Allelic Exchange Plasmid Maps for the Nebraska Transposon Mutant Library (NTML) Genetic Toolbox

Catalog No. NR-49947

Allelic Exchange Instructions

Electroporate or transduce plasmid into the transposon (Tn) mutant strain, keeping plates and cultures at 30°C. Create freezer stocks of transductants.

Recombination with Allelic Exchange Plasmid

Day 1: At the end of day, streak freezer stock for confluency onto Tryptic Soy agar with 10 µg/mL chloramphenicol plate and incubate at 44°C.[1]

Day 2: In the morning, check for colonies that are resistant to chloramphenicol. Large colonies are likely to be single-recombinants (SRs).[2] Select several of these large colonies and re-streak for isolated colonies on Tryptic Soy agar with 10 µg/mL chloramphenicol and incubate at 44°C.

Day 3: Re-streaks with good growth are usable SRs. Pick several colonies from each SR and inoculate in 3 mL of Tryptic Soy broth. Incubate at 30°C with shaking. Additionally, inoculate SRs into Tryptic Soy broth with 10 µg/mL chloramphenicol and grow at 44°C to make freezer stocks of SRs to come back to if necessary

Day 4: Subculture into a fresh tube of 3-mL Tryptic Soy broth and incubate at 30°C with shaking.

Day 5: Subculture into a fresh tube of 3-mL Tryptic Soy broth and incubate at 30°C with shaking.

Day 6: Subculture into a fresh tube of 3-mL Tryptic Soy broth and incubate at 30°C with shaking. Additionally, inoculate a 10^{-7} final dilution on Tryptic Soy agar with 100 ng/mL Anhydrotetracycline (ATc). ATc is a counter-selection that inhibits growth of cells carrying the plasmid. Do not use with pTET. Alternatively, plate on Tryptic Soy agar at 10^{-8} and patch more colonies on next day.

Day 7: Replica patch large colonies on Tryptic Soy agar, Tryptic Soy agar with 10 µg/mL chloramphenicol and Tryptic Soy agar with 5 µg/mL erythromycin.

Day 8: Check for double recombinants (DRs). Colonies which are erythromycin-sensitive, chloramphenicol-sensitive are the desired DRs. Colonies which are erythromycin-sensitive, chloramphenicol-resistant are DRs that have desired replacement but have not lost the plasmid. Colonies which are erythromycin-resistant, chloramphenicol-sensitive do not have the desired replacement.

Days 6 to 8 should be repeated for 2 more days if the desired mutant is not immediately found. If the desired mutant is not obtained in this time frame, start over or inoculate Tryptic Soy broth from frozen SRs.

[1]The frequency of recombination is partially dependent on the length of the homologous DNA fragment used. Because the length of homologous DNA used in this system is about 500 base pairs and less than typically used for allelic exchange, expect to get fewer single recombinants. To increase chances of finding single recombinants, streaking on several plates is recommended.
[2]Increased incubation times will make it difficult to discern large colonies from small colonies.

Other considerations

1. The plasmids are temperature-sensitive. 30°C is the replicative temperature while 44°C is non-permissible; therefore, good growth on chloramphenicol occurs when the plasmid has incorporated into the chromosome.
2. Obtaining chloramphenicol-sensitive DRs relies on a combination of the plasmid excising from the chromosome and then loss of plasmids during replication at 30°C due to the lack of antibiotic selection. The timing of these two events are unpredictable, resulting in varying lengths of time to obtain the correct DR.

Confirmation of Allelic Exchange

To confirm proper exchange of the *bursa aurealis* Tn with the desired replacements, design and use gene-specific primers that flank the *bursa aurealis* insertion site (Figure 1). For plasmid containing a selectable marker (pKAN, pTET or pSPC), screen for appropriate antibiotic resistance.

