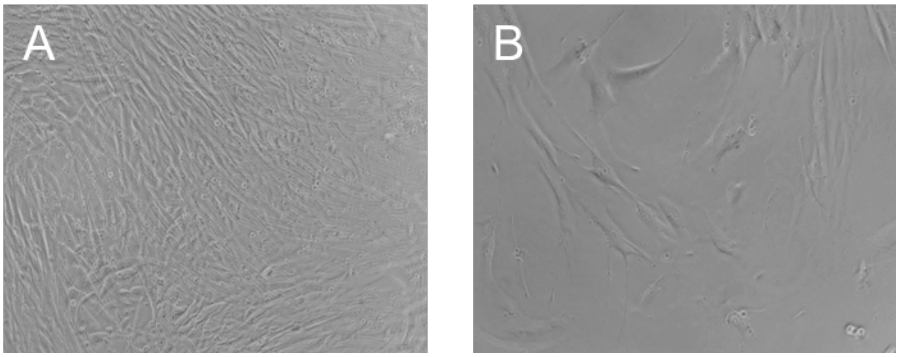


Figure 10. Optimal range of cell densities for genomic DNA extraction using MightyPrep Reagent. Primary fibroblasts were seeded at different cell densities—from 2 x 10^5 (**Panel A**) to 2 x 10^4 (**Panel B**) cells per well—and were tested successfully for the presence of the SNP (*DMP1* c.274C>G).

1. 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 its lid or optical film 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.

VII. Protocol: Amplify the Target Site by PCR

A. Design Primers

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

- The optimal amplicon size should be 200–700 bp, with the SNP 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.

NOTES:

- We recommend testing the primer pairs on sample genomic DNA extracted with MightyPrep Reagent using Terra polymerase with the suggested PCR conditions (Section VII.B) to ensure correct amplification and absence of primer dimers.
- Please 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 www.ncbi.nlm.nih.gov/tools/primer-blast/.

B. Amplify the Target Site

1. Prepare a master mix of the PCR reaction mixture depending on the number of samples to be analyzed as follows (the volumes provided below are for the exact number of wells as your samples, prepare 5–10% extra to account for pipetting errors). Thaw all the reagents on ice.

Table 6. 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 5–10% extra to account for any 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 VI.B. 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:

98°C	2 min	
35 cycles:		
98°C	10 sec	⎫
68°C	1 min	⎭
4°C	hold	

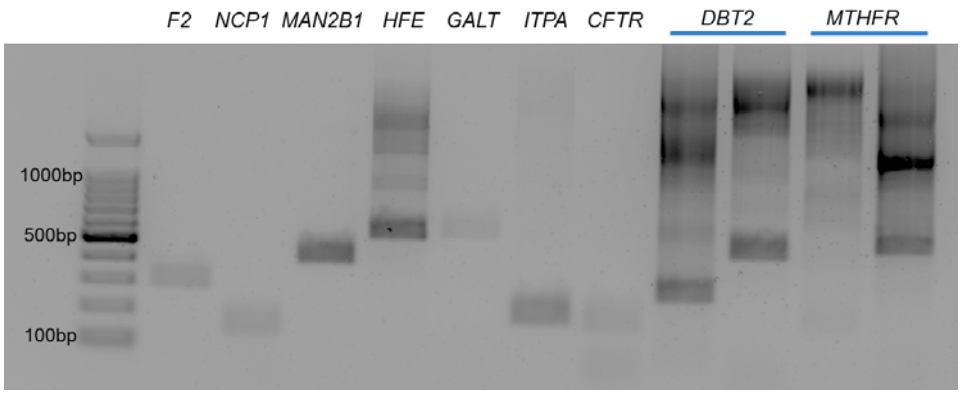


Figure 11. Examples of PCR-amplified target DNA used as samples with the Guide-it SNP Screening Kit. Please note that the production of multiple amplicons during the PCR step does not negatively affect the enzymatic reaction. The size of the amplified target can range between 200 bp and 700 bp. For SNP detection assays involving the genes *DBT2* and *MTHFR*, two different sized amplicons (ranging in size between 200 bp—450 bp) were successfully used for the enzymatic assay.

VIII. Protocol: Perform the Enzymatic Assay

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

1. Take the PCR products generated in Section VII.B and dilute them 1/40 with Dilution Buffer (i.e., 2 µl of each PCR reaction in 78 µl of Dilution Buffer).
2. Thaw the oligos encoding for the wild-type and SNP target sequences ("wild-type control oligo" and "SNP control oligo") if negative/positive controls are desired. The concentration of each oligo should be 1 nM in water (the same concentration that was used for testing in Section V.B).
3. Pipette 10 µl of the diluted PCR product per well of a 96-well PCR plate (Plate #1). If negative and positive controls are desired, add 10 µl of the wild-type or SNP control oligos (1 nM in water) into two separate wells.
4. Prepare the annealing master mix in a 1.5-ml PCR tube as follows (the volumes provided below are for the exact number of wells as your samples, prepare 5–10% extra to account for pipetting errors):

Table 7. Annealing master mix preparation guidelines

Number of wells (96-well plate)	Annealing Buffer (µl)	Flap-probe oligo [20µM] (µl)	Displacement oligo [1µM] (µl)
96	336	48	96
n*	3.5 x n	0.5 x n	1 x n

*Where n is the number of wells containing samples (prepare 5–10% extra master mix to account for pipetting errors).

