Subcloning of Tol2-pU6-gRNA pCAG-Cas9-2A-GFP integrative plasmid

Virtual cutting with Serial cloner> **pCAG is in the Tol2-mCherry plasmid**

Restriction analysis of **Tol2 mCherry**.xdna [Circular] Incubated with **AgeI + BsrGI**

1: **4 779 bp** - From BsrGI[5492] To AgeI[4779]

2: 713 bp - From AgeI[4779] To BsrGI[5492]

Restriction analysis of **PX458-Cas9-GFP**.xdna [Circular] Incubated with **AgeI + BsrGI + SspI**

1: **5 056 bp** - From AgeI[1239] To BsrGI[6295]

2: 2 939 bp - From SspI[7589] To AgeI[1239]

3: 908 bp - From BsrGI[6295] To SspI[7203]

4: 386 bp - From SspI[7203] To SspI[7589]

AgeI

Activity in NEBuffers
NEBuffer 1.1: 100%
NEBuffer 2.1: 75%
NEBuffer 3.1: 25%
CutSmart® Buffer: 75%

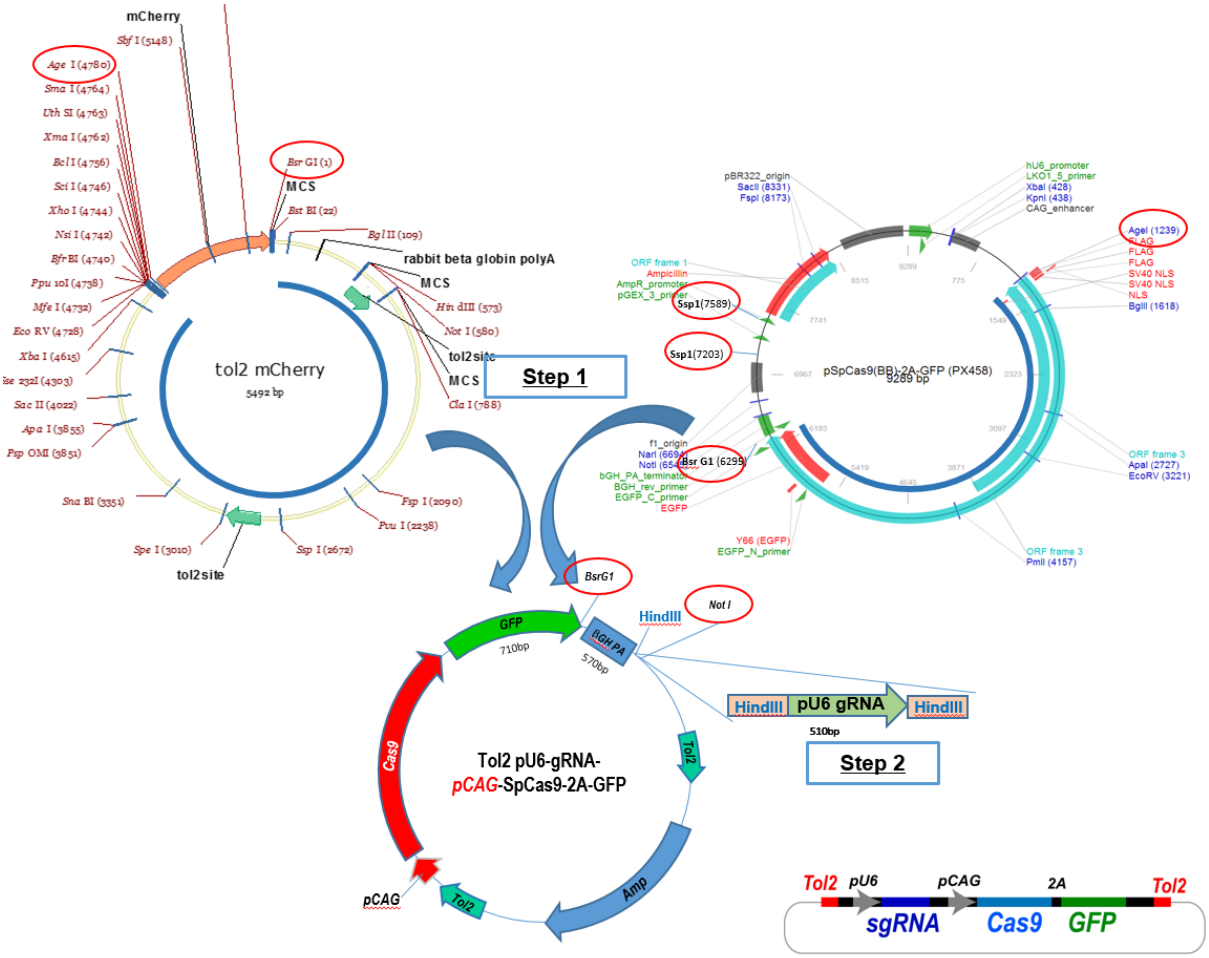
BsrGI

Activity in NEBuffers
NEBuffer 1.1: 25%
NEBuffer 2.1: 100%
NEBuffer 3.1: 100%
CutSmart® Buffer: 25%

SspI

Activity in NEBuffers

NEBuffer 1.1: 50%
NEBuffer 2.1: 100%
NEBuffer 3.1: 50%
CutSmart® Buffer: 50%



pU6 BbsI/BbsI gRNA

```
1     gagggcctat ttcccatgat tccttcatat ttgcatatac gatacaaggc 50
