

VIGS PROTOCOL

Selection of trait: The trait for which VIGS experiment needs to be done, source gene can be orthologous. Hence first step for using this orthologous gene in the crop of interest requires bioinformatics' tools to find out the true orthologous from the available public databases (Such as NCBI, Gramene, Harvest etc.).

Preparation of insert for VIGS

1. Dissolve the oligos cloned in vector using 20 μL 1mM Tris (pH-8.0) and keep overnight at 4 $^{\circ}\text{C}$.
2. Take 1 μL vector with oligos and transform into DH5 α and grow it on LBA plate with ampicilline and keep for overnight at 37 $^{\circ}\text{C}$ in an incubator.
3. Pick the isolated colonies and grow it in 2mL LB medium with ampicilline for overnight at 37 $^{\circ}\text{C}$ in an incubator shaker.
4. Take 16-18 hrs grown culture and do the Mini preparation to isolate the plasmid.
5. Load 1 μL the isolated plasmid on 0.8% agarose gel along with different concentration of λ uncut DNA (10ng/ μL) to estimate the concentration.
6. Double digest the plasmid with *NotI* and *PacI* enzyme to take out the cloned oligos from the above given template.

Double digestion

Plasmid DNA-	15 μL (~ 15ug)
Buffer 2 (10X) -	3.0 μL
Not I	- 3.5 μL
Pac I	- 3.0 μL
BSA (100X)	- 0.3 μL
Water	- 5.2 μL
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Total	- 30.0 μL

Keep at 37 $^{\circ}\text{C}$ in water bath overnight.

7. Load 30 μL the double digested reaction on 0.8% agarose gel along with MW marker.
8. Cut the desired fragment from the gel under UV light with the help of surgical blade and transfer it into 2.0 ml micro centrifuge tube (MCT).
9. To know the weight of the gel piece initial and final weight of the MCT must be recorded.
10. Use GENE CLEAN III Kit for isolating the oligo fragment from the gel piece.
11. Load 1 μL of isolated oligo fragment on 1% agarose gel along with λ DNA (10ng/ μL) to estimate the concentration.

Preparation γ Vector for VIGS:

Double digest the plasmid vector with *NotI* and *PacI* enzyme to take out the PDS gene cloned (200 bp).

Double digestion

Plasmid DNA-	10 μ L (~ 15ug)
Buffer 2 (10X)	- 2.0 μ L
<i>Not I</i>	- 2.0 μ L
<i>Pac I</i>	- 2.0 μ L
BSA (100X)	- 0.2 μ L
Water	- 3.8 μ L
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Total	- 20.0 μ L

Keep at 37 °C in water bath overnight.

12. Load the 20 μ L double digested reaction on 0.8% agarose gel along with MW marker.
13. Cut the desired fragment (linearized vector) from the gel under UV light with the help of surgical blade and transfer it into 2.0 mL micro centrifuge tube (MCT).
14. To know the weight of the gel piece initial and final weight of the MCT must be recorded.
15. Use GENECLEAN III Kit for isolating the linearized vector fragment.
16. Load 1 μ L of isolated linearized plasmid on 0.8% agarose gel along with λ DNA (10ng/ μ L) to estimate the concentration.

Ligation of Vector and Insert

17. Ligation ratio for Insert: Vector (3:1). Set the ligation reaction in 200 μ l PCR tube as follows:

Vector-	1.0 μ L (25 ng/ μ L)
Insert-	3.0 μ l (25 ng/ μ L)
Buffer (10X)	- 0.5 μ L
Ligase enzyme-	0.5 μ L
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Total	- 5.0 μ L

Put the reaction in PCR machine with programme set for 16°C for 18 hrs and store at 4 °C forever.

Transformation of Ligated Reaction:

18. Take 1 μ L of ligated reaction and transform into DH5 α (using transformation protocol) and grow it on LBA plate with ampicilline and keep for overnight at 37 °C in an incubator.
19. Pick the colonies and grow it in 2 mL LB medium with ampicilline for overnight at 37 °C in an incubator shaker.
20. Take 16-18 hrs grown culture and do the Mini preparation to isolate the plasmid (Mini preparation protocol).
21. Load 1 μ L of isolated plasmid on 0.8% gel along with λ uncut DNA (10ng/ μ L) to estimate the concentration.

22. To check the proper cloning, the insert needs to be released from recombinant plasmid. To check this digest recombinant plasmid with *NotI* and *PacI* enzyme (for double digestion see Step 19).
23. Load the digested product on 1% agarose gel the desired cloned fragment band will appear on the gel along with linear vector DNA.
24. Now γ construct is ready for VIGS experiment.

