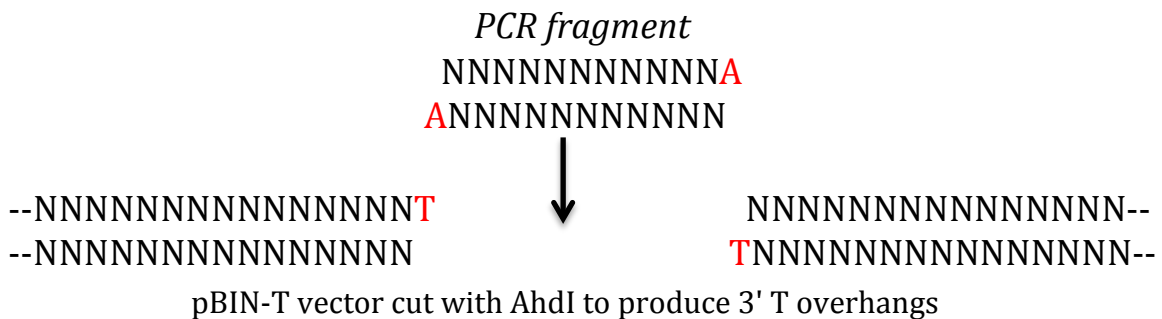
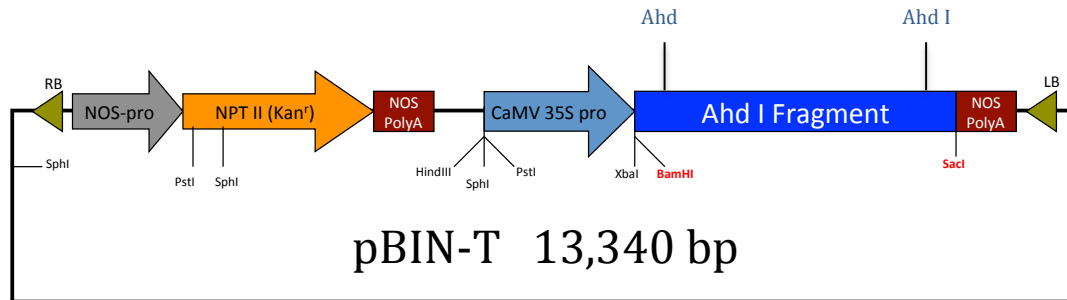


pBIN-T Cloning Protocol

Modified July 2015 by David Neece

pBIN-T is a modified version of the pBIN-m-gfp5-ER vector, in which the GFP gene has been replaced with an AhdI cloning cassette to allow for direct cloning of PCR products.

1. Vector Preparation - vector pBIN-T is cut with AhdI* to yield the cloning site for PCR fragment.



*Ahd I cuts pBIN-T twice, resulting in two fragments: 440 bp and 12,900 bp.

Minipreps to obtain pBIN-T vector

Use the Qiagen miniprep kit, following the standard protocol. Plasmid prep yield is low, so you need to grow enough culture for several minipreps (4-6 preps). Use 3-5ml culture for each prep and warm elution buffer EB to 70° C to maximize elution efficiency.

AhdI Digest of pBIN-T vector

Set up 2 identical reactions:

DNA:	20-30 ul (appx 1-2 ug)
10x Buffer:	10ul
100x BSA:	1 ul
AhdI:	3 ul
H ₂ O:	57 ul
Total:	100 ul

Incubate at 37° C for 2 hours.

Heat-kill enzyme at 65-70° C for 20 minutes

Cool on ice and then add 1/10 volume of 3M Sodium Acetate

Add 3 volumes of 100% ethanol

Incubate at -20° C for 1 hour

Spin @13,000 rpm, 4° C, for 10 minutes, then discard supernatant

Wash with 300 ul of 70% Et-OH

Spin @13,000 rpm for 5 minutes, then discard supernatant

Air dry the pellet (will be difficult to see)

Resuspend each pellet in 20 ul sterile H₂O

Gel purification of pBIN-T

Pour a 0.8% agarose 0.5 X TBE mini-gel.

Run 1-2 ug of the digested pBIN-T vector per well.

Run at appx. 80V until the loading dye band is 3/4 way down the gel.

