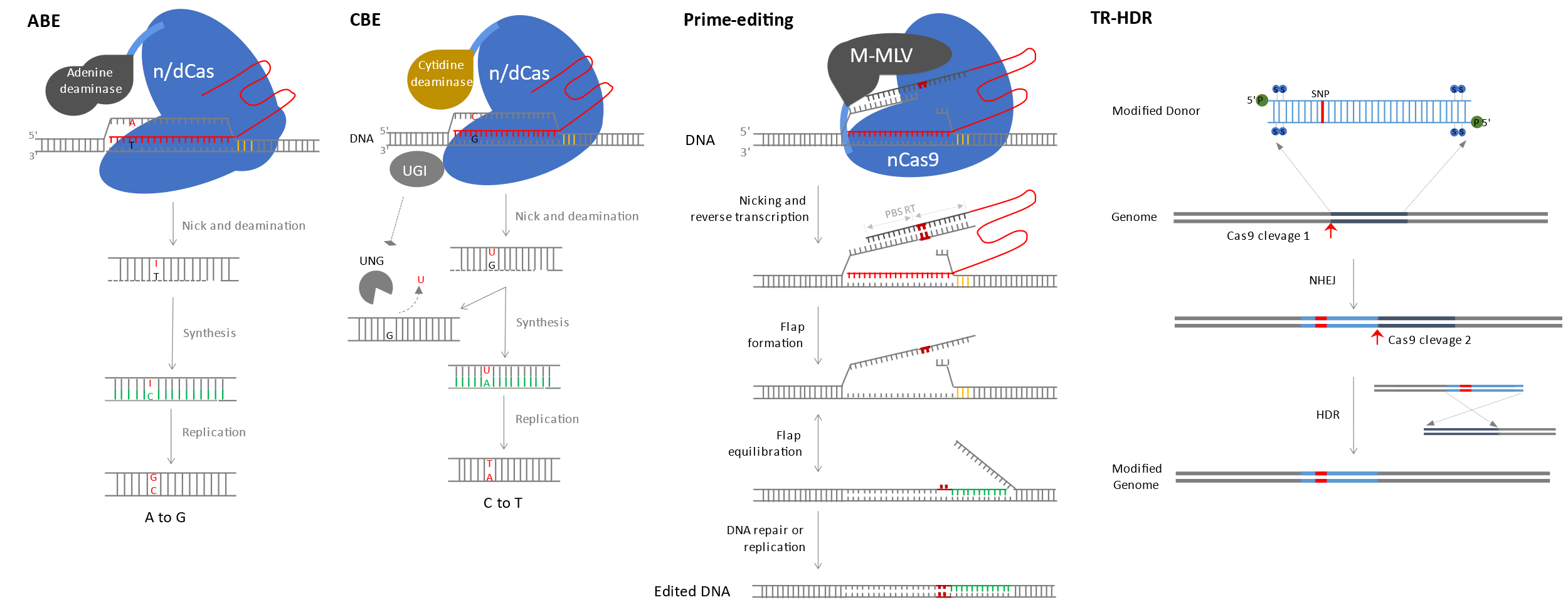
[Supplemental Note1](C:\\Users\\18757\\Documents\\WeChat Files\\wxid_dkhlkl6kirzw22\\FileStorage\\File\\2021-09\\FIG\\图片8.svg)