5. Add 5 µl of the annealing master mix prepared in Step 4 to each sample-containing well.
6. Cover the plate with optical film or cap-strips.
7. Centrifuge the plate at 700*g* for 1 min.
8. 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

B. Perform Enzymatic Reaction

1. While the oligos are being annealed, prepare the enzymatic reaction master mix described in Table 8 (the volumes provided below are for the exact number of wells as your samples, please prepare 5–10% extra to account for pipetting errors). Thaw all the reagents on ice.

Table 8. Enzymatic reaction master mix preparation guidelines

Number of wells (96-well plate)	Flapase Buffer (µl)	Guide-it Flap Detector (µl)	Guide-it Flapase (µl)
96	336	48	96
n*	3.5 x n	0.5 x n	1 x n

*Where n is the number of wells containing samples (prepare 5–10% extra master mix 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 VIII.A.1., Step 8 (above). If the 10-min incubation at 63°C has completed and the machine is on hold at 63°C, stop the reaction. If the 10-min

incubation at 63°C 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:

63°C	75 min
4°C	hold

9. After the reaction is complete (on hold at 4°C), take a 96-well black/clear-bottom plate (Plate #3) and add 35 µl of RNase-free Water to each well (to achieve the minimum final volume required for the plate reader to perform an accurate reading upon addition of the reactions from Plate #2).

   NOTE: 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 volume of reaction that will be added to each well from Plate #2 (20 µl).

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 briefly to eliminate any air bubbles (700*g* for 1 min).
12. Proceed to read the fluorescence in a plate reader with a filter set that allows for the detection of a dye with an excitation maximum of 485 nm and an emission maximum of 535 nm (i.e., the same plate reader with the exact settings used in Section V.B).
13. A positive fluorescent signal corresponds to the existence of the desired SNP in a clonal cell sample.
14. Pick out clonal cells from your master cell plate (Section VI.A) corresponding to those that generated a positive fluorescent signal and confirm the results using Sanger sequencing if desired.

Appendix A. Troubleshooting Guide

Table 9. Troubleshooting Guide for the Guide-it SNP 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 at 1 nM concentration should be diluted in RNase-free Water and not in Dilution Buffer
	Configuration of the plate reader	• Use Guide-it SNP Control Set to check for the proper filter set in the plate reader
	Not enough volume in the well for a correct readout	• Check the minimum volume required for the plate reader to get an accurate measurement

	Error in the oligo design	• Check and correct any errors in the oligo design
		  • Presence of the fixed sequence in 5' end of flap-probe oligo
		  • Addition of the noncomplementary extra nucleotide in the 3' end of displacement oligo

Fluorescence values of the designed oligos are much lower than the Guide-it Control Mix or their ratio of positive vs negative signal is lower than 6	Need of design optimization	• Increase the Tm of the flap-probe oligo or displacement oligo (you can use an RT-PCR machine to detect the kinetics of the reaction) • Change to alternative design (Section V.A.4) • Presence of several repeats in the target sequence immediately 3' relative to the interrogated base, may affect the hybridization of the flap-probe oligo, use alternative design (Section V.A.4) to design your oligos
Negative control has the same fluorescence as positive control and NTC	Oligos diluted in Dilution Buffer (there is only background signal being detected)	• Control Oligos at 1 nM concentration should be diluted in RNase-free Water and not in Dilution Buffer
	Background signal due to the presence of the sequence 5'-GGAGX-3' or 5' GGAGNX 3' at the 3' end of the displacement oligo	• Change to alternative design (Section V.A.4)
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 oligo control (SNP control oligo)	Oligos diluted in Dilution Buffer	• Oligos should be diluted in RNase-free water
	Problems with the plate reader	• Check the proper function of your plate reader
	Not enough volume in the well for a correct readout	• Check the minimum volume required for the plate reader to get an accurate measurement using Guide-it SNP Control Set

Appendix B. Glossary of Terms

Table 10. Glossary of terms

Interrogated base	Nucleotide at the target site 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
Displacement oligo	User-generated oligo that anneals to the denatured PCR product 3’ relative to the interrogated base
Flap-probe oligo	User-generated oligo that anneals to the denatured PCR product 5’ relative to the interrogated base 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 is not active against gapped structures
Guide-it 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

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com	web: takarabio.com
e-mail: ordersUS@takarabio.com	e-mail: techUS@takarabio.com

Notice to Purchaser

Our products are to be used for research purposes only. They may not be used for any other purpose, including, but not limited to, use in drugs, *in vitro* diagnostic purposes, therapeutics, or in humans. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without prior written approval of Takara Bio USA, Inc.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product’s web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.

This document has been reviewed and approved by the Quality Department.