51    tgttagagag ataattggaa ttaatttgac tgtaaacaca aagatattag 100
101   tacaaaatac gtgacgtaga aagtaataat ttcttgggta gtttgcagtt 150
151   ttaaaattat gttttaaaat ggactatcat atgcttaccg taacttgaaa 200
201   gtatttcgat ttcttggctt tatatatctt GTGGAAAGGA CGAAACACCg 250
251   gGTCTTCgaG AAGACCtgtt ttagagctaG AAAtagcaag ttaaaataag 300
301   gctagtccgt tatcaacttg aaaaagtggc accgagtcgg tgcTTTTTTg 350
351   ttttagagct agaaatagca agttaaaata aggctagtcc gtTTTTagcg 400
401   cgtgcgccaa ttctgcagac aaatggctct agaggtaccc gttacataac 450
451   ttacggtaaa tggcccgcct ggctgaccgc ccaacgaccc ccgcccattg 500
501   acgtcaatag
HindIIsite>A/AGCTT

              TTCGA/A
```

PCR product using the PX459 Cas9-gRNA plasmid

HindIII PCR primers pU6 BbsI/BbsI gRNA

```
taataagcttgagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataat
tggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggta
```

gtttgcagttttaaaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatttctt
ggctttatatatcttGTGGAAAGGACGAAA**CACCggGTCTTCgaGAAGACct**gttttagagctaGAAAtagcaag
ttaaaataaggctagtccgttatcaacttgaaaaagtggcac**cgagtcggtgcTTTTTTgtt**aagcttatta

