pMADRID Restriction Enzyme Cloning

Purpose

This protocol was used to construct pMADRID, a version of the autonomous INTEGRATE plasmid with BsaI restriction sites in its spacer for fast and easy spacer cloning. To construct pMADRID, we used the pSPAIN backbone gifted to us by Sam Sternberg's lab at Columbia university. We used pSL1142, a pSPIN construct from Addgene, as the source of our BsaI spacer.

Materials

- pSPAIN backbone (1 μg)
- pSPIN plasmid PCR template (< 1,000 ng)
- PCR primers for pSPIN BamHI-DR-BsaI-spcr-BsaI-DR-SalI amplicon (IDT)
- Q5 2x MasterMix with standard buffer (New England BioLabs)
- Monarch PCR and DNA clean up kit (New England BioLabs)
- BamHI and SalI restriction enzymes with CutSmart buffer (New England BioLabs)
- Thermosensitive Alkaline Phosphatase (TSAP) (Promega)
- DpnI (New England BioLabs)
- Microcon YM-100 Centrifugal Filters
- T4 DNA ligase with ligase buffer (New England BioLabs)

Protocols

Prepping the insert

1. PCR amplify **BamHI-DR-BsaI-gRNA-BsaI-DR-SalI** region from pSL1142 (pSPIN) plasmid
   (a) Set up 4, 25 μL PCR reactions using pSPIN template (50 ng), 10 μM PCR primers (500 nM final primer concentration), and Q5 2x master mix with standard buffer.
   (b) Amplify DNA using a touchdown cycle starting at 72°C annealing temperature and touching down to 67°C.
   (c) Verify the presence of a 227 base pair band on a 2% agarose gel.
2. DpnI treat remaining PCR products by adding 0.5 μL DpnI to the PCR tubes and incubating at 37°C for 2 hours in a thermocycler.
3. Perform DNA clean up using Monarch PCR clean up kit:
   (a) Pool four 25 μL PCR products together into a sterile, 1.5 mL microfuge tube.
   (b) Add 500 μL DNA binding buffer to 100 μL sample from step #2 (5:1 DNA, buffer:sample for this amplicon size).
   (c) Insert column into collection tube and load sample onto column and close the cap. Spin for 1 minute at 16,000 X G, then discard flow-through.

(d) Re-insert column into collection tube. Add 200 μL DNA Wash Buffer and spin for 1 minute at 16,000 X G. Discarding flow-through is optional.

(e) Repeat wash (Step d).

(f) Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column does not come into contact with the flow-through. If in doubt, re-spin for 1 minute at 16,000 X G to ensure traces of salt and ethanol are not carried over to next step.

(g) Add 20 μL of MilliQ water to the center of the matrix. Wait for 1 minute, then spin for 1 minute at 16,000 X G to elute DNA.

(h) Store and nanodrop the elution, as it contains desired DNA.

4. Set up the restriction enzyme double digest with BamHI and SalI on the cleaned up PCR product according to the table below:

Table 1: pMADRID insert double digestion reaction table

Component	**50 μL reaction**
pSPIN PCR product	13.5 μL (1 μg)
10X CutSmart Buffer	5 μL
BamHI-HF	1 μL
SalI-HF	1 μL
MilliQ H_2O	Fill to 50 μL (29.5 μL)

(a) In a thermocycler, incubate restriction enzyme double digest at 37 °C for 15 minutes.

(b) Heat at 80 °C for 1 hour to inactivate enzymes (SalI, DpnI) in the thermocycler.

5. Using the Microcon YM-100 sentrifugal filters, spin out the digested ends from the insert. These centrifugal filters will retain DNA molecules over 100 bases and pass molecules under 100 bases.

(a) Add 50 μL MilliQ water to a Microcon YM-100 spin column and centrifuge 14,000 X G for 3 minutes.

(b) Add all 50 μL of sample to a Microcon YM-100 spin column placed on top of a microfuge tube and centrifuge at 500 X G for 15 minutes.

(c) Wash spin column with 250 μL MilliQ water and centrifuge again at 500 X G for 15 minutes. Discard filtrate.

(d) Flip the filter column around, and place into a clean collection tube. Add 50 μL of MilliQ water to the other side of the filter and centrifuge at 1,000 X G for 3 minutes.

(e) Nanodrop and store the final elution, as it contains the digested insert with no digested ends.

Prepping the backbone

1. Set up the restriction enzyme double digest on the pSPAIN backbone with BamHI and SalI according to the table below:

Table 2: pMADRID backbone double digestion reaction table

Component	**50 μL reaction**
pSPAIN midiprep	4 μL (1 μg)
10X CutSmart Buffer	5 μL
BamHI-HF	1 μL
SalI-HF	1 μL
MilliQ H_2O	Fill to 50 μL (39 μL)

   (a) In a thermocycler, incubate restriction enzyme double digest at 37°C for 15 minutes.

   (b) Add 1 μL of TSAP for up to 1 μg of DNA. Incubate mixture in the thermocycler for another 15 minutes.

   (c) Heat inactivate enzymes at 74°C for 20 minutes in the thermocycler.

2. Perform DNA clean up using Monarch PCR clean up kit:

   (a) Add 100 μL DNA binding buffer to the 50 μL sample from step #1 (2:1 DNA, buffer:sample for this backbone size).

   (b) Insert column into collection tube and load sample onto column and close the cap. Spin for 1 minute at 16,000 X G, then discard flow-through.

   (c) Re-insert column into collection tube. Add 200 μL DNA Wash Buffer and spin for 1 minute at 16,000 X G. Discarding flow-through is optional.

   (d) Repeat wash (Step C).

   (e) Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column does not come into contact with the flow-through. If in doubt, re-spin for 1 minute at 16,000 X G to ensure traces of salt and ethanol are not carried over to next step.

   (f) Add 20 μL of MilliQ water to the center of the matrix. Wait for 1 minute, then spin for 1 minute at 16,000 X G to elute DNA.

   (g) Store and nanodrop the elution, as it contains desired DNA.

Setting up the ligation reaction

1. Prepare the ligation reaction **on ice** according to the table below:

   (a) Add ligase last to prevent unwanted ligation.

   (b) Incubate overnight at 16°C in a thermocycler.

   (c) Heat inactivate ligase at 65°C for 10 minutes.

   (d) Chill the reaction on ice, then transform 5–10 μL of the ligation reaction into competent DH5α *E. coli* (see heat-shock transformation protocol for more details).

Table 3: pMADRID ligation reaction table

Component	**20 μL reaction**
10X T4 DNA ligase buffer	2 μL
pSPAIN backbone digest	9 μL (265.5 ng)
pSPIN insert PCR product	1 μL (10.26 ng)
MilliQ water	7 μL
T4 DNA ligase	1 μL