**Open Genome design guidelines and experiment protocols**

1. **Background**

Precise genome editing has become a more demanding issue for precision plant breeding since the emerging and rapid development of CRISPR methods. Methods such as adenine and cytosine base editors, prime editing, and recently developed TR-HDR all have equipped this field. Base-editing, based on the CRISPR/Cas9 systems, containing two types of base editors-cytosine base editor (CBE) and the adenine base editor (ABE). The cytosine base editor (CBE) converts C•G to T•A, adenine base editor (ABE) empowers the conversion of A•T to G•C, enabling the direct, irreversible conversion of one target DNA base into another in a programmable manner, without requiring dsDNA backbone cleavage or a donor template (Komor et al. 2016) (Gaudelli et al. 2017). Prime editing, developed in 2019, have been making huge improvement of precise genome editing. It directly writes new genetic information into a specified DNA site using a catalytically impaired Cas9 endonuclease fused to an engineered reverse transcriptase, programmed with a prime editing guide RNA (pegRNA) that both specify the target site and encodes the desired edit, enabling targeted insertions, deletions, and all 12 types of point mutation, without requiring double-strand breaks or donor DNA templates. (Anzalone et al. 2019). Targeted insertion and TR-HDR (tandem repeat-HDR strategy), developed in 2020, based on the design of the phosphorothioate-linkage modification to stabilize the oligos in cells and the 5ʹ-phosphorylation to facilitate non-homologous end joining (NHEJ): Targeted insertion strategy could insert sequences of up to 2,049 base pairs (bp), into the rice genome at an efficiency of 25%. TR-HDR is mainly based on the preview formation of tandemly arranged repeat elements (such as the YFFP and GUUS reporters that contain a tandem repeat of the middle part (F and U, respectively) of the YFP and GUS reporters) using modified donor insertion in plant cells. The oligo is designed to also form a target site for the same sgRNA upon insertion. All 12 types of point mutation, highly efficient targeted insertions, deletion could be achieved through TR-HDR.

**Figure 1: Four different precise genome editing methods.**

1. **Web guidance:**

The following list provides a quick overview of the steps involved in precise genome editing design (Figure 2). Note: Read the step-by-step guide to ensure optimal results.

Input sequences:

1. Please access the "Design" page to start a job. Select a genome you are studying, input your DNA sequence in FASTA format or you could upload a FASTA file on your local computer, then fill the edited/desired DNA sequence as you wish. Note: the DNA sequences between your desired edits should be at least more than 100 bps so that the computer could design the best primers for your further mutation detection experiments. Substitution could directedly edit on the sequence without any symbols; the insertion should use the format as [AACCT]; the deletion should use the format {TCCTT}.

Examples:

Base substitution using ABE: (A-G)

WT>CTTCTCATCC AACCTCGCTT CCCAACCCTG GATCCAAATC CCAACCTATC CCAAAGCCGA AACCGAGGAG AGGAAAAAGGTTACGCGCAATTATTACTAGCTATGGCTAG GTAGGTTTGGGGGAGGCGAGATCATGAAGCGCGAGTACCAAGAAGCCGGCGGGAGCAGCG GCGGCGGGAG CAGCGCCGAT ATGGGGTCGT GCAAGGACAA GGTGA

Edited>CTTCTCATCC AACCTCGCTT CCCAACCCTG GATCCAAATC CCAACCTATC CCAAAGCCGA AACCGAGGAG AGGAAAAAGGTTACGCGCAATTATTACTAGCTATGGCTAG GTAGGTTTGGGGGAGGCGAGATCATGAAGCGCGAGTACCAAGAAGCCGGCGGGAGCAGCG GCGGCGGGAG CAGCGCCGAT ATGGGGTCGT GCAAGGACAA GGTGA

Base substitution, insertion and deletion using TR-HDR: (A-G)