5'primer taataagcttgagggcctatttcccatgat

3' primer ta**ataagcttaacAAAAAAgcaccgactcg**

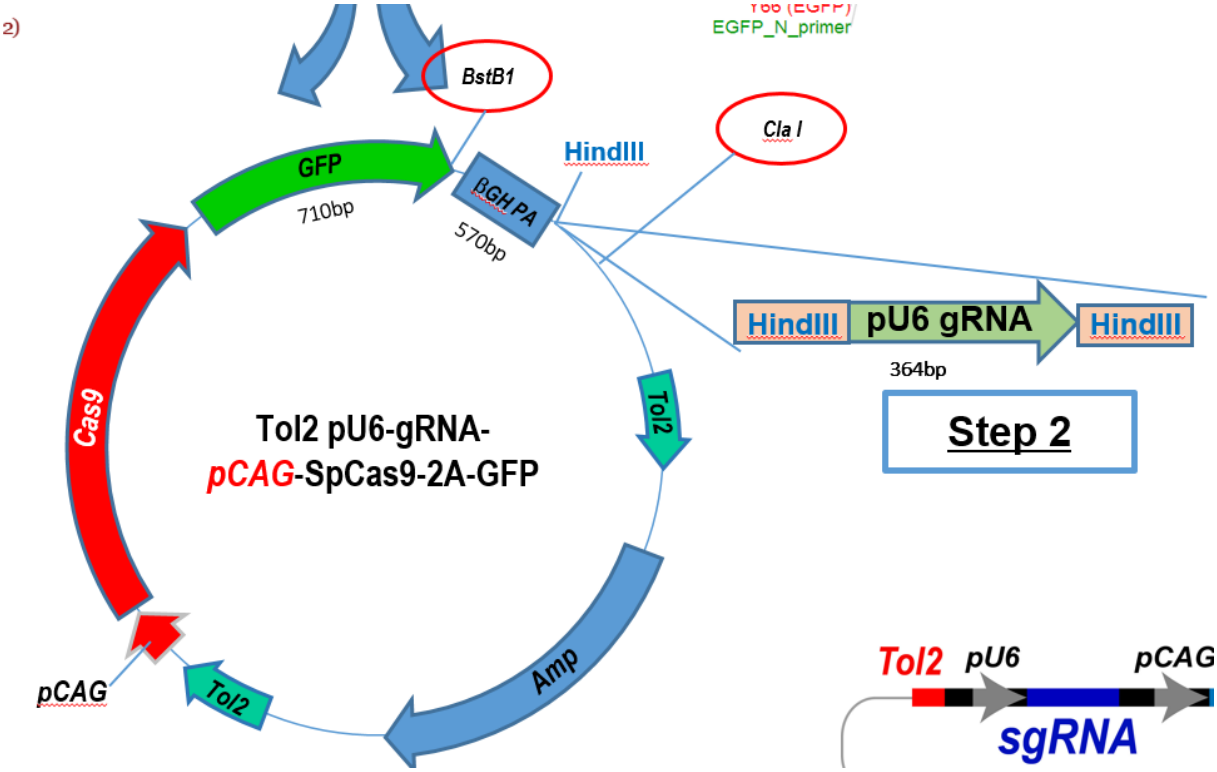
3'-5' **cgagtcggtgcTTTTTTgtt**aagcttatta

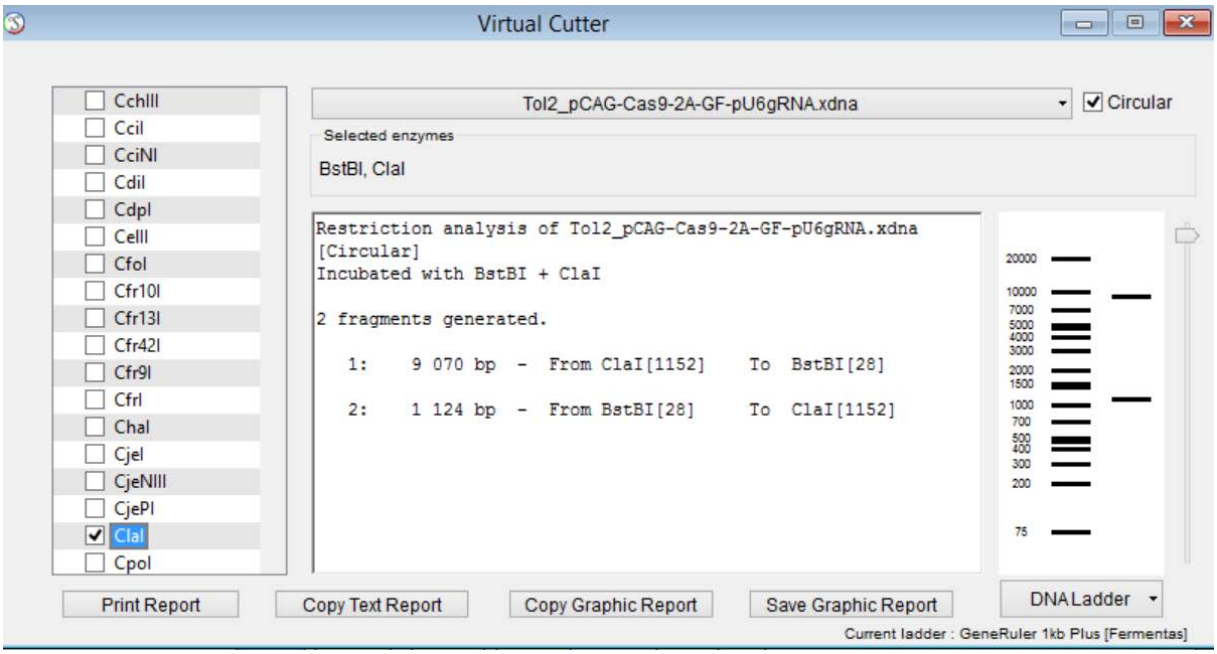
PCR product **352** bp Tm- 57 C

Care: HindIII do not cut well with only 2 extra nt. Better 3-4.