Figure 1: Design of Gene-Specific Primers for Confirmation of Proper Exchange

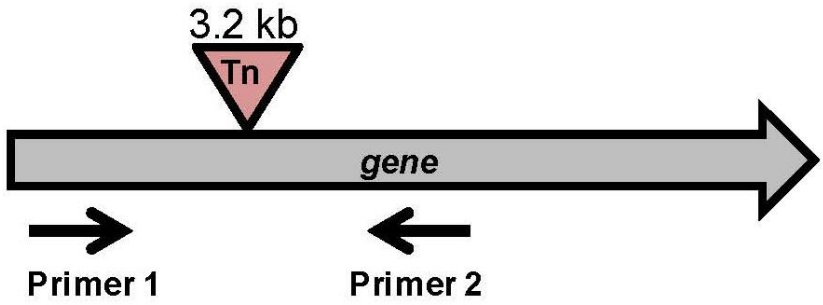


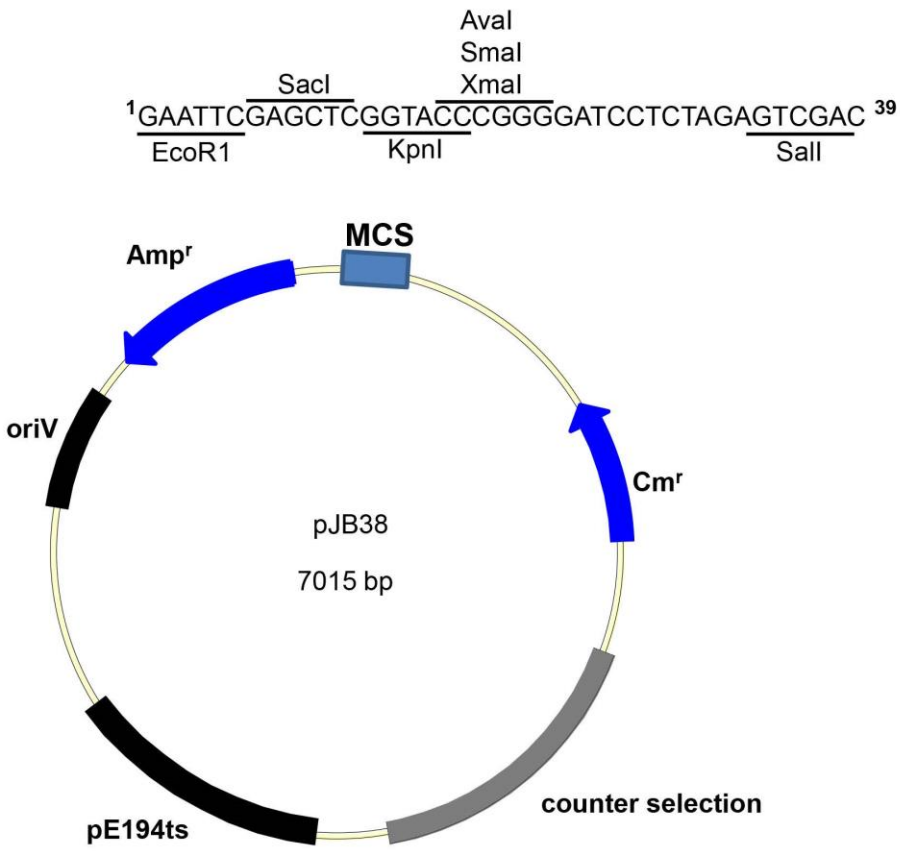
Table 1 provides the expected sizes of the PCR products that would be generated with each of the plasmids when used with primers positioned 250 base pairs away from the Tn insertion.

Table 1: Expected PCR Products Sizes in Kilobases (kb)

Component Number	Plasmid	Difference from *bursa aurealis** (~3.2 kb)	Expected size* (if WT=0.5 kb)
NRC-49933	pTnT	-2.1 kb	1.6 kb
NRC-49934	pSPC	-1.0 kb	2.7 kb
NRC-49935	pKAN	-0.9 kb	2.8 kb
NRC-49936	pTET	+0.25 kb	3.9 kb
NRC-48837	pGFP-F	-1.4 kb	2.3 kb
NRC-48838	pGFP-R	-1.4 kb	2.3 kb
NRC-49939	pYFP-F	-1.4 kb	2.3 kb
NRC-49940	pYFP-R	-1.4 kb	2.3 kb
NRC-49941	pBFP-F	-1.4 kb	2.3 kb
NRC-49942	pBFP-R	-1.4 kb	2.3 kb
NRC-49943	pRFP-F	-1.4 kb	2.3 kb
NRC-49944	pRFP-R	-1.4 kb	2.3 kb
NRC-49945	pFP650-F	-1.4 kb	2.3 kb
NRC-49946	pFP650-R	-1.4 kb	2.3 kb

