ENABLE® Gene Editing *in planta* toolkit

Extended protocol for new users

This extended protocol is designed for new users of the ENABLE® Gene Editing *in planta* toolkit. The user must have ordered the ENABLE® toolkit and have access to basic molecular biology equipment and reagents. For a shortened protocol for experienced users and further information, see:

https://www.growmorefoundation.org/enable-crispr-kits

PROTOCOL

The CRISPR/Cas9 technology allows you to induce mutations in regions of choice in any given genome by expressing a SpCas9 nuclease (a DNA cutting enzyme from *Streptococcus pyogenes*) and a small RNA molecule called single guide RNA (sgRNA), which directs Cas9 to its intended target site via base pairing. In most plant genome editing experiments, plants are transformed with DNA vectors containing the genes for Cas9 and sgRNAs, as well as a transformation selection marker. The ENABLE® toolkit allows you to generate such a vector to target your gene of interest in monocot and dicot species and this extended protocol will guide you through that process.

Contents of the ENABLE® Gene Editing kit

The kit provides all necessary plasmids for cloning a binary T-DNA vector containing genes for expression of Cas9, two sgRNA and a transformation selection marker (Hygromycin for stable plant transformation or eGFP for protoplasting and transient expression experiments).

- Order the following plasmids from Addgene using the Addgene kit ID **1000000270**:
  - **pGMF1-M**: sgRNA subcloning vector for **M**onocot species, *OsU6-2p::gRNA1*
  - **pGMF2-M**: sgRNA subcloning vector for **M**onocot species, *OsU6-2p::gRNA2*
  - **pGMF1-D**: sgRNA subcloning vector for **D**icot species, *AtU6-26p::gRNA1*
  - **pGMF2-D**: sgRNA subcloning vector for **D**icot species, *AtU6-26p::gRNA2*
  - **pGMF3**: Golden Gate Level 2 Binary T-DNA Backbone vector
  - **pGMF4**: *CAS9* cassette, *AtUBQ10p::SpCas9:Pea3At*. Also known as **pFH54.**
  - **pGMF5**: Hygromycin resistance marker (*35Sp*::*HPTII*:*35St*). Also known as **pICSL11059**.
  - **pGMF6**: Nuclear localized eGFP marker (*35Sp*::*eGFP*:*35St*).

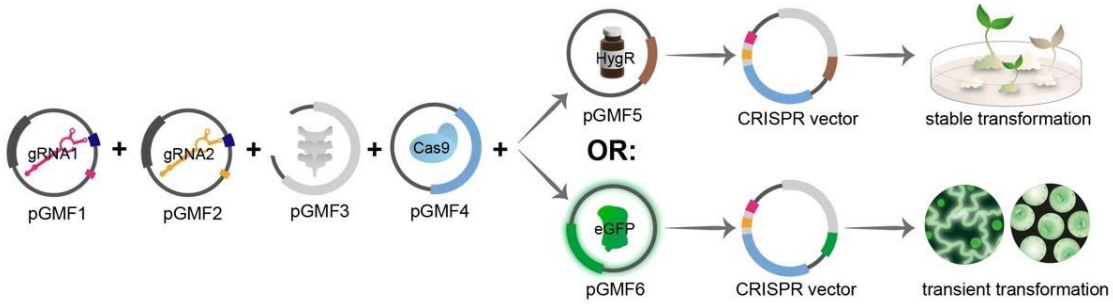

Figure 1: The vectors included in the ENABLE® toolkit allow cloning of binary T-DNA vectors for stable or transient plant transformation. Note that there are monocot and dicot specific versions of pGMF1 and pGMF2.

- Additional reagents to be ordered:
  - BsaI-HF®v2 (20 U/ µL, New England Biolabs, Cat. No: R3733S)
  - BbsI-HF® (20 U/ µL, New England Biolabs, Cat. No: R3539S)
  - T4 DNA Ligase (400 U/ µL, New England Biolabs, Cat. No: M0202S)
  - Recombinant Albumin (1 mg/ mL, diluted down from New England Biolabs, Cat. No: B9200S)
  - Double-distilled nuclease-free water

Note: Reagents can also be obtained from other providers when needed, though their performance was not tested in this study.

- Optional primers for verification of plasmid integrity (not provided) :
  - Primer GMF001: GAACCCTGTGGTTGGCATGCACATAC
  - Primer GMF002: CTGGTGGCAGGATATATTGTGGTG
  - Primer GMF003: AGATAAGGGAATTAGGGTTC

- Store plasmid DNA filter paper at 4 ˚C and enzymes, buffers and primers at - 20 ˚C.

The user will need to provide primers for subcloning their experiment-specific target sequence of interest (see protocol). The user will also need to have access to standard molecular biology equipment (e.g. pipettes, PCR tubes, thermocycler, Miniprep reagents) and ideally methods to verify integrity of the cloned CRISPR vector (Sanger sequencing services, PCR based methods or restriction digest based methods, see protocol). Similarly, reagents for *E. coli*, *A. tumefaciens*, or plant/protoplast transformation are not supplied with this kit.

Procedure

The ENABLE® gene editing kit is designed for expression of two sgRNA targeting two genomic loci in either monocot or dicot species. These target sites for the user's gene(s) of interest need to be chosen and their sequences need to be cloned into the binary T-DNA vector by the user. Expression of two sgRNA increases the chances of knockout in a single gene or allows targeting two genes at the same time. It also allows targeted introduction of large deletion between two sgRNA target sites which provides a low cost method for mutation detection post transformation.

The following protocol supports the user in finding a suitable sgRNA target site and subsequently in cloning a binary T-DNA vector containing their sgRNAs of choice. If gene(s) of interest and sgRNA sequences are known (e.g. from literature/previous experiments) proceed to PreStep 2 but please ensure that your target site does not contain BbsI or BsaI restriction sites, as this will interfere with the cloning procedure. We highly recommend taking advantage of freely available online resources for target gene identification and sgRNA design and selection, such as CRISPOR, CCTOP or CRISPR-P2.0 and usage of free molecular biology tools such as Benchling, ApE or SnapGene Viewer for sequence annotations/alignments/visualization. All of these tools provide extensive documentation on how to use them.

Design Steps

PreStep 1: Designing sgRNA target sites with high efficiency and minimal off target activity for gene knockout.

Finding suitable target site in your genome of interest is a key step in every CRISPR experiment. In case of using the SpCas9 nuclease, a target site is usually 20 bp long. The only restriction on targets in a given genome is that the 20 bp target site is directly followed by a so called protospacer adjacent motive (PAM) on the DNA strand (not on the sgRNA sequence!), which is NGG in case of SpCas9. However, a lot more restrictions are imposed by requirements regarding efficiency and specificity of your sgRNAs. The following section should help you designing

suitable sgRNAs for creating knockout mutations in your gene of interest. In general, these design criteria are universal between species.

To design sgRNAs for your gene of interest, you need to know the exact sequence of the gene. Nowadays, sequence information for most genes are available in public databases.

- Determine 5' to 3' DNA sequence of target gene (start codon to stop codon). Online databases such as Phytozyme or Ensembl Plants contain the genomic information of many plant species. You can find sequences for your gene of interest either by searching for the name or gene ID of your gene of interest or by BLAST search using protein sequences from closely related, well annotated model species as input sequence.

  Note: A lot of crop plants exist in various local varieties which are not 100% identical on DNA level to reference sequences of that plant in public databases. While those nucleotide polymorphisms are rare, they can render a CRISPR experiment nonfunctional. If working with non-model organisms, it is therefore good practice to PCR amplify your gene of interest using primers designed on the database sequence and Sanger sequence the PCR amplicon to ensure that the gene sequence in the variety that you are working with is identical to the reference sequence in the public database.
- Determine exonic and intronic regions and transcript variants. Exon/Intron predictions are generally available in the same public databases and can be exported together with the DNA sequence information. If not available, you can consider using RNA sequencing or utilizing prediction algorithms and/or alignments with homologous exonic regions of closely related species to determine exonic regions.

Genes can be several kilobases in length, so it is important to consider which areas of the gene one wants to target. In some cases, the target region can be very narrow, e.g. if one wants to create mutations in specific active residues of a protein. In the following, we describe a general approach for the most common use case, the generation of a functional knockout of a given protein.

