**Protocol for cloning a 4kb transcript in soybean**

1. **Reverse transcription**

SuperScript IV reverse transcriptase was used. I exactly followed the protocol below. 

Notes:

* Use 50uM Oligo d(T)20
* Add ~2ug clean RNA template treated with DNase
* Use RNase Inhibitor, Murine (NEB)
* After RNase H treatment, I pooled 2 reactions together and performed a clean-up using DNA Clean & Concentrator (ZYMO RESEARCH). The final elution volume is 30uL.
* It’s better to use fresh synthesized cDNA immediately. If not, store in -20C.
1. **PCR and electrophoresis**
2. PCR: Phusion High Fidelity DNA Polymerase was used.

**Component**  50 ul reaction

Nuclease-free water to 50 ul

5X Phusion HF buffer 10 ul

10 mM dNTPs 1 ul

10 uM Forward Primer 2.5 ul

10 uM Reverse Primer 2.5 ul

Template DNA 3 ul

Phusion DNA Polymerase 0.7 uL

**Program**

Step 1 98 C 30 s (Matt suggested 3min for this)

Step 2 98 C 10 s

Step 3 60 C 20 s

Step 4 72 C 2 min 30 s

Repeat steps 2-4 for 38 cycles

Step 5 72 C 10 min

Step 6 4 C hold

Notes:

* My transcript is not expressed at a high level. So I added 3 uL of cDNA (~90ng) as template in a 50 uL reaction
* Set annealing temp based on the NEB Tm Calculator for Phusion polymerase specifically
* I usually do four tubes of 50uL reaction because the downstream gel cutting will lose a lot
1. Electrophoresis

0.8% agarose

Cut and extract DNA from gel using GeneJET GEL Extraction Kit (thermo scientific)

Notes:

* Use the minimum elution buffer volume because the gel extraction efficiency is low for long fragments.
1. **Restriction enzyme treatment**
2. Find customized protocol for double digestion in NEBcloner

<http://nebcloner.neb.com/#!/redigest>



1. Incubate at 37 C for 60 min
2. Heat inactivate the enzyme by 65 C for 20min
3. Clean up the reaction with DNA Clean & Concentrator (ZYMO RESEARCH)
4. **Ligation**
5. T4 DNA ligase (NEB)

Component 20 uL Reaction

T4 DNA ligase buffer 2ul

Vector DNA (3kb) 50 ng

Insert DNA (4kb) 200 ng

Nuclease-free water to 20 ul

T4 DNA ligase 1 ul

\*based on a molar ratio of 1:3 vector to insert

1. Gently mix and ant incubate under room temperature for 1 hour
2. Heat inactivate at 65 C for 10min
3. **Transformation and PCR verification**
4. Take 5 ul ligation reaction and do heat-shock transformation using NEB Turbo Competent *E. coli* cells. The protocol

<https://www.neb.com/protocols/0001/01/01/high-efficiency-transformation-protocol-c2984>

1. After the bacteria grow, pick at least 30 colonies on the plate and do colony-PCR using Taq polymerase. The pair of primer used can amplify a short specific region in my transcript of interest.

Note:

* I only identified two out of 30 colonies that have PCR band. So the efficiency of transformation for long fragments are low.
1. Design primers every 500 ~ 600 bp for walking through the 4kb transcript by Sanger sequencing.