*sizes are approximate

Figure 2: Plasmid Map of pJB38 (NR-49932)

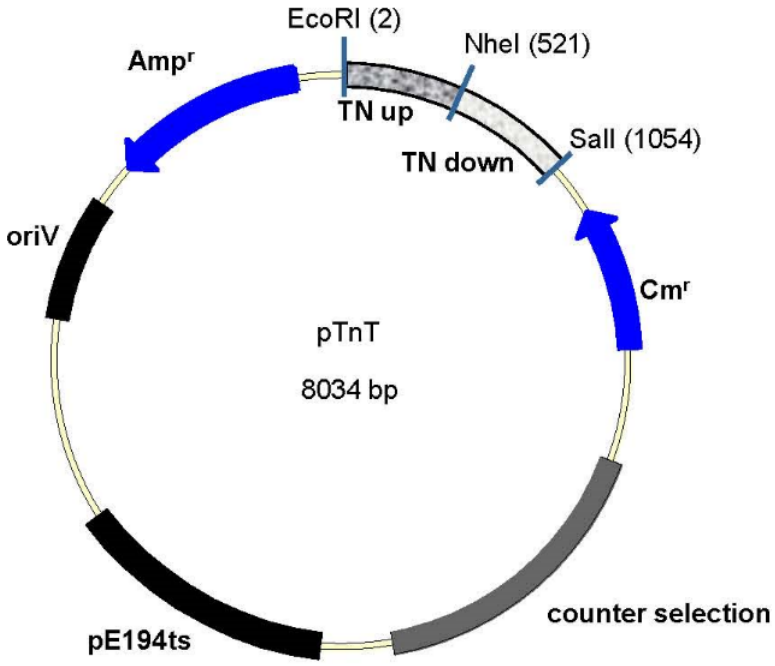


E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})
-MCS: shown multi-cloning site

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})

Note: inserts into the multi-cloning site can be confirmed with:
Forward primer: CCCGAAAAGTGCCACCTGACGTC
Reverse primer: CGAAAATGCCTCACATTTGTGCCACC

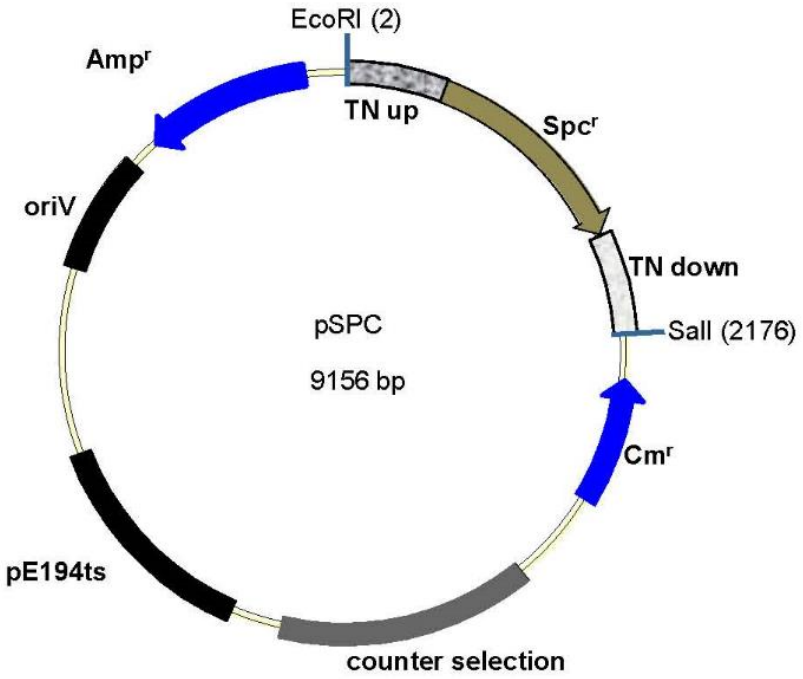
Figure 3: Plasmid Map of pTnT (NRC-49933)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TN up/down: homologous DNA to *bursa aurealis*