VIGS Inoculation Procedure

Materials:

1. Restriction Endonuclease:
 - Swal
 - BssHII
 - Mlu I
 - Spe I
2. Ambion T7 mMessage mMachine Kit:
 - 2X dNTP/Cap
 - 10X Buffer
 - 10X enzyme
 - nuclease free water
3. 10X GP:
 - 18.77 g Glycine
 - 26.13 g K_2HPO_4
 - Bring Volume to 500 ml with ddH₂O
 - Autoclave 20 min
4. FES:
 - 50ml 10X GP
 - 2.5 g Sodium Pyrophosphate
 - 2.5 g Bentonite
 - 2.5 g Celite

Bring volume to 250 ml w/ ddH₂O

Autoclave 20 minutes

(note: Bentonite and celite do not dissolve)

5. 10-12 day old Wheat or Barley plants, depending on your objective
6. All tips and tubes used are DEPC treated and autoclaved.

Procedure:

1. For each plant to be inoculated, linearize 1µg of 1 plasmid for each the of the 3 genomic RNAs:
 - a. pα42: use Mlu I
 - b. pβΔβa (or pβ42.sp1, depending on the host and experimental conditions): use Spe I
 - c. pγ42 or experimental γRNA plasmid : use Mlu I for wt γRNA, BssHIII or Swa I for the experimental plasmid, depending on the vector backbone (BssHIII is preferable because of more stability)
 - d. Check the linearization of each of the three plasmid on 0.8% gel along with the unlinearized plasmid as control.
 - e. Purify the linearized plasmids with following steps:
 - Add 1µL of 0.5M EDTA, 2.1 µL of 3M sodium acetate (pH-5.2) and 46.2 µL of ethanol (stored at -20 °C) to 20 µL of digested plasmid DNA.
 - Mix well and keep at -20 °C for 20 minutes.
 - Centrifuge at 14,000 rpm for 15 minutes. Discard the supernatant.
 - Add 20 µL of 70% ethanol and centrifuge at 14,000 rpm for 2 minutes.
 - Air dry the sample and re-suspend the DNA in 15 µL of 1mM Tris solution.

Adjust volume so the final template concentration should be 125ng/µL for each reaction. A 2.5 µL reaction volume/plant is generally a good rule of thumb.

(All the linearized plasmids should be strictly of equal concentrations.)

2. Infectious BSMV RNAs is prepared from linearized plasmid by in vitro transcription using T7 DNA-dependent RNA polymerase (Ambion kit no. AM1344) according to manufacturer's instructions.

Transcription Reaction:

80ng template (@125 ng/µL)	0.625
Water	0.125
10X Buffer	0.25
2X Nucleotides	1.25
10X Enzyme	0.25
Total	2.5µL

Reaction should proceed at 37 °C for 2 hours. Place on ice immediately or freeze at -80 °C until ready to use. Do not freeze/thaw transcripts more than 2 times.

3. Transcripts of each of the BSMV genomes are mixed in a 1:1:1 ratio (2.5µL each for a total of 7.5µL /plant). Place the transcripts in ice until FES is added into it.
4. Label and water the plants you intend to inoculate immediately before inoculation.
5. Add 45µL of FES for each 7.5 µL aliquot of the transcription mix immediately before use and apply to the lower 2 leaves.
 - a. Wearing gloves place the tip of your forefinger and thumb together
 - b. Apply 50µl of the freshly mixed FES/ Transcript mix between your forefinger and thumb.
 - c. Place forefinger and the thumb on either side of the base of the leaf to be inoculated.
 - d. Holding the stem of plant with other hand, using light pressure, rub the leaf with the inoculating hand from the base to the tip in a single motion.
 - e. Change gloves, every time using a different construct.
 - f. When finished spray the plants with water and return to the greenhouse. Cover the plant with plastic bag or dome overnight to maintain moisture.
 - g. Symptoms (if knockdown phenotype is observable) start appearing in 7-10 days and reach at the pick between 15-18 days.

Plant growth conditions:

- 1) Grow wheat (one seed in each pot) in sunshine mix (with 14 g Nutricote 14-14-14 100-day timed release per 1 L of sunshine mix).
- 2) Plants should be grown at 22°C day and 18°C nights, with 23-50% RH and 16 hours light.
- 3) Use normal fertilizer regime for wheat (nutrient water once a week).