- For generation of a functional knockout, you need to target exonic coding regions within the gene. Choose coding exons which form part of all known transcript variants (see the negative example of exon 3 in figure below, which is not part of all mature transcripts) and avoid targeting untranslated regions, introns, intergenic regions, and intron-exon borders.
- Avoid the very beginning or end of the coding region (ideally, the target site is located between 5-60% of coding region as rule of thumb). Mutations at the start of the gene might have no effect if alternative start codons can be used by the translation machinery and mutations towards the end of the coding region might keep many functional domains of the corresponding protein intact and functional.
- You can predict functional domains of your encoded protein using tools such as InterPro. This helps you to understand which areas of the gene encode highly important functional domains. Ideally you want to target regions upstream of most of the functional domains.

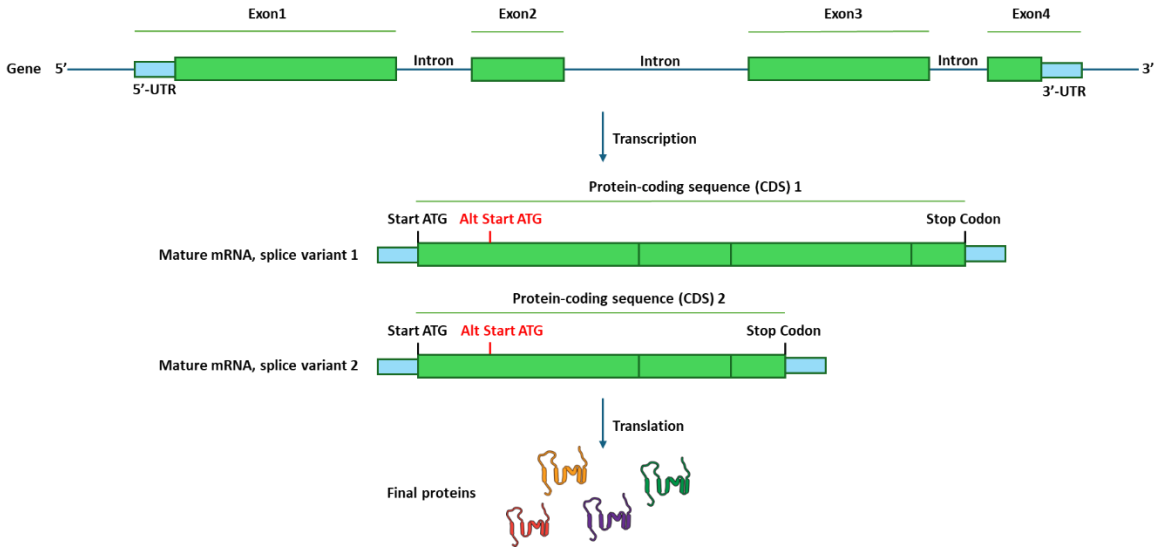

Figure 2: Choosing the right target region in a given gene. By selecting target regions in the gene which are conserved in all possible protein outcomes and are disrupting as many functional regions as possible in case of being mutated, one has a good chance of establishing a functional gene knockout in a CRISPR experiment.

Once you have identified a wider region in your gene that you want to target, you need to find suitable sgRNA target sites in that region. In general, sgRNAs guiding the Cas9 nuclease can target any 20 bp stretch of DNA that is followed by a protospacer adjacent motif (PAM) NGG (with N = A or C or T or G). Therefore, lots of potential sgRNA target sites exist in most genes. One aims to find sgRNA target sites with **high on target efficiency** and **minimal off target activity**. We highly recommend taking advantage of freely available online resources for sgRNA design and selection, such as CRISPOR or CCTOP.

- If utilizing an online program such as CRISPOR or CCTOP, copy/paste DNA sequence of target region(s) to search for sgRNA candidates. Make sure to select the NGG PAM site, SpCas9 nuclease, and the correct reference genome as options (contact the tool providers to add new reference genomes if your plant reference genome of choice is not available). If choosing sgRNA manually (**not recommended**), highlight potential target sites (a target site is any 20 bp stretch which is followed by an NGG PAM at its 3'-end, with N = T or A or G or C) within the chosen exonic target regions. Note that target sites can be on both DNA strands, though a target site will always be in 5'-3' direction with the NGG PAM at its 3' end (see design example in pre-step 2). Then pick suitable target sites according to the following constraints:
- The target site should not contain any restriction enzyme sites that will interfere with cloning processes during your vector assembly. In case of the ENABLE® Gene Editing Kit, these would be BsaI (5'...GGTCTC...3') or BbsI (5'...GAAGACNN...3') restriction enzyme sites.
- The target site should not contain highly similar sequences in your genome of interest to avoid off targeting. We recommend that your sgRNA target site does not have off targets with fewer than three mismatches to your target site candidate and also no off targets without any mismatches within the seed region, 8 - 12 bp adjacent to PAM (off targets with mismatches outside the seed region are more likely to be targeted by Cas9). Off targets and mismatch locations can e.g. be found using CRISPOR or CCTOP online tools. Most of these tools also provide various specificity scores, which are overall scores predicting the influence of all possible off targets and which can also be used to find the most specific target sites for your CRISPR experiment.

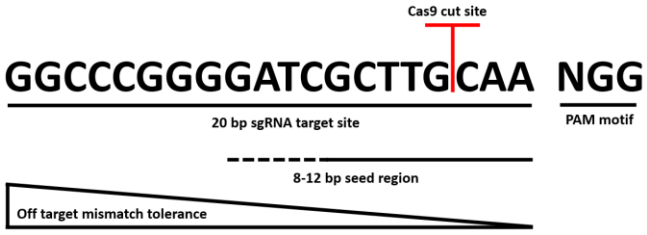

Figure 3: Exemplary sgRNA target site with relevant features.

Note: Most of the sgRNA design tools are not optimized for polyploid organisms which can cause problems when looking for suitable target sequences in certain plants. This is especially relevant for off target search as homeologues of a given gene are considered as off targets and therefore, those sgRNA sites are ranked badly even though in most cases, you want to target all homeologues of a given gene on all genomes to generate functional knockouts. For some polyploid plants, specific sgRNA design tools exists (e.g. WheatCRISPR) but in most cases, you will need to manually curate the rankings of the potential sgRNA target sites from the web tools and cannot use combined specificity scores.

- Most online sgRNA design tools give you some type of efficacy score which is supposed to be an indicator on how well a sgRNA will work on your intended target. Similarly, various models predict the likelihood of generating out of frame mutations at your target site (e.g. the Lindel- and Out-of-frame scores in the CRISPOR online tool). While a high score is certainly nice to have, it is worth to keep in mind that most of these scores are based on experiments in mammalian cell lines, so the scores might not always align with mutation efficiencies in plants.
- When you plan to express your sgRNA using a Polymerase III promoter, such as U3 or U6 promoters (as it is the case in the ENABLE® Gene Editing Kit), avoid Poly-T stretches in your sgRNA target sites (>3 T in a row, serve as stop signal for Polymerase III)
- Avoid a GC content of less than 25 % or more than 75 % in your 20 bp target sequence.

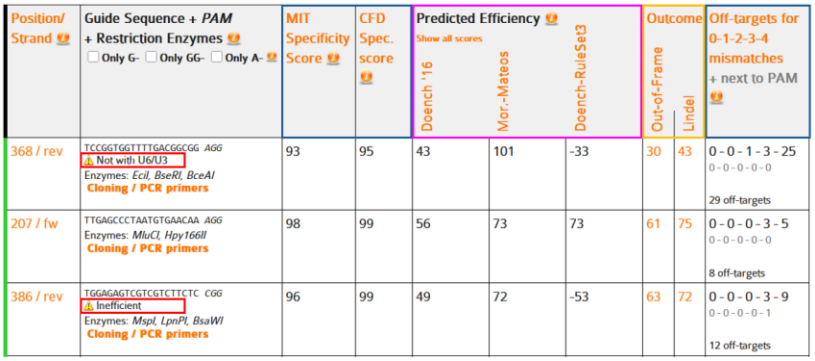

Figure 4: Online tools such as CRISPOR (depicted, screenshot from March 2025) are useful in finding suitable target sites in your gene of interest by providing ***predictions*** *about sgRNA specificity and potential off targets (blue), mutation efficiencies (pink), mutational outcome (orange) as well as potential issues in certain target sites, such as poly-T stretches (red).*

- Avoid any sgRNA target sites that would lead to mutations close to intron/exon borders as this can lead to unintended effects. We recommend a distance of at least 5 bp between the Cas9 cut site (the Cas9 cut site is always 3 bp upstream of the PAM motif in a given target site) and the intron exon border.
- When designing multiple sgRNAs for one target gene, ensure that they are not too close to each other to ensure diversity in the targeting space (we recommend that the cut sites of a given sgRNA is at least 5 bp away from the cut site of the other sgRNA). Similarly, it might complicate downstream mutant analysis if multiple target sites in one gene are too far apart, as you ideally want to amplify the whole targeting space

in one PCR. We recommend in case of designing two sgRNAs (as it is the case in the ENABLE® Gene Editing Kit) that they are ideally less than 400 bp apart from each other.