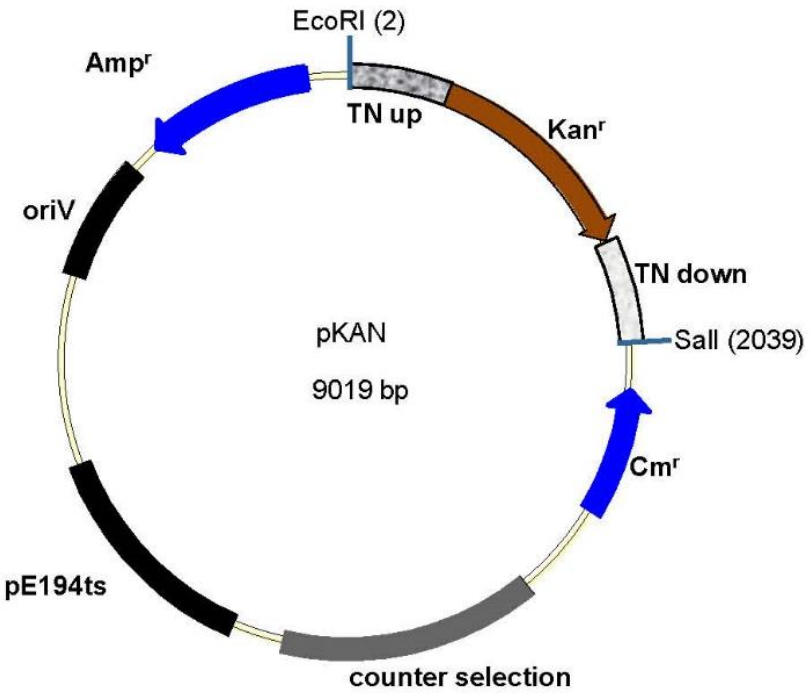
Figure 4: Plasmid Map of pSPC (NRC-49934)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-Spc^r: Spectinomycin resistance (1000 µg ml^{-1})
-TN up/down: homologous DNA to *bursa aurealis*

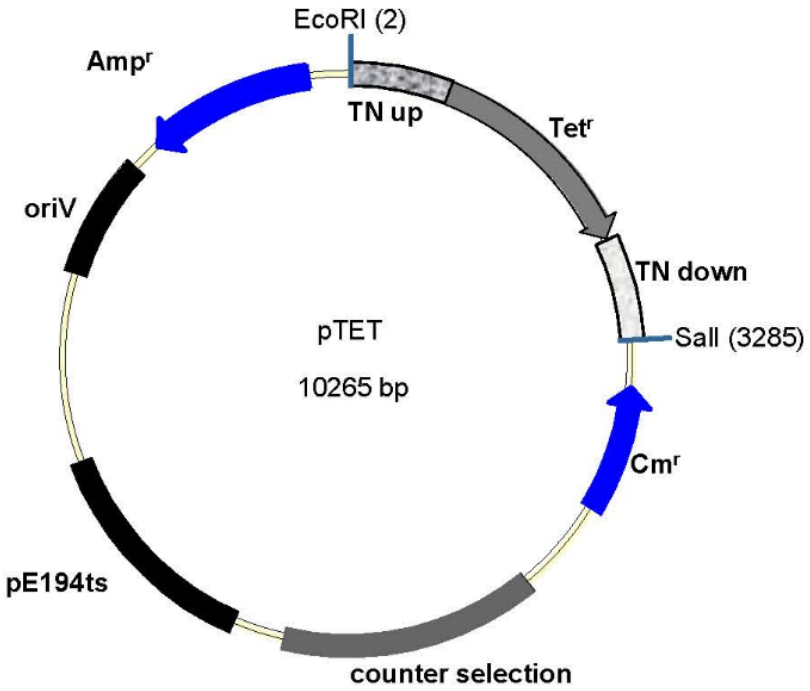
Figure 5: Plasmid Map of pKAN (NRC-49935)