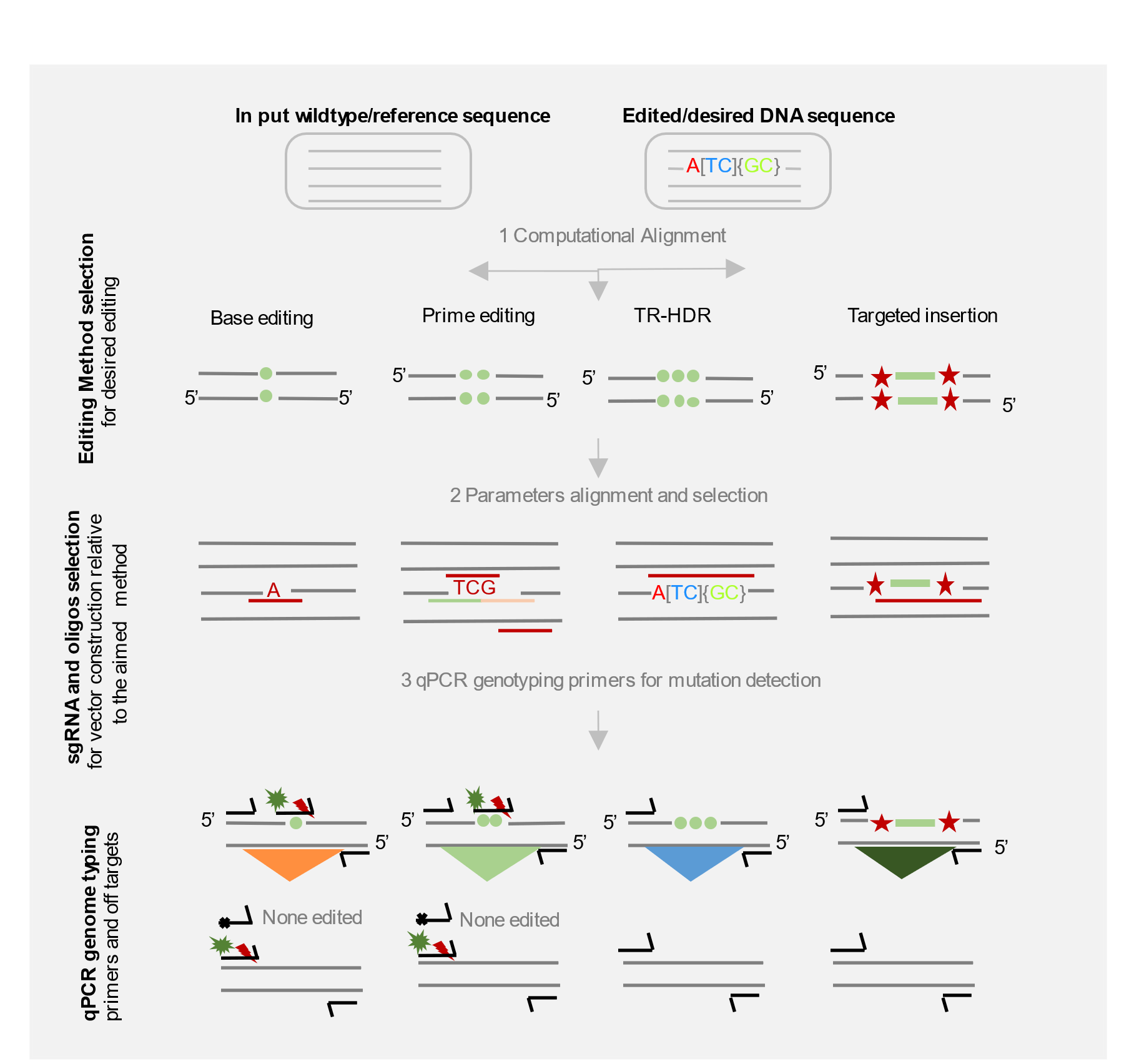
WT>GTTTTGTGAGAACAAATACAATCGCTACGGAGGTAGTATCCTGTTTCCTCTCAGCCGTTAGATCAAGCAATCGAGTGGTTTTTCTCCGTTGATTCGAGGCTGTGTTCGGCTCCTGGGGTTGGGAACCCATCTCCCTGCACGGAAAAAGGAGTGGTTTATTAGCGCGTGATTAATTAAGTATTAACTATTTTTTTAAAATATATATTAATTTGAATTTTTTAAGCAACTTTCGTATAGAAACTTTTTACAAAAAAAA

Edited>GTTTTGTGAG AACAAATACA ATCGCTACGG AGGTAGTATC CTGTTTCCTC TCAGCCGTTA GATCAAGCAA TCGAGTGGTT TTTCTCCGTT GATTCGAGGCTGTGTTCGGC TCCTGGGGTT GGGAACCCAT CTCCCTGCAC GGAAAAACC[G] {A}GTGGTTTAT TAGCGCGTGA TTAATTAAGT ATTAACTATTTTTTTAAAAT ATATATTAAT TTGAATTTTT TAAGCAACTT TCGTATAGAA ACTTTTTACA AAAAAAA

Targeted insertion using Targeted-KI ：(TGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTC)