- At this step, you probably have narrowed down the number of suitable sgRNAs in your gene of interest to a handful of sgRNAs. As an optional step to increase you chance of finding the best sgRNAs, you can now predict the secondary structure of each potential sgRNA. For that, add each potential 20 bp sgRNA target sequence to the sgRNA scaffold sequence below and predict RNA secondary structure using the ViennaRNA RNAfold server, ensuring the predicted sgRNA structure contains 3 stem loops, which have been shown to be important for an optimal function of the sgRNA.

  *Note: The PAM sequence is **not** part of the final sgRNA.*

  exemplary sgRNA target sequence + Scaffold
  GGCCCGGGGATCGCTTGCAAGTTTCAGAGCTATGCTGGAAACAGCATAGCAAGTTGAAATAAG
  GCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

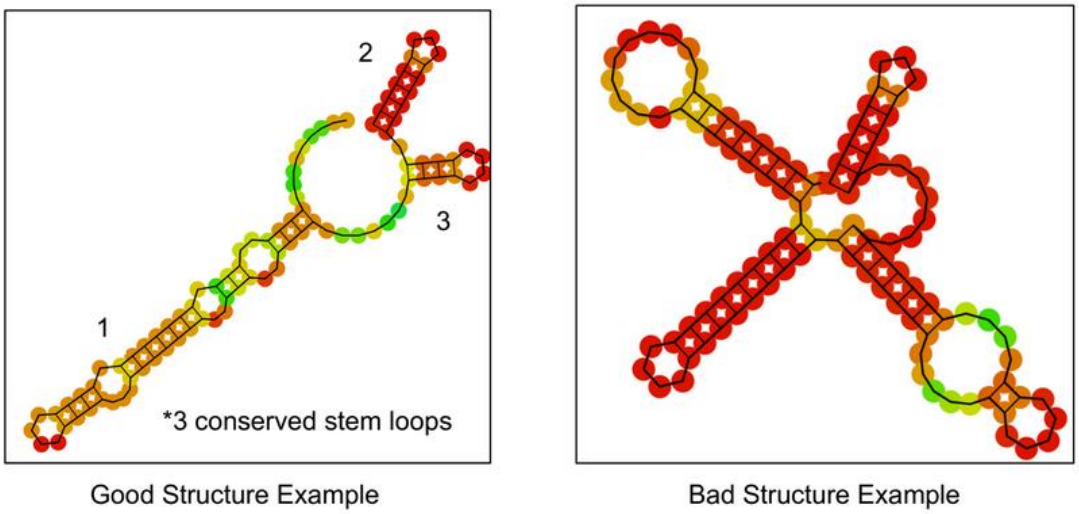

Figure 5: Structure predictions of two different sgRNAs.

With all the information gathered for the target sites, it is time to take a decision. Usually there are no perfect sgRNAs which perfectly tick all the boxes. Often one has to compromise on things like the efficacy scores, the secondary structures or others. We always recommend to use multiple sgRNAs per gene to increase chances for a successful gene knockout, hence the ENABLE® Gene Editing toolkit allows you to clone two sgRNAs for your target gene.

PreStep 2: Designing complementary oligo pairs for subcloning of target sites

To clone your two unique 20 bp target sites into the ENABLE® vector system, you will need to design and order two oligo pairs (four oligos in total) encoding your target sequence as well as overhangs relevant for cloning. The PAM site is **not** part of the sgRNA and therefore not included in the oligo sequences with overhangs. We recommend noting down the 20 bp target site sequence with the PAM NGG at its end when designing the oligos as it is important that the target site is cloned into the correct orientation (see examples below). Design tools such as CRISPOR or CCTOP will provide target sites in the correct 5'-3' orientation with the PAM site NGG at the end.

- Design two complementary oligos by adding **GTTG** (if working in a monocot species) or **ATTG** (if working in a dicot species) to the 5'-3' 20 bp target sequence (all 20 bases up until the PAM NGG) of sgRNA target site 1 for oligo 1 and AAAC to the reverse complement of sgRNA target site 1 for oligo 2.

- Design two complementary oligo by adding **GTTG** (if working in a monocot species) or **ATTG** (if working in a dicot species) to the 5'-3' 20 bp target sequence (all 20 bases up until the PAM NGG) of sgRNA target site 2 for oligo 3 and AAAC to the reverse complement of sgRNA target site 2 for oligo 4.
- Order all four oligos (two complementary oligos per sgRNA target site).

Exemplary DNA sequence of a monocot gene with two target sequences of interest.
Both complementary DNA strands are depicted.

```
5′GCTTCCATGAGTCGTAGCCGTAGCGTAAGTGCTAGTTTGTGTACCATCGCGTATGGAT 3′
3′CGAAGGTACTCAGCATCGGCATCGCATTCACGATCAAACACATGGTAGCGCATACCTA 5′
```

In case of sgRNA1 (light gray), the target site is already written down in 5'-3' direction with the PAM site (green) at the 3'-end, which makes oligo design straightforward. **Please note that if working with a dicot species, you will need to add an ATTG overhang to Oligo 1 instead of the GTTG one.**

```
                    N1                    N20
sgRNA1+PAM      5′  AGTTTGTGTACCATCGCGTATGG  3′
Oligo 1         GTTGAGTTTGTGTACCATCGCGTA      Overhang+sgRNA1
Oligo 2         AAACTACGCGATGGTACACAAACT      Overhang+sgRNA1 RC
```

In case of sgRNA2 (dark gray), the target site is on the reverse strand of the DNA. We therefore reverse the sequence (note: not reverse complement!) while noting the target site down so that it is written in 5'-3' direction with the PAM site NGG is the 3'-end of the sequence for oligo design.
Please note that if working with a dicot species, you will need to add an ATTG overhang to Oligo 1 instead of the GTTG one.

```
                    N20                   N1
sgRNA2+PAM      3′  GGTACTCAGCATCGGCATCGCAT  5′
Reversed                TACGCTACGGCTACGACTCATGG
Oligo 3         GTTGTACGCTACGGCTACGACTCA      Overhang+sgRNA2
Oligo 4         AAACTGAGTCGTAGCCGTAGCGTA      Overhang+sgRNA2 RC
```

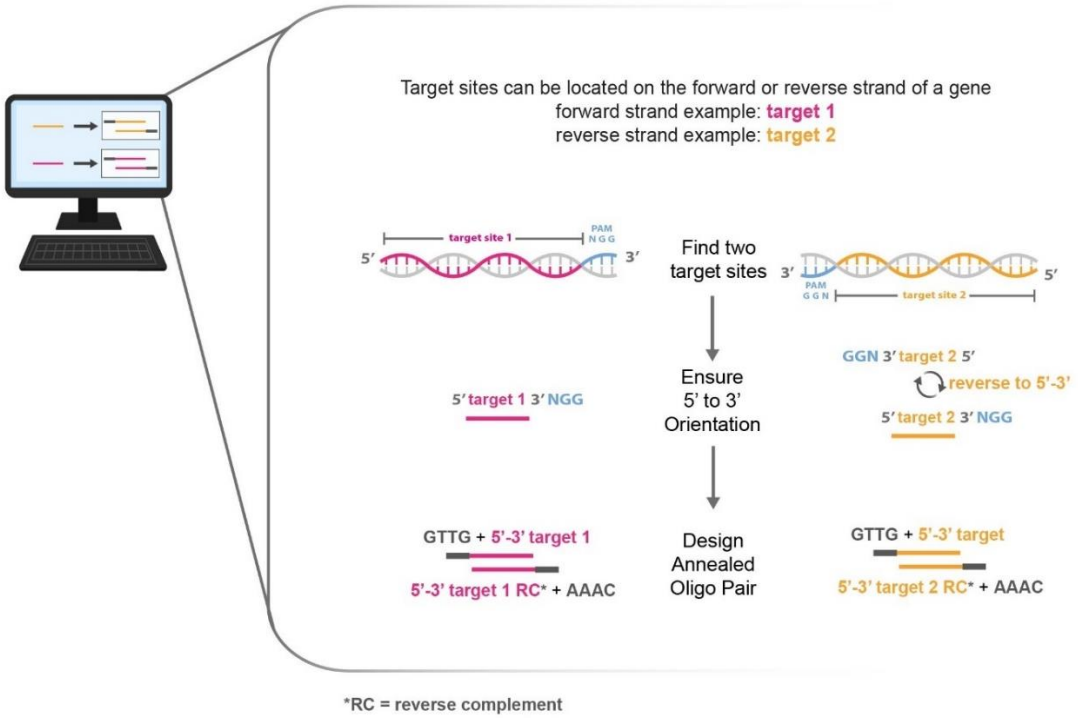

Figure 6: Visual explanation of oligo pair design for a monocot species. For dicot species, add ATTG overhangs instead of GTTG overhangs.