E. coli Features
-oriV: high copy origin
-AmpR: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-CmR: chloramphenicol resistance (10 µg ml^{-1})
-KanR: Kanamycin resistance
-250 µg ml^{-1} plasmid replicating
-75 µg ml^{-1} on chromosome
-TN up/down: homologous DNA to *bursa aurealis*

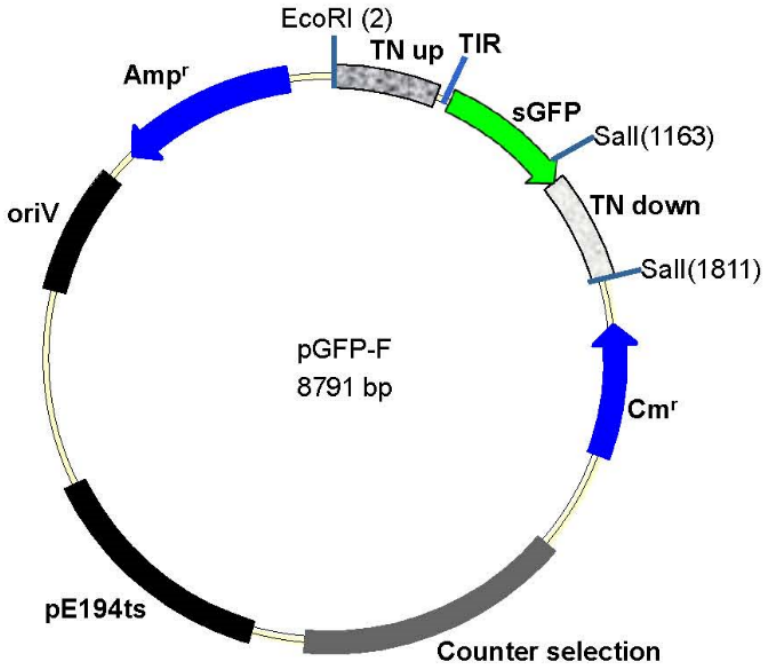
Figure 6: Plasmid Map of pTET (NRC-49936)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-Tet^r: Tetracycline resistance
-5 µg ml^{-1} plasmid replicating
-0.625 µg ml^{-1} on chromosome
-TN up/down: homologous DNA to *bursa aurealis*

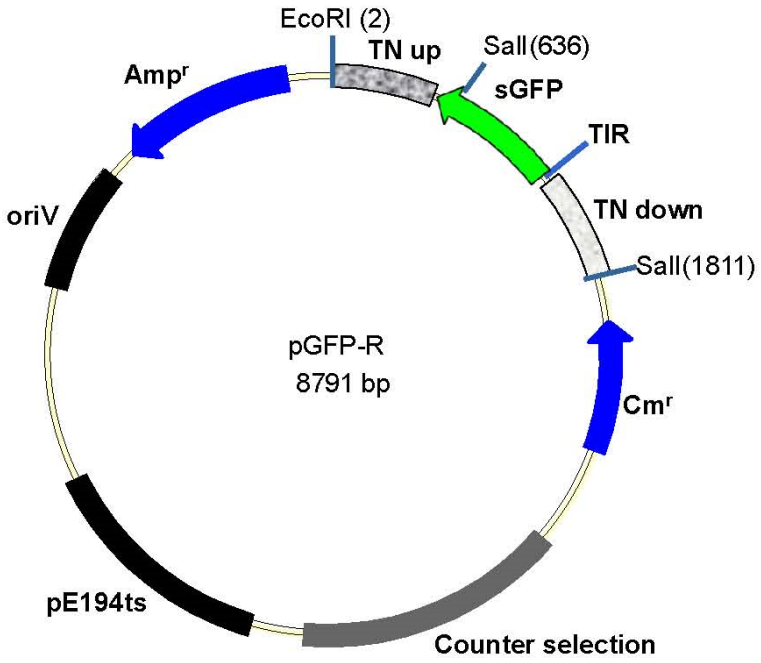
Figure 7: Plasmid Map of pGFP-F (NRC-49937)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
- sGFP : encodes superfolder GFP
-TN up/down: homologous DNA to *bursa aurealis*