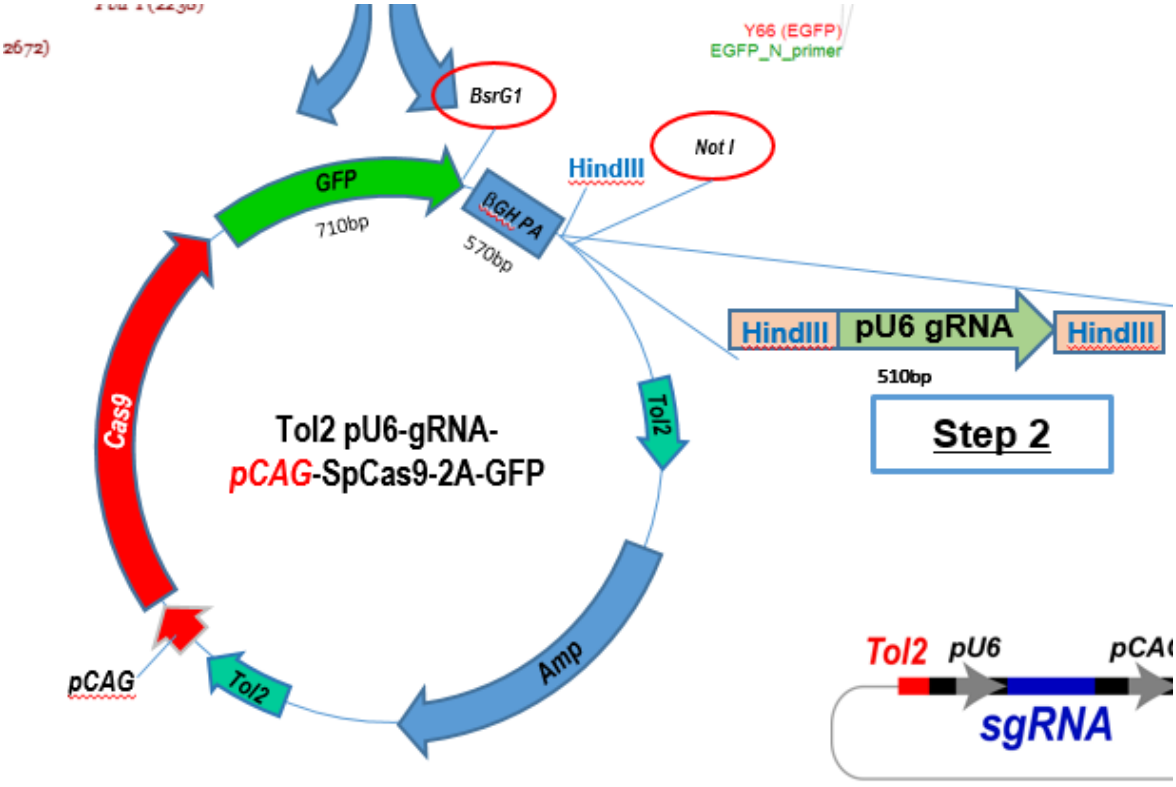
Verifying the insertion of pU6-gRNA fragment