Cloning Steps

Step 1: Plasmid recovery

As a first step, you will need to recover the ENABLE® vector set (pGMF1 - pGMF6) depending on the format in which they are sent to you. Addgene provides plasmids as glycerol stock, liquid DNA or filter paper. In the following, we describe plasmid recovery from filter paper, as this tends to be the procedure which is the most challenging. If your plasmids are sent in another format, please refer to Addgene's accompanying protocols on plasmid recovery.

- Cut out each filter paper disk using a sterile blade and place separately into labelled tubes with 20 µL of double-distilled nuclease-free water.
- Incubate for 10 minutes at room temperature, mixing every 2 minutes.
- Store eluted plasmid DNA at - 20 ˚C
- Recommended: To prepare larger quantities of DNA, transform 2 µL of each plasmid elution into competent *E. coli* following standard protocols. Select pGMF1-M, pGMF1-D, pGMF2-M, pGMF2-D, pGMF4, pGMF5, and pGMF6 on LB plates containing **Ampicillin** (100 µg/mL) and inoculate a single colony overnight for subsequent DNA extraction. We recommend blue-white selection by using **IPTG** (final concentration 0.5 mM) and **X-Gal** (final concentration 80 µg/mL) on top of Ampicillin for pGMF1-M, pGMF1-D, pGMF2-M and pGMF2-D, colonies should appear blue. Note that pGMF4/5/6 do not contain a lacZ cassette, therefore colonies transformed with pGMF4/5/6 should always appear white, independent of presence or absence of IPTG/X-Gal. Select pGMF3 on LB plates containing **Kanamycin** (50 µg/mL; no IPTG/X-Gal selection needed) and select red colonies for inoculation and subsequent DNA extraction. Store plasmid DNA at - 20 ˚C.
- *Note: For all E.coli transformations here and in the following, please follow manufacturer's instructions or standard protocols. For all plasmid preparations, please use commercial plasmid preparation kits following manufacturer's instructions or use standard protocols.*

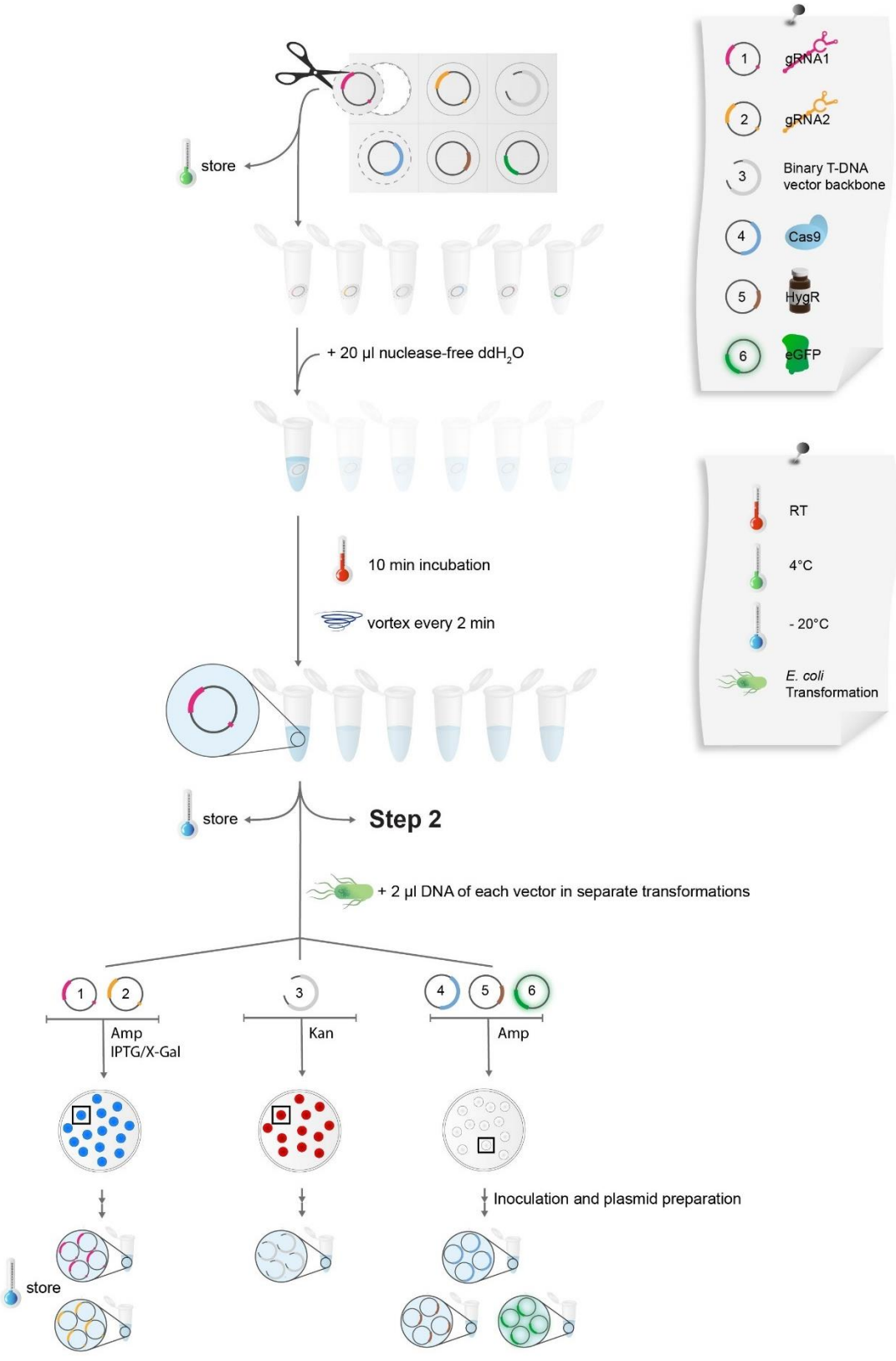

Figure 7: Graphic depiction of plasmid recovery. Note that monocot and dicot versions of pGMF1 and pGFM2 are not distinguished in this graphic for simplicity.

Step 2: Subcloning of target sequences

In this step, you will subclone your two target sequences into the vectors pGMF1 and pGMF2, which contain the conserved sgRNA scaffold sequence as well as a Polymerase III promoter to express a functional sgRNA *in planta*.

Important: Depending on the use case, the user will have to choose between using pGMF1-D and pGMF2-D, or pGMF1-M and pGMF2-M for subcloning their target sequences. pGMF1-M and pGMF2-M are recommended for CRISPR editing in Monocot species while pGMF1-D and pGMF2-D are recommended for work in Dicot species.

Note: If you want to express just one sgRNA instead of two, you will need to subclone the same annealed oligo pair into both pGMF1 and pGMF2 at this step, as the later cloning steps require presence of pGMF1 and pGMF2 with subcloned target sequences.

- Adjust oligo concentration to 10 µM with double-distilled nuclease-free water.
- In two separate tubes for each sgRNA target sequence, mix 2 µL of the corresponding complementary oligos and incubate for 5 minutes at room temperature.

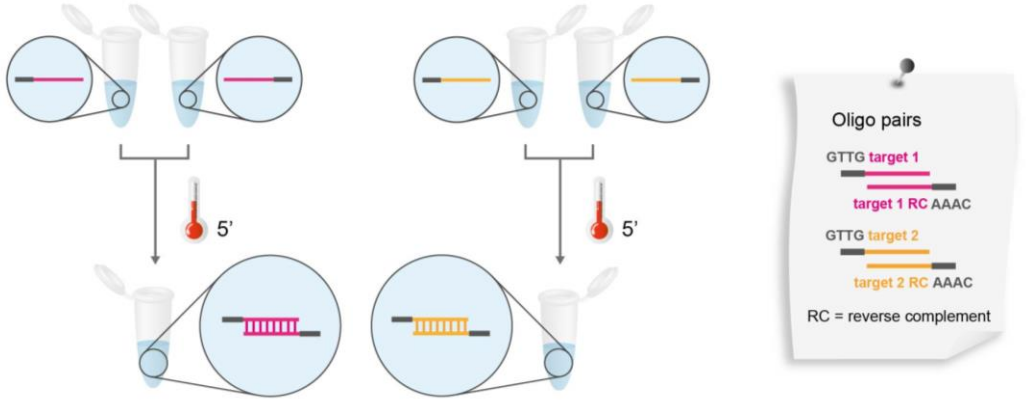

Figure 8: Annealing of oligo pairs. Note that in case of working in dicot species, the oligo overhangs will be ATTG instead of GTTG.

- Choose between monocot and dicot version of pGMF1 and pGMF2 depending on use case (see "Important" note above) and set up the following two restriction-ligation reactions in two separate PCR tubes, resulting in two vectors: pGMF1::annealed_oligo_pair1 and pGMF2::annealed_oligo_pair2.

Reaction 1:	
1 µL	pGMF1 (-M **or** -D, adjusted to 100 ng/ µL)
1 µL	Annealed Oligo Pair 1 (10 µM)
1.5 µL	T4 DNA Ligase buffer (10 x)
1.5 µL	Recombinant BSA (1 mg/ mL)
1 µL	BsaI-HF® (20 U/ µL)
1 µL	T4 DNA Ligase (400 U/ µL)
8 µL	Double distilled water

Reaction 2:	
1 µL	pGMF2 (-M **or** -D, adjusted to 100 ng/ µL)
1 µL	Annealed Oligo Pair 2 (10 µM)
1.5 µL	T4 DNA Ligase buffer (10 x)
1.5 µL	Recombinant BSA (1 mg/ mL)
1 µL	BsaI-HF® (20 U/ µL)
1 µL	T4 DNA Ligase (400 U/ µL)
8 µL	Double distilled water

- Run the following program for restriction-ligation reactions in a thermocycler.

Step	Temperature	Time	
First restriction	37 ˚C	20 s	
Restriction	37 ˚C	3 min	50 x
Ligation	16 ˚C	4 min	
Heat inactivation I	50 ˚C	5 min	
Heat inactivation II	80 ˚C	5 min	
Hold	16 ˚C	until use	

Note 1: This program runs 7+ hours. You can shorten the cycle number to 30 cycles, this might reduce the number of positive colonies though.

Note 2: After running the program, you can store the restriction ligation reaction at 4 ˚C for up to one week or at -20 ˚C long term.

- Transform 2 µL of each restriction-ligation reaction **separately** in two aliquots of competent *E. coli*. Grow overnight at 37 °C on LB plates supplemented with **Ampicillin** (100 µg/mL), **IPTG** (0.5 mM), **X-Gal** (80 µg/mL) and inoculate a white E. coli colony from each transformation plate separately in LB media supplemented with **Ampicillin**. Grow overnight at 37 °C/ 300 rpm and extract plasmid DNA using standard methods.

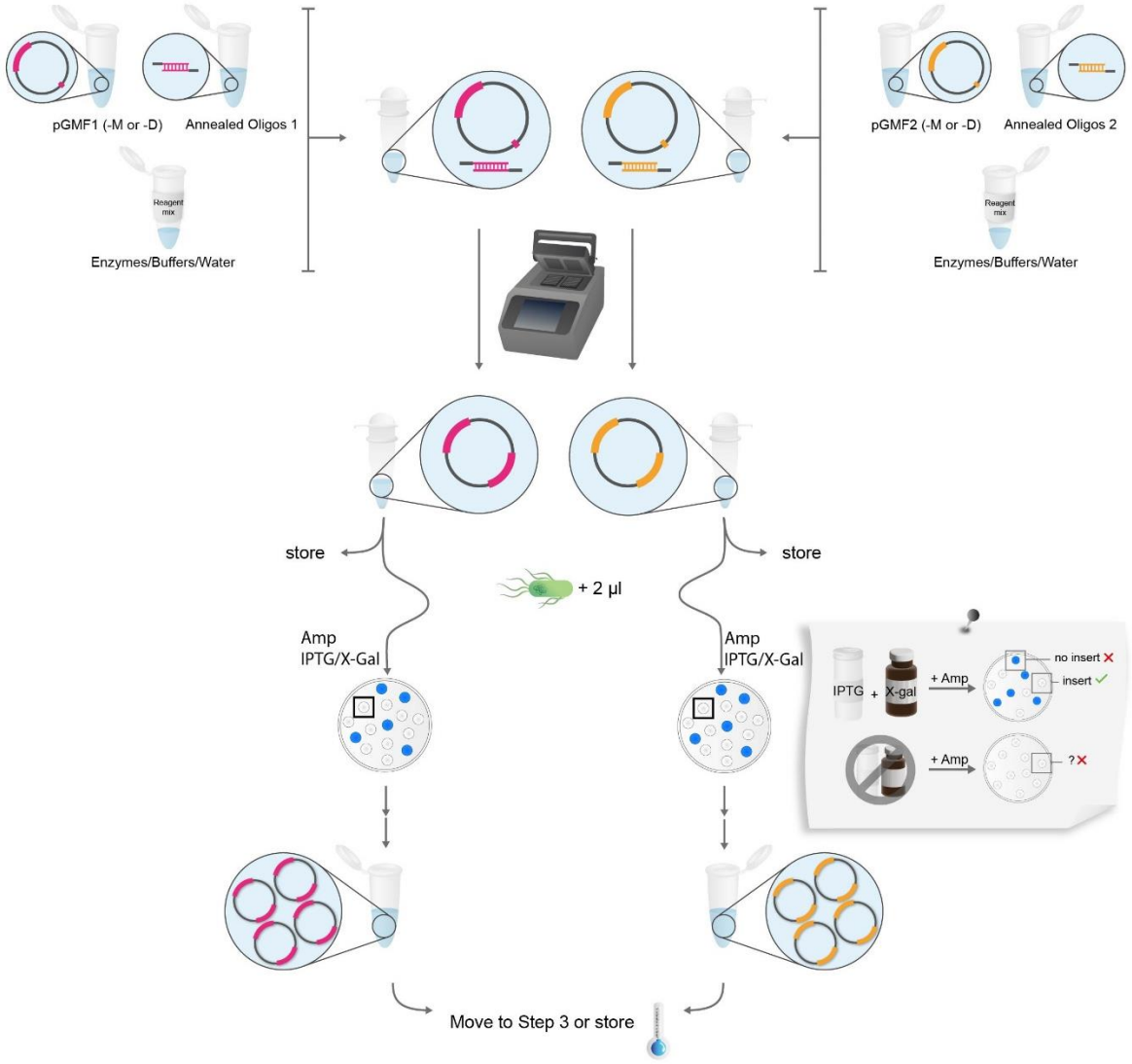

Figure 9: Subcloning of target sequences into pGMF1 and pGMF2.

- Verify both plasmids using one of the following methods:

  Recommended: Sequence both plasmids with primer GMF001 to verify correct integration of target sequence into plasmid or sequence full plasmid. Reference sequences are provided in Suppl. File 1 of our preprint to facilitate alignment with sequencing results.

  Verify integration of target site into pGMF1 and pGMF2 via PCR (either on colony or on extracted plasmid DNA) using primers GMF001/GMF002 (Expected product size for pGMF1/2-M based constructs: 670 bp if successful, 1242 bp if no integration occurred. Expected product size for pGMF1/2-D based constructs: 872 bp if successful, 1444 bp if no integration occurred. Note that this method might not reveal small issues around the cut-ligation sites which can still influence downstream experiments.

Restriction digest of plasmid using BbsI-HF®v2 (cut site GAAGACNN). Expected pattern for pGMF1/2-M based constructs: if positive 3147, 390 bp, if negative: 3147, 962 bp. Expected pattern for pGMF1/2-D based constructs: if positive 2370, 592 bp, if negative: 2370, 1164 bp. Note that this method might not reveal small issues around the cut-ligation sites which can still influence downstream experiments.

- Adjust plasmid concentration to 100 ng/µL.

Step 3: Assembly of binary T-DNA vector

In this step, you will assemble a full binary T-DNA vector containing your sgRNAs, Cas9 and a selection marker. This vector is suitable for subsequent plant transformation by A. tumefaciens/biolistic bombardment or for protoplast transformation. It is important to note that this step combines both plasmids extracted from Step 2 into one final restriction-ligation reaction. Note that depending on your downstream application, you need to choose between adding pGMF5 vector (confers Hygromycin resistance *in planta*) or pGMF6 (confers eGFP expression, e.g. for protoplast experiments) into the final restriction-ligation reaction.

- Set up the following restriction-ligation reaction in a PCR tube to assemble the final binary T-DNA vector. pGMF5 vector (confers Hygromycin resistance *in planta*) can be substituted with pGMF6 (confers eGFP expression for protoplast experiments).