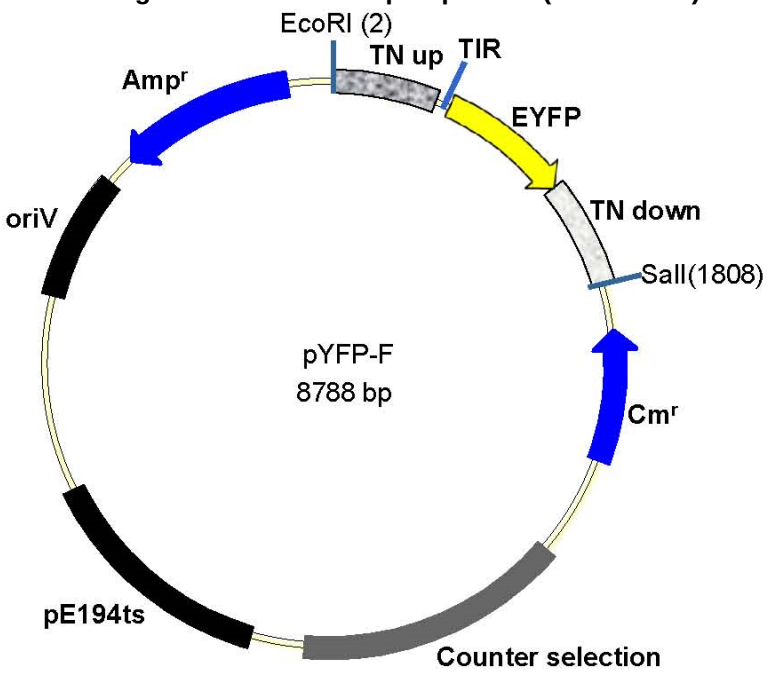
Figure 8: Plasmid Map of pGFP-R (NRC-49938)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-sGFP : encodes superfolder GFP
-TN up/down: homologous DNA to *bursa aurealis*

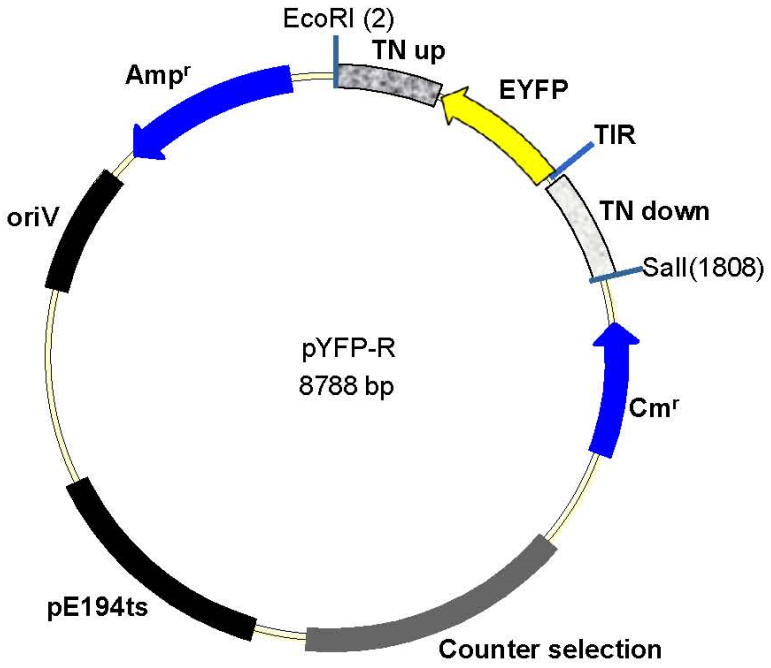
Figure 9: Plasmid Map of pYFP-F (NRC-49939)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-EYFP: encodes EYFP
-TN up/down: homologous DNA to *bursa aurealis*