WT>CGATTAGTTTGAAACGTGTATGGAAAGATTGCTTTCCTAAATATCTATTCAACAAAATCAGCCAGATTAAACTGCACGAATTACGTACGCCCAATTAATTACATGCCAAATGAAGCAACTGCACGCCGGAGAGATGACATATATAGCACCATGTAAACCTGCCATATGCCACCCTCTTGCAGCCCAACCCAACCCAATAAGGATATAAAACCACTCGCGGTTTTGCACAGCTAGCGCGGCGCAGTGTGTAACCGTCCAGGGTGACACAATTGACTTAT

Edited> CGATTAGTTTGAAACGTGTATGGAAAGATTGCTTTCCTAAATATCTATTCAACAAAATCAGCCAGATTAA ACTGCACGAATTACGTACGC CCAATTAATTACATGCCAAAT[TGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTC]GAAGCAACTGCACGCCGGAGAGATGACATATATAGCACCATGTAAACCTGCCATATGCCACCCTCT TGCAGCCCAA CCCAACCCAA TAAGGATATA AAACCACTCG CGGTTTTGCA CAGCTAGCGC GGCGCAGTGT GTAACCGTCCAGGGTGACACAATTGACTTAT

1. Select the appropriate editing strategy for the mutation you want to instill.
2. Customize the appropriate Cas protein to ensure the PAM compatibility among the sequence you want to edit.
3. For each of these editing methods, there are different optimal parameters ranges. Follow the website to fill these parameters.

**Figure 2: The overall workflow of OpenGenome Toolkit.**

Result page guidelines:

1. After users have completed the selection of the best editing solution, we will then validate the input format to fit the construction we require and compare the edited and original sequences with specific patterns to examine the different editing types.
2. A few seconds after submission, the website will automatically go to the results page for your chosen design.
3. For each design method, all edits within the user-defined sequence range are automatically constructed. And designs are generated for the installation of these edits: including the recommendation of a candidate sgRNA combination, the generation of an oligo synthesis solution based on our laboratory validated vectors, and the most efficient vectors based on those previously validated by other researchers; as well as vector acquisition ways and linkages to CRISPR-GE to detect off targets.
4. Open Genome has ranked each editing solution according to the optimized parameters for a specific design solution, shows the top three designs and their sequence visualization results.

Vector construction(Figure 3) 、primer design and mutation detection(Figure4) protocols:

1. Cloning overview

ABE and CBE vectors construction using Open Genome design

1. Digest ABEmax-nCas9; CBEmax-nCas9 plasmid (#1 vector) with *BsaI* and isolate the plasmid fragment containing the origin of replication, U6 promoter, U6 poly-T termination sequence, ABEmax-NLS-linker-nCas9-NLS-NOS (or NLS-Anc698-linker-nCas9-NLS-NOS)and HPT gene.
2. Order oligonucleotides designed by Open Genome with overhangs :

UP: 5’-TGTGGNNNNNNNNNNNNNNNNNNNN-3’

LW: 5’-AAACNNNNNNNNNNNNNNNNNNNNC-3’

1. Anneal top and bottom oligonucleotides and ligation:
2. Dissolve UP and LW oligos in ddH2O to 10uM, take 1ul each to 18 ul Anneal Buffer(TE + 50mM NaCl) and mix by pipetting.
3. Run the annealing programs using a slow cool-down process from 95℃ to 16℃ in PCR machine.
4. Add 180ul ddH2O to the product and mix by pipetting,set up a 10ul ligation reaction with *BsaI*(NEB) digested #1 vector .

Annealing product 1ul

BsaI digested vector 1ul

10ⅹT4 buffer 1ul

T4 DNA ligase (NEB) 0.5ul

Add ddH2O to 10ul, ligation at 25℃for 1-2hour.

1. Verify by colony PCR or sanger directed sequencing.

Prime editing vector construction using Open Genome design

1. Digest pCXPE3 plasmid (#2 vector) with *BsaI* and isolate the plasmid fragment containing the origin of replication, U6 promoter, U6 poly-T termination sequence, gRNA-35S:NLS-nCas9-linker-MMLV(Os)-NLS-NOS and HPT gene.
2. Amplified the suitable AtU6 adaptors(#3 adaptors ,~ 300bp)and isolated as the for the following template and using .

AtU6-Forward: ATTTTTTTCTCGTGGCGTCAGCATTCGGAGTTTTTGTATCT

AtU6-Reverse: CCAATCACTACTTCGACTCTAG

1. Order oligonucleotides designed by Open Genome to amplify the appropriate connector contains *BsaI* digestion sites with different adaptors .