Place plastic wrap on UV lightbox, then place gel on plastic wrap.

Quickly take a photo (UV light will degrade DNA, so do not expose very long).

Using a glass cover slip, cut out the upper (12.9 Kb) fragments and transfer to two 1.5ml tubes.

Note: when cutting, press straight down with the coverslip. Do not cut back-and-forth like when using a knife, because this will damage the UV lightbox screen.

Proceed with Zymoclean gel purification.

Zymoclean gel purification

Use Zymoclean Gel DNA Recovery kit, according to standard protocol with the following modifications:

Dissolve gel slice at 42°C (higher may damage DNA).

After 42°C incubation, add one additional volume (= gel slice volume) of water.

Elute using 12ul of elution buffer (protocol says use 6ul).

When measuring on Nanodrop, elution buffer must be used as blank.

Check the quantity of DNA on the Nanodrop, and then run 100-200 ng on a 0.8% agarose gel to check quality.

Ligation of PCR product into pBIN-T vector

For maximum efficiency, the PCR reaction should be performed the same day as the ligation. Perform PCR as normal, followed by a Qiaquick PCR Cleanup step.

Ligation Reaction:

The NEB Quick Ligation Kit is used for the ligation. This kit uses concentrated DNA ligase, which is essential for the inefficient T/A cloning of PCR products. We also supplement the reaction with additional ATP, to maximize the efficiency of the ligase. Ligation reaction volumes should be kept as small as possible (10-15ul is best). Adjust vector and insert volumes below according to your sample concentrations.

pBIN-T vector	1 ul (30-50 ng)
PCR Insert	4-5 ul (100-200 ng total)
2X Ligase Buffer	7.5 ul
10mM ATP*	1 ul
Quick T4 DNA Ligase	1 ul
Total	15 ul

Add ligase last, mix thoroughly, then quick-spin.

Incubate at room temperature for 5 minutes, then put on ice or store at -20° C until transformation.

* Fresh ATP is crucial to ligase function, and is very susceptible to thermal breakdown. Thaw at room temperature and then put immediately on ice. ATP is aliquoted in 0.5ml tubes and kept with the Quick Ligase.

Transformation

Transform into NEB Turbo Competent *E. coli* (High Efficiency) according to the NEB protocol. Use **5 μ l** of the ligation reaction for the transformation.

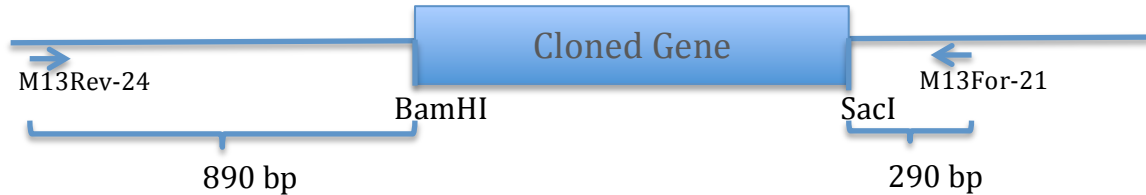
After 1 hour shake, transfer cells to a 1.5ml tube and spin 5000 rpm for 2 minutes. Decant supernatant and resuspend cells in 200 μ l of LB or SOC.

Plate out onto 2 LB/Kan plates; you may want to plate a low volume and a high volume plate (e.g. 50 μ l and 150 μ l, if total volume is 200 μ l); avoid plating volumes less than 50 μ l because spreading will be uneven. Incubate overnight @37° C.

There is no blue/white selection with this vector, so transformants may be screened by colony PCR using your original PCR primers.

Checking for proper orientation of PCR product

PCR cloning is bi-directional, so you must screen transformants for proper orientation in the vector



Using your cloned gene Forward primer and the M13For-21 primer as a Reverse primer for PCR, transformants in the proper orientation will yield a PCR product, whereas transformants with a reverse orientation insert will not. You should also run a [cloned gene Reverse primer + M13For-21 primer] PCR reaction as a control to identify reverse orientation clones.

Transformation into Agrobacterium

After your clone is obtained, it may be transfected into Agro strain GV3101 using the heat shock protocol. After PCR sequencing confirmation, your Agro clone is ready to transform Arabidopsis by the Floral Dip method.