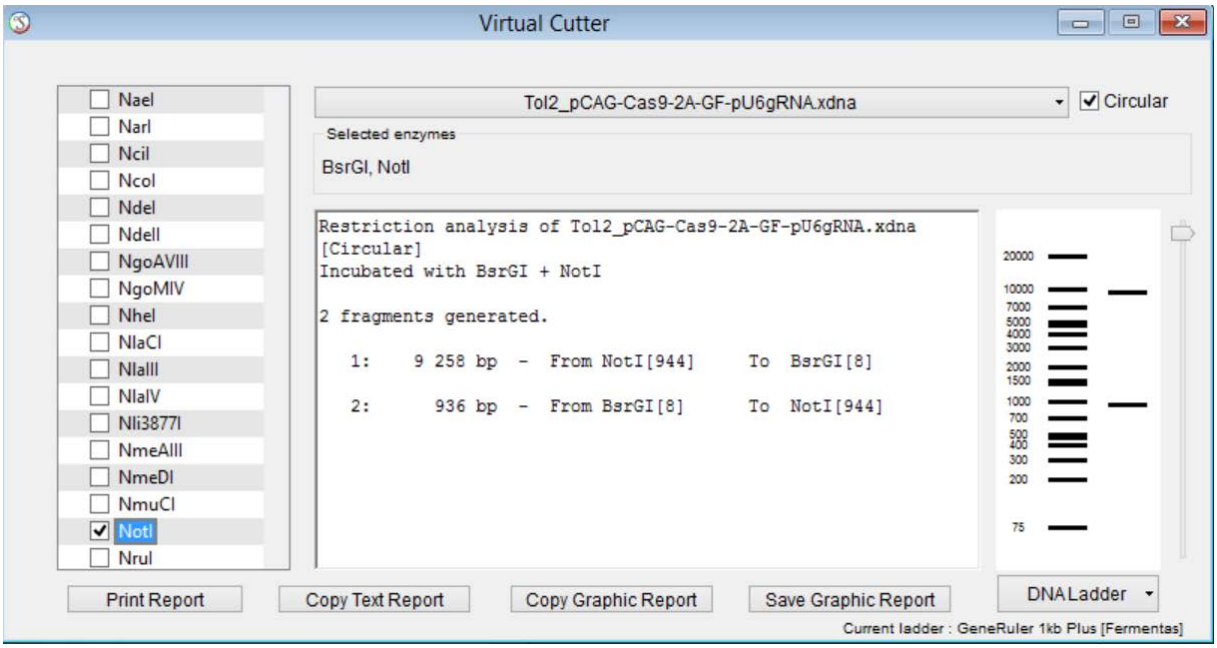
Cutting with **BstB1** (after GFP) and **Cla I** (after pU6-gRNA) > fragment of 1126 bp.





Cutting with **BsrG1** (end of GFP) and **NotI** (after pU6-gRNA) > fragment of 1 Kb.





Primer3Plus

pick primers from a DNA sequence

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Pair 1: Primer

Left Primer 1: gagggcctatttcccatgat

Start: 1 Length: 20 bp Tm: 56.7 C GC: 50.0 % Any: 0.3 End: 0.0 TB: 7.0 HP: 46.4 3' Stab: 2.5 Penalty: 3.285

Problems: Temperature too low;

Right Primer 1: aacAAAAAAgcaccgactcg

Start: 352 Length: 20 bp Tm: 57.5 C GC: 45.0 % Any: 0.0 End: 0.0 TB: 9.0 HP: 0.0 3' Stab: 4.2 Penalty: 2.469

Problems: Contains too-long poly nucleotide tract;

Pair: Product Size: 352 bp Any: 0.0 End: 0.0 TB: 14.0 Penalty: 5.753

Send to Primer3Manager | Reset Form

```
1     gagggcctat  ttcccatgat  tccttcatat  ttgcatatac  gatacaaggc
51    tgttagagag  ataattggaa  ttaatttgac  tgtaaacaca  aagatattag
101   tacaaaatac  gtgacgtaga  aagtaataat  ttcttgggta  gtttgcagtt
151   ttaaaattat  gttttaaaat  ggactatcat  atgcttaccg  taacttgaaa
201   gtatttcgat  ttcttggctt  tatatatctt  GTGGAAAGGA  CGAAACACCg
251   gGTCTTCgaG  AAGACctgtt  ttagagctaG  AAAtagcaag  ttaaaataag
301   gctagtccgt  tatcaacttg  aaaaagtggc  accgagtcgg  tgcTTTTTTg
351   ttttagagct  agaaatagca  agttaaaata  aggctagtcc  gtTTTTagcg
401   cgtgcgccaa  ttctgcagac  aaatggctct  agaggtaccc  gttacataac
451   ttacggtaaa  tggcccgcct  ggctgaccgc  ccaacgaccc  ccgcccattg
501   acgtcaatag
```

Select all Primers

Statistics:	
Primer Pair:	considered 1, unacceptable product size 1, primer in pair overlaps a primer in a better pair 12, ok 1