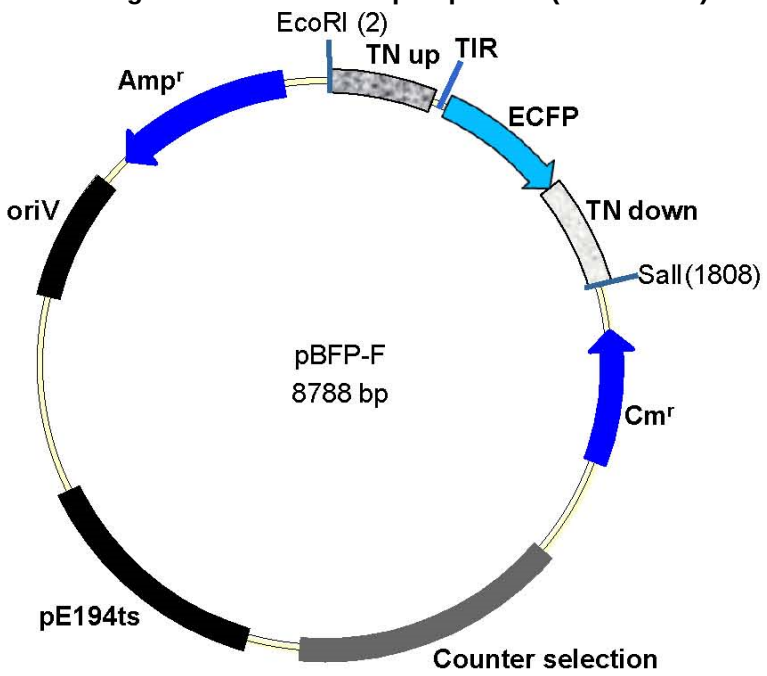
Figure 10: Plasmid Map of pYFP-R (NRC-49940)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-EYFP: encodes EYFP
-TN up/down: homologous DNA to *bursa aurealis*

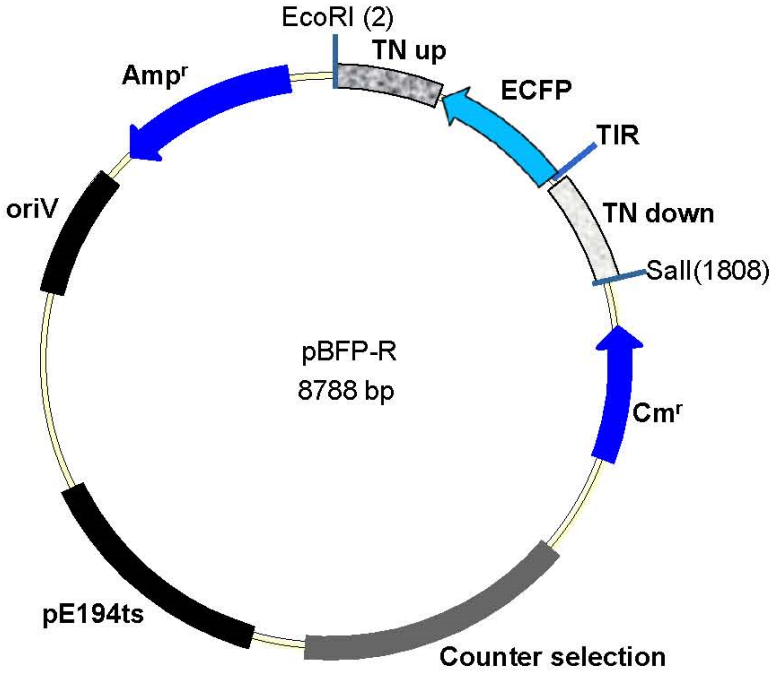
Figure 11: Plasmid Map of pBFP-F (NRC-49941)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-ECFP: encodes ECFP
-TN up/down: homologous DNA to *bursa aurealis*