Spacer-gRNA-RTT-F:

AATAGGTCTCATGTGTGCAAGATGGATACTAAGGGTCGTTTTAGAGCTAGAAAT

Spacer-gRNA-RTT-R:

ATGGTCTCACTACCTATGATTCCGCACCGACTCGGTGCCACTTTTTC

PBS-OsU3-nickSG\_F:

TAGGTCTCATTCCGGAATCATAGGTAGTTTTTTGTTTTAGAGCTCC

PBS-OsU3-nickSG\_R:

ATGGTCTCAAAACTACATCCAACAAGTATGGCCCGCCACGGATCATCTGCACAA

1. Amplified SG-gRNA-RT and PBS-AtU6-rSG fragments respectively .
2. Dissolve primers in ddH2O to 10uM,and mix by pipetting.
3. Amplified the SG-gRNA-RT(#4, ~150bp) and PBS-AtU6-rSG(#5, ~350bp) fragments respectively and isolated.

#3 adaptors 0.2ul

Forward primer 1ul

Reverse primer 1ul

KOD-201(TOYOBO ) 0.5ul

10ⅹKOD buffer 5ul

10ⅹdNTPs 5ul

Mg2+  3ul

Add ddH2O to 50ul, and mix by pipetting then amplified using the normal PCR programs.

1. SG-gRNA-RT and PBS-AtU6-rSG Golden Gate assembly reaction with vectors

Digested pCXPE3 plasmid-vector(#2,pre-cut and isolated) 1ul (30-50ng/ul)

SG-gRNA-RT(#4, isolated) 0.3ul(30-50ng/ul)

PBS-AtU6-rSG(#5, isolated) 0.3ul(30-50ng/ul)

*BsaI*-HFv2 (NEB) 0.3ul

T4 DNA ligase (NEB) 0.50 µL

10x T4 DNA ligase buffer (NEB) 1ul

ddH2O 6.6ul

Total reaction volume:10ul

Incubation in 37 ˚C for 5min, then follow the cycle between 3 min at 37 ˚C and 1 min at 20 ˚C, 2 min at 10 ˚C for 15 cycles, hold at 12 °C. Verify by colony PCR and sanger directed sequencing.

Targeted insertion and TR-HDR vector construction using Open Genome design

1. Digest pCBSG032 plasmid (#6 vector) with BsaI and isolate the plasmid fragment containing the origin of replication, U6 promoter, U6 poly-T termination sequence, gRNA-UBI: 3 x Flag-NLS-Cas9-NOS and HPT gene.
2. Order oligonucleotides designed by Open Genome with overhangs :

UP: 5’- TGTGTGGGCGTGCAGTTGCTTCATT-3’

LW: 5’- AAACAATGAAGCAACTGCACGCCCA-3’

1. Anneal top and bottom oligonucleotides and ligation:
2. Dissolve UP and LW oligos in ddH2O to 10uM, take 1ul each to 18 ul Anneal Buffer(TE + 50mM NaCl) and mix by pipetting.
3. Run the annealing programs using a slow cool-down process from 95℃ to 16℃ in PCR machine.
4. Add 180ul ddH2O to the product and mix by pipetting,set up a 10ul ligation reaction with *BsaI*(NEB) digested #1 vector .

Annealing product 1ul

BsaI digested vector 1ul

10ⅹT4 buffer 1ul

T4 DNA ligase (NEB) 0.5ul

Add ddH2O to 10ul, ligation at 25℃for 1-2hour. Verify by sanger directed sequencing.

