**Oligo Designs for Mutagenesis of RNA1 promoter and +1 region**

1. Conduct Inverted PCR on pSB1A2-BR plasmid (with whatever insert) using the following primers

J-GGA\_E\_rev; gcatggtctcaCTGTAGCACCGCCTACAT

J-GGA\_F\_for; CcagggtctctTGGTATCTGCGCTCTGCT

1. Anneal top and bottom strand Oligos and use J-GGA to insert them Between Junctions E and F.

 Below is the design of the top and bottom strands for the wild type (WT; original) region. Change bases on top and bottom strands to introduce mutations.

RNA1\_WT\_Top; acagagttcttgaagtggtggcctaactacggctacactagaagGacagtatt

RNA1\_WT\_Bot; ACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT

Oligos Annealed

acagagttcttgaagtggtggcctaactacggctacactagaagGacagtatt

 TCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTCCTGTCATAAACCA

Complete Promoter Change to P5 (highest):

RNA1\_P5\_Top; acagttgacaattaatcatccggctcgtaatttatgtgga

RNA1\_P5\_Bot; ACCATCCACATAAATTACGAGCCGGATGATTAATTGTCAA

Oligos Annealed

acagttgacaattaatcatccggctcgtaatttatgtgga

 AACTGTTAATTAGTAGGCCGAGCATTAAATACACCTACCA

Single-Point Mutation:

RNA1\_HT\_Top; acagagttcttgaagtggtggcctaactacggctacactagaag**N**acagtatt

RNA1\_HT\_Bot; ACCAAATACTGT**N**CTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT

Oligos Annealed

acagagttcttgaagtggtggcctaactacggctacactagaag**N**acagtatt

 TCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTC**N**TGTCATAAACCA

Initiation-Site Change:

RNA1\_IT\_Top; acagagttcttgaagtggtggcctaactacggctacactagaagG**GGTT**tatt

RNA1\_IT\_Bot; ACCAAATA**aacc**CCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT

Oligos Annealed

acagagttcttgaagtggtggcctaactacggctacactagaagG**GGTT**tatt

 TCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTCC**CCAA**ATAAACCA

>pSB1A2 Part-only sequence (2079 bp)

tactagtagcggccgctgcaggcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgcccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttct**t**gaagtggtggcctaactacggcta**c**actagaaggacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggtttttttgtttgcaagcagcag**a**ttacgcgcagaaaaaaaggatctc**a**agaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatggcagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttcctttttcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaataggggttccgcgcacatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacgaggcagaatttcagataaaaaaaatccttagctttcgctaaggatgatttctggaattcgcggccgcttctagag

The box in the above sequence is the pMB1 replication origin region. Within this region are coding sequences for the synthesis of species I RNA and species II RNA which regulate the plasmids copy number by a negative control element, more specifically RNA I and the origin of replication itself.

The key is as follows:

* Sequence highlighted in gray codes for RNA II (\*most but not all).
	+ RNA I sequence is within RNA II sequence.
* Sequence highlighted in yellow codes for RNA I.
* Nucleotide G highlighted in dark gray is the origin of replication.
* Sequences highlighted in red are possible -10 and -35 promoter elements for RNA I or RNA II predicted by bacterial promoter prediction software.
	+ RNA I uses forward promoters.
	+ RNA II uses backwards promoters.
* Sequence highlighted in magenta are possible -10 and -35 promoter elements for RNA I predicted by another bacterial promoter prediction software.
* Sequence highlighted in green is the -35 promoter for RNA II.
* Nucleotides highlighted in blue are the +1 sites for RNA I and RNA II sites, respectively.
* Nucleotides in bold are at exactly -10 or -35 positions of the promoters.

GTA GCT CTT GAT CCG GCA AAC AAA CCA CCG CTG GTA GCG GTG GTT TTT TTG TT