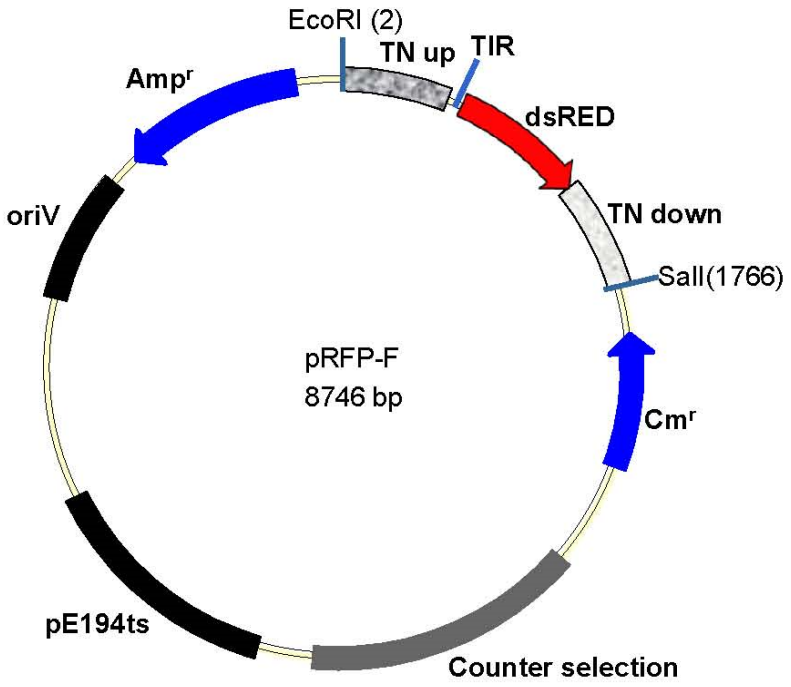
Figure 12: Plasmid Map of pBFP-R (NRC-49942)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-ECFP: encodes ECFP
-TN up/down: homologous DNA to *bursa aurealis*

Figure 13: Plasmid Map of pRFP-F (NRC-49943)

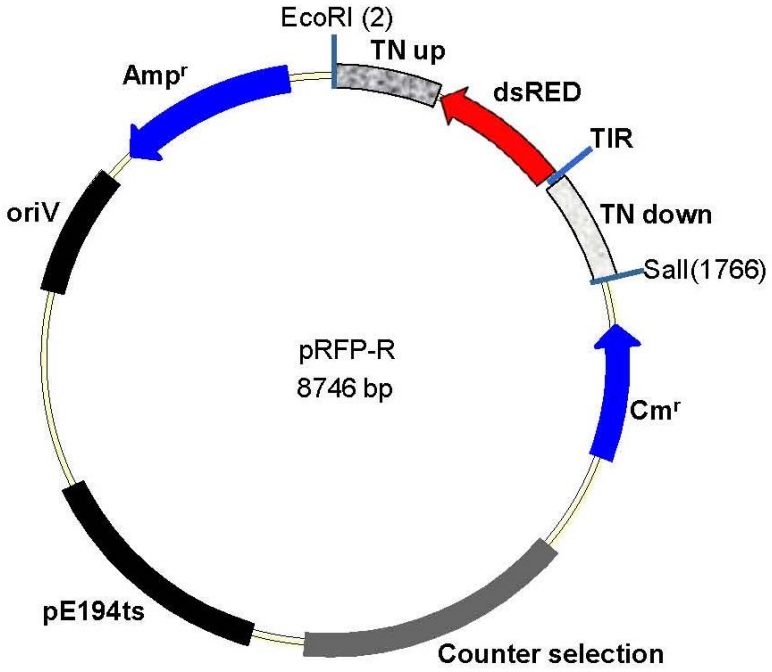


E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-dsRed: encodes DsRed.T3(DNT)
-TN up/down: homologous DNA to *bursa aurealis*

DsRed.T3 spectra can be found in:
Bevis,B.J., and B.S. Glick. 2002. Rapidly maturing variants of *Discosoma* red fluorescent protein (DsRed). Nature Biotechnology. 20:83-87.

Figure 14: Plasmid Map of pRFP-R (NRC-49944)