1 µL	pGMF1::annealed_oligo_pair1 (adjusted to 100 ng/ µL)
1 µL	pGMF2::annealed_oligo_pair1 (adjusted to 100 ng/ µL)
1 µL	pGMF3 (adjusted to 100 ng/ µL)
1 µL	pGMF4 (adjusted to 100 ng/ µL)
1 µL	pGMF5 or pGMF6 (adjusted to 100 ng/ µL)
1.5 µL	T4 DNA Ligase buffer (10 x)
1.5 µL	Recombinant BSA (1 mg/ mL)
1 µL	BbsI-HF®v2 (20 U/ µL)
1 µL	T4 DNA Ligase (400 U/ µL)
5 µL	Double distilled water

- Run the following program for restriction-ligation reactions in a thermocycler.

Step	Temperature	Time	
First restriction	37 ˚C	20 s	
Restriction	37 ˚C	3 min	50 x
Ligation	16 ˚C	4 min	
Heat inactivation I	50 ˚C	5 min	
Heat inactivation II	80 ˚C	5 min	
Hold	16 ˚C	until use	

Note 1: This program runs 7+ hours. You can shorten the cycle number to 30 cycles, this might reduce the number of positive colonies though.

Note 2: After running the program, you can store the restriction ligation reaction at 4 ˚C for up to one week or at -20 ˚C long term.

- Transform 2 µL of the restriction-ligation reaction into competent *E. coli* following standard protocols. Grow cells overnight at 37 °C on LB plates supplemented with Kanamycin (50 µg/mL) and inoculate a white colony in LB media supplemented with Kanamycin (50 µg/mL). Grow overnight at 37 °C/300 rpm and extract plasmid DNA using standard methods.

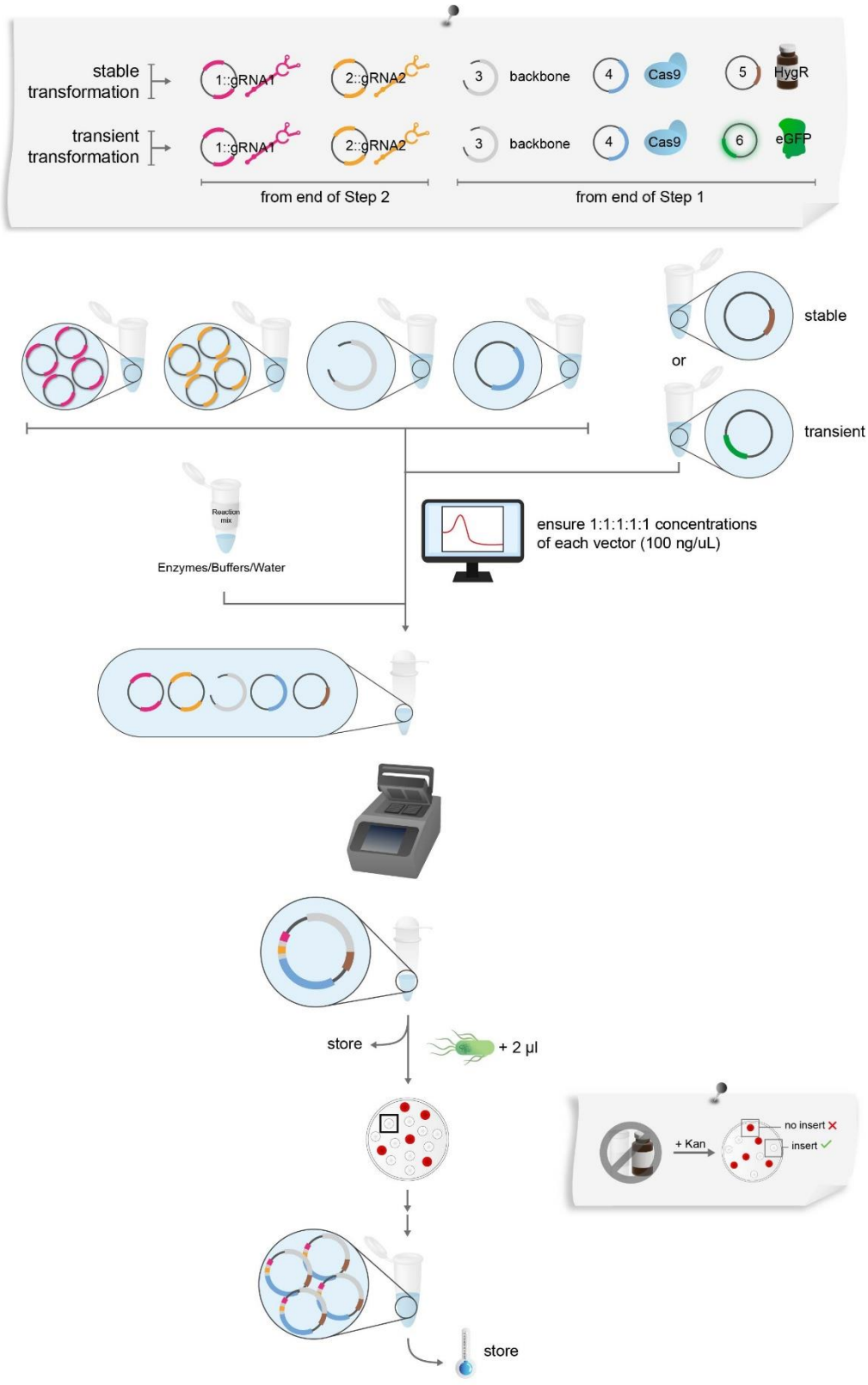

Figure 10: Assembly of binary T-DNA vector

- Verify both plasmids using one of the following methods:

  Recommended: Sequence full plasmid or perform Sanger sequencing using primers GMF001, GMF002 and GMF003 to verify correct integration of target sequences into plasmid. Reference sequences are provided in Suppl. File 1 of our preprint to facilitate alignment with sequencing results.

Restriction digest of plasmid, e.g. using HincII (cut site GTYRAC). Expected pattern for final vector containing pGMF1/2-D and pGMF5 if positive 436, 678, 1537, 2128, 2206, 3227, 4520 bp. Expected pattern for final vector containing pGMF1/2-M and pGMF5 if positive 436, 678, 1537, 2128, 2206, 2823, 4520 bp. Expected pattern for final vector containing pGMF1/2-D and pGMF6 if positive 678, 1744, 2128, 2206, 3227, 4520 bp. Expected pattern for final vector containing pGMF1/2-M and pGMF6 if positive 678, 1744, 2128, 2206, 2823, 4520 bp. Note that if HincII cut site is present in chosen 20 bp target site, the restriction pattern will change.

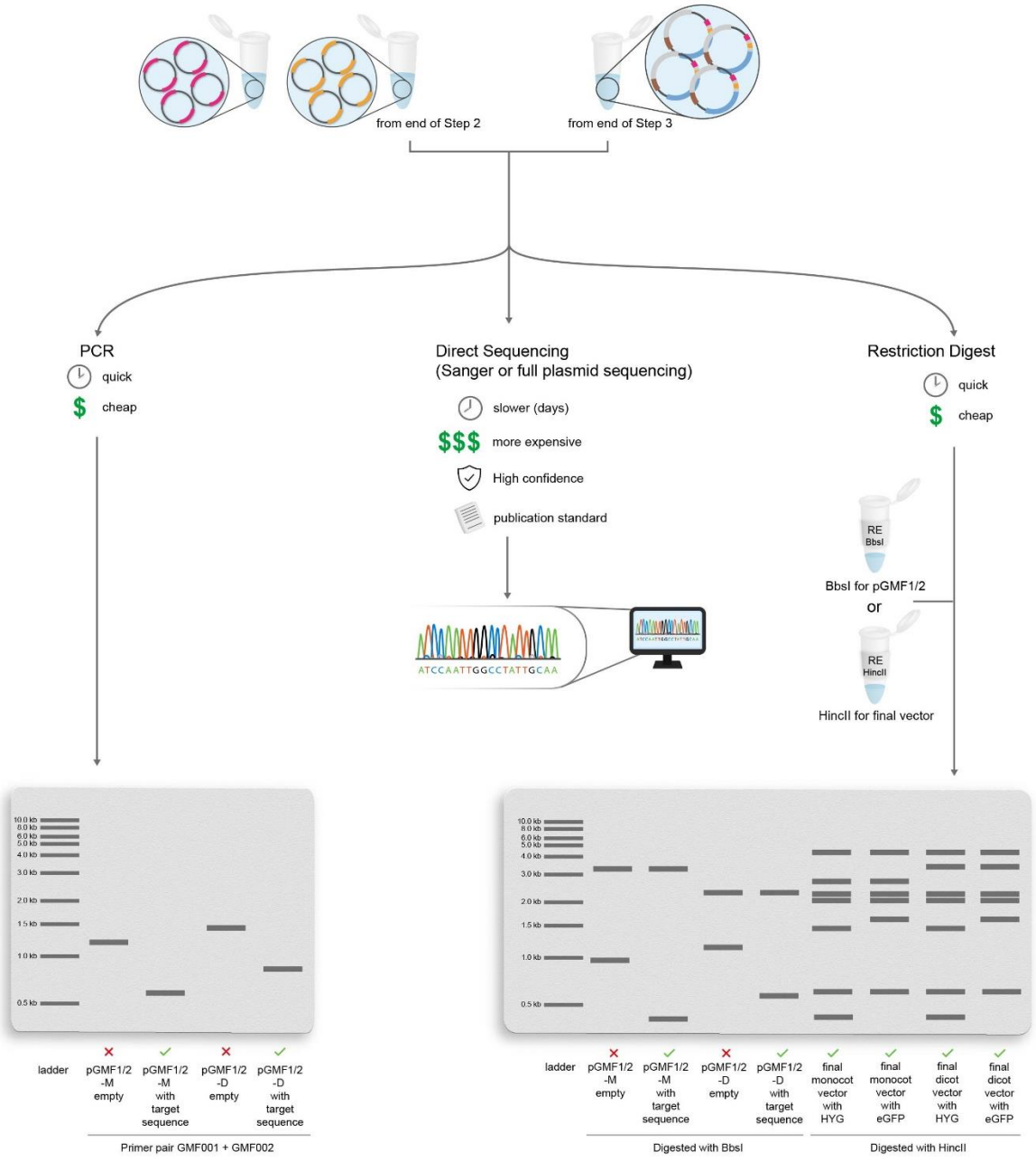

Figure 11: Summary of verification methods for successful cloning of your constructs.

Plant transformation

This step is dependent on the plant species you are working with. In general, for stable plant transformation, follow established protocols for *A. tumefaciens* mediated transformation or biolistic bombardment and select for transformed plants on **Hygromycin**. For protoplast or transient transformation, follow protocols for protoplast/transient transformation and verify successful transformation by checking for **eGFP** expression using fluorescence microscopy.

Editing verification

After successful plant (or protoplast) transformation with your CRISPR vector, you will need to verify that editing of your target gene(s) took place. There are a variety of methods used for that, we will give a short overview on some commonly used ones in the following.

- Phenotype: If a mutation in your target gene leads to a visible phenotype, you can use that to screen for plants where editing was successful. However, to avoid any uncertainties, it is important to still verify editing on DNA level using some of the following methods.
- Sequencing of target genes: PCR amplification of your target genes followed by sequencing allows you to get accurate sequence information of your target sites and is the gold standard for mutation analysis. Sanger sequencing is the go-to method in most cases and especially suitable for stably transformed plants. Sequencing histograms can be analyzed for mutant alleles using tools such as TIDE or ICE. Please note that Sanger sequencing cannot accurately detect low frequency mutation events. This can be a problem e.g. in transient transformation experiments, where frequency of transformed and edited cells can be low compared to non-edited cells. Similarly, it is not suitable to get accurate sequence information for multiple, differently edited alleles, as it can be the case for highly polyploid plants (it can still provide you an idea if gene editing happened or not). In those cases, we suggest amplicon next generation sequencing approaches, albeit them being pricier. Tools such as CRISPResso allow you to analyze NGS data for CRISPR induced mutations.
- PCR amplicon length difference: When targeting a gene with two sgRNAs, large deletions between both sgRNA target sites can occur. When amplifying your target gene by PCR, these can be easily detected as the PCR amplicon in edited plants will be shorter than that of a corresponding wild type plant. While being a very straightforward way of finding mutant plants, please note that large deletions are just one possible outcome of CRISPR edits. Small insertions or deletions (InDels) at each target sites are common outcomes as well and will not be detected using this method despite providing functional gene knockouts. Similarly, if your target sites are too close to each other, the PCR amplicon size difference might be too small to be detected on a standard agarose gel. We recommend choosing two target sites which are at least 50 bp apart from each other if you plan to use this method.
- T7 Endonuclease assay: The target sites are amplified via PCR. If different InDel mutations occurred e.g. in different cells in a protoplast cell culture or on individual alleles in a polyploid plant, you will actually have a variety of slightly different PCR amplicons after the PCR reaction which just differ in a few base pairs around the target site. In a next step, the PCR amplicons are denatured and reannealed. As all PCR amplicons are highly similar, single stranded DNA of one amplicon can reanneal with single stranded DNA of another amplicon. If two non-identical single strands anneal, they will form a heteroduplex structure. T7

Endonuclease I is a DNA cleaving enzyme which recognizes these heteroduplexes and cleaves those amplicons, which can be detected on an agarose gel. In contrast, if only wild type or unedited DNA is present, all amplicons will be perfectly reannealed without heteroduplexes, and no digest will occur. Please note that this method requires access to the T7 Endonuclease I enzyme. Also, this method cannot easily detect homozygous mutants, which can be problem especially when screening stably transformed plants.

- Restriction fragment length polymorphism: This method relies on having a restriction enzyme recognition site in your target sequence, spanning the Cas9 cleavage site (3 bp in front of the PAM). After amplification of the target site using PCR, the PCR amplicon can be digested with the corresponding restriction enzyme. In case of wild type or unedited DNA, the digest will be successful, in case of CRISPR induced mutations, the restriction enzyme recognition site will be altered, hence no digest of the PCR amplicon will occur. This method is very efficient in screening many plants in a short amount of time. However, please note that this method requires access to the restriction enzyme of choice. Also, the need for having a restriction enzyme recognition site spanning the Cas9 cleavage site heavily restricts the choice of target sites in a given gene.

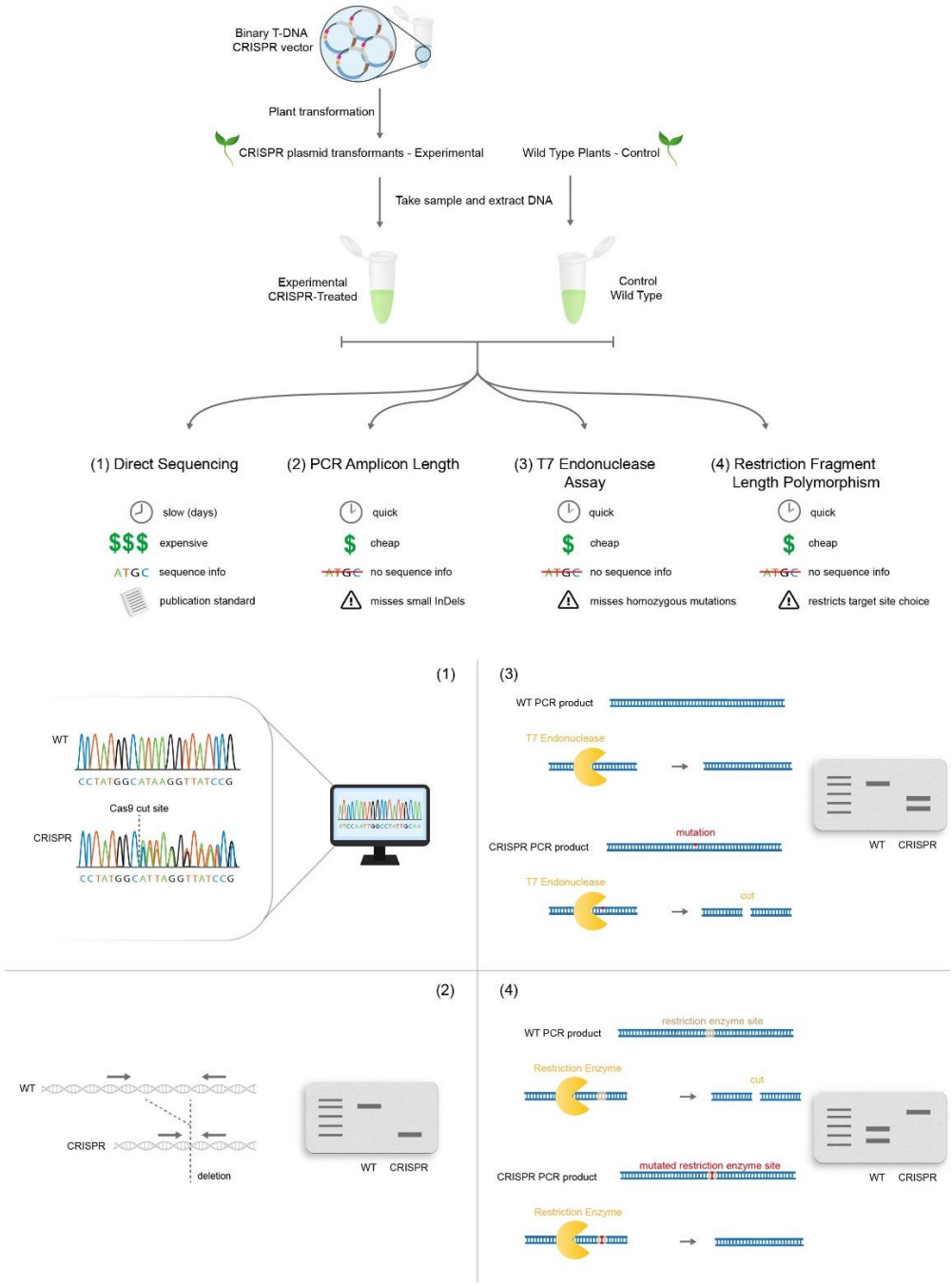

Figure 12: Commonly used methods for detecting CRISPR induced mutations

- In summary, we suggest using methods like PCR amplicon length difference, T7 Endonuclease assay or the Restriction Fragment Length Polymorphism assay to narrow down the numbers of plants of interest and then use sequencing approaches on those plants to get accurate sequence information.
- When trying to create stable mutant lines, be aware of chimerism. Our toolkit uses ubiquitous strong promoters for Cas9/sgRNA expression which are active throughout the life cycle of a plant. That means that editing can happen in all cells of the plant with potentially different editing outcomes in all of those cells, leading to a chimeric plant. To create a stable mutant line, it is important that mutations happen in germline cells so that these mutations are passed on to progeny generations. Given that editing verification is usually performed on DNA taken from leaves (and not from germline cells), there is a chance that you will detect mutations which are not passed on. It is therefore good practice to also verify any type of editing from your

initial mutants in their progeny generations where possible. Any stable mutant allele should be passed on in a mendelian segregation pattern and it should be possible to obtain homozygous mutants in your gene of interest. We also highly recommend to screen for progeny lines which lost the T-DNA containing the CRISPR components through mendelian segregation, as loss of the gene editing systems gives your certainty that no new CRISPR editing can have occurred in this plant and that all edits were passed on stably through the germline.

Limitations of the ENABLE® Gene Editing Kit

The ENABLE® Gene Editing Kit is designed to be an easy and relatively straightforward start into the world of plant gene editing. Its goal is teaching about necessary components of a plant CRISPR project (Cas9 nuclease, sgRNAs, plant selection marker) and making those components accessible for researchers all over the world, as well as training the user in sgRNA design and molecular assembly of a CRISPR vector. In the end, the user is provided with a fully functional genetic construct, ready to be used *in planta*. However, given the scope of this toolkit as a gateway into plant gene editing, there are some limitations of this kit that we want to discuss in the following.

- The ENABLE® toolkit provides you with vectors which have been tested thoroughly in plants such as rice and Arabidopsis. However, when working in your crop of choice, there is a chance that e.g. our Cas9 and sgRNA modules are not as efficient as in some of the tested plants, e.g. due to expression issues with non-native promoters. If you encounter this problem, it is worth to use the knowledge gained through the work with the ENABLE® toolkit to find or design more suitable CRISPR modules in the scientific literature. Our ENABLE consulting service can support you with this.
- Other selection markers might be needed for certain plant transformation protocols instead of Hygromycin or eGFP. We refer to plasmid databases, such as Addgene, where various Golden Gate based selection marker cassettes are available to order, which can be used instead of pGMF5 and pGMF6 if necessary.
- Our ENABLE® toolkit is designed for gene knockouts or creation of deletions between two target sites. Over the years, the CRISPR/Cas system has developed into a universal tool to create even more sophisticated modifications, such as gene insertions, base exchanges or epigenetic changes. We encourage users to dive deeper in the vast possibilities of gene editing approaches after having gained a solid foundation in this technology using this toolkit.

Troubleshooting

While the ENABLE® toolkit was designed for providing a straightforward cloning experience and was tested in plants and protoplast, every experiment can run into problems. This section should provide possible solutions for issues you can encounter. If problems persist, please contact the Grow More Foundation for further troubleshooting advice.

Cloning

- No colonies after transformation: During transformation, ensure that correct antibiotics were used during selection on plates. Also transform a positive control vector with the same antibiotic resistance if available to ensure that *E. coli* cells are still competent and the bacterial transformation protocol is working. If so, repeat the restriction-ligation reaction ensuring all components are included in the reaction. If using DNA straight after filter paper elution for any restriction-ligation reaction, ensure that DNA elution was successful, e.g. by checking DNA presence in your eluate using a Nanodrop or an agarose gel.

- Bacterial growth on selection plate but no formation of distinct colonies: Ensure that plate contains antibiotic at indicated concentration and repeat bacterial transformation. Also ensure that you do not plate out too many cells, e.g. try plating different volumes of bacteria after transformation.
- Colonies appear, but no/few white colonies: Repeat restriction-ligation reaction ensuring all components are included in the reaction.
- White colonies appear but sequencing results/plasmid verification negative: Pick 2-3 other white colonies from the plate and verify their integrity. If still negative, repeat restriction-ligation reaction ensuring all components are included in the reaction.
- Problems with PCR verification of plasmids: If PCR amplicons are not present or at the wrong size, repeat PCR reaction on 2-3 other white colonies from the plate. Use positive control if possible to ensure all components are included in the PCR mix (e.g. pGMF1 empty vector for the target sequence subcloning step). If still negative, repeat restriction-ligation reaction ensuring all components are included in the reaction.
- Problems with restriction digest verification of plasmids: If no bands are visible, repeat digest ensuring that you add vector DNA. If DNA band is visible but no digestion visible, repeat digest ensuring that all components are included in the preparation of the digest reaction and that the reaction is performed for a sufficient amount of time at the right temperature. Include a positive control if possible, e.g. pGMF1 empty vector for the target sequence subcloning step). If digest occurs but digest bands are at the wrong size, pick 2-3 other white colonies and verify their integrity. If still negative, repeat restriction-ligation reaction ensuring all components are included in the reaction.
- If problems persist with cloning, request fresh batches of enzymes and buffers. Please ensure that all components of the ENABLE® toolkit are stored at the right temperatures as e.g. buffers and enzymes are heat sensitive.

Plant transformation and genotyping

- No transformed plants/protoplasts recovered: Ensure that every vial of plasmid that is used for plant/protoplast transformation is verified, e.g. through verification via a restriction digest. Ensure that hygromycin selection pressure for stable transformation is applied using a suitable hygromycin concentration. Ensure that fluorescence microscopy settings are all set for detection of GFP in case of transient transformation, e.g. by using a positive control GFP emitting sample if available. In case of protoplast transformation, work with various vector DNA amount/protoplast number ratios during the transformation step to find a ratio that gives high transformation efficiencies. In general, further trouble shooting for plant transformation is out of the scope of the ENABLE® toolkit.
- Problems with PCR on target gene in extracted plant DNA: If your target gene cannot be amplified via PCR, you can try some of the following: Use positive controls during PCR, such as amplifying a gene from your extracted plant DNA with primers used in the past/from literature to ensure PCR setup as well as DNA extraction is not the root issue. Design new primer pairs to amplify your target gene. Consult the troubleshooting section of your PCR polymerase provider regarding suggestions on PCR setup for difficult to amplify genes, such as working with different annealing temperatures or PCR additives.
- No/low editing efficiencies in stably transformed plants: Ensure that the plants you are working with are actually transformed with your CRISPR construct e.g. by PCR based verification of presence of the Cas9

gene in the plant. Ensure presence of your intended target site in your gene of interest by Sanger sequencing your target region. Consider designing new sgRNAs for your target gene as not every sgRNA provides high efficiencies. You can consider testing sgRNAs *in vitro* by digesting a PCR product containing your target sites with a Cas9/sgRNA complex before starting the cloning/plant transformation procedure, however, that requires obtaining sgRNA synthesis kits (or synthesized sgRNA molecules) as well as Cas9 protein, assay optimisation and does not guarantee successful editing in plants either.

- No/low editing efficiencies in transiently transformed plants/protoplasts: Ensure high transformation efficiencies in transient expression experiments, based on GFP expression. It can be challenging to verify editing in transient expression experiments as many cells might not be transformed so you have a lot of unedited background DNA in your DNA extraction. NGS based amplicon sequencing might be necessary to pick up editing events. Otherwise, we recommend the same troubleshooting steps as for stably transformed plants (see above).