1. Order oligonucleotides designed the forward and reverse primers of TR-HDR or KI modified donor.

TR-HDR

TR-HDR modified donor forward: **P**\*T\*GCAGGTATTTTAAAAAAATAGTTAATACTTAATTAATCACGCGCTAATAAACCACCGGTTTTTCCGT\*G\*C

TR-HDR modified donor reverse:

**P**G\*C\*ACGGAAAAACCGGTGGTTTATTAGCGCGTGATTAATTAAGTATTAACTATTTTTTTAAAATACCTG\*C\*A

Targeted KI

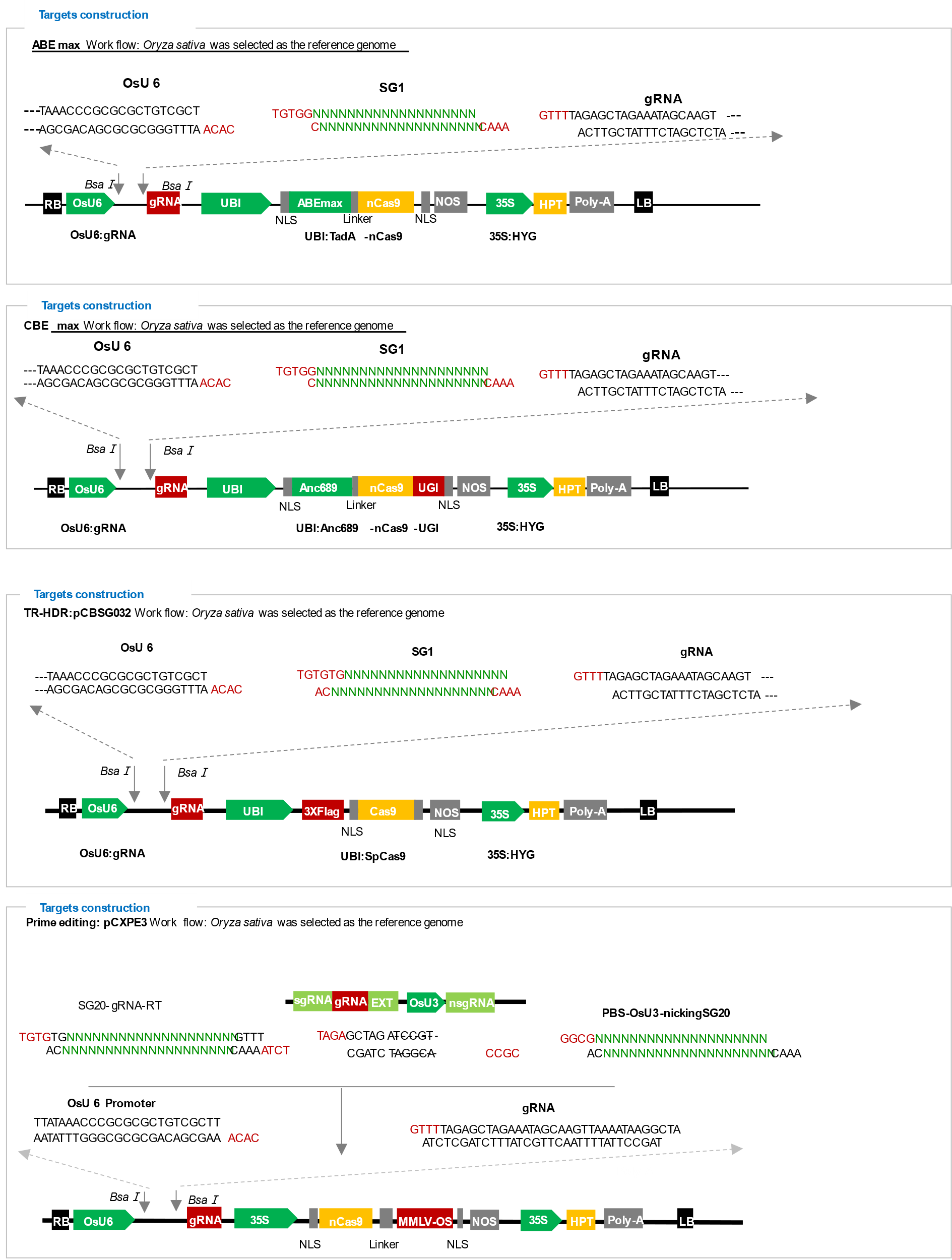
Knock in modified donor forward:

**P**G\*A\*AGGATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGAGATGTCACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCA

Knock in modified donor reverse:

**P**T\*G\*GACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTC

Then transfer via gene gun.

Figure 3: Vector construction using ABEmax-nCas9; CBEmax-nCas9; pCBSG032; pCXPE3

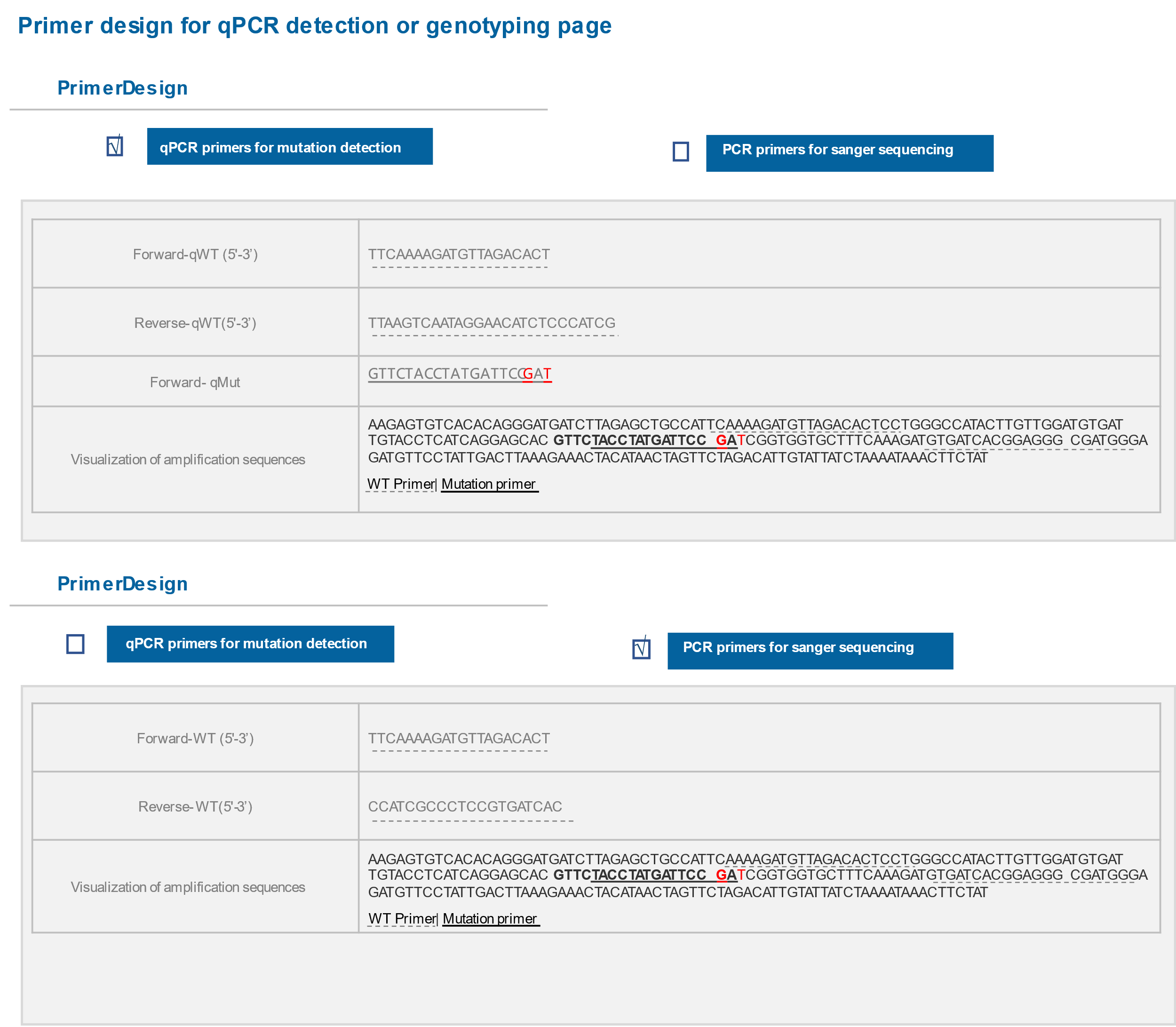
1. Overview of mutation detection using qASPCR for BE and PE.
2. Design mutation detection primers using Open Genome web tools.

Figure 4: Primer design using the Open Genome web tool.

1. Using qPCR primers for qASPCR mutation detection.
2. Extracted the genomic DNA of your samples through CTAB. Diluted the gDNA in to ~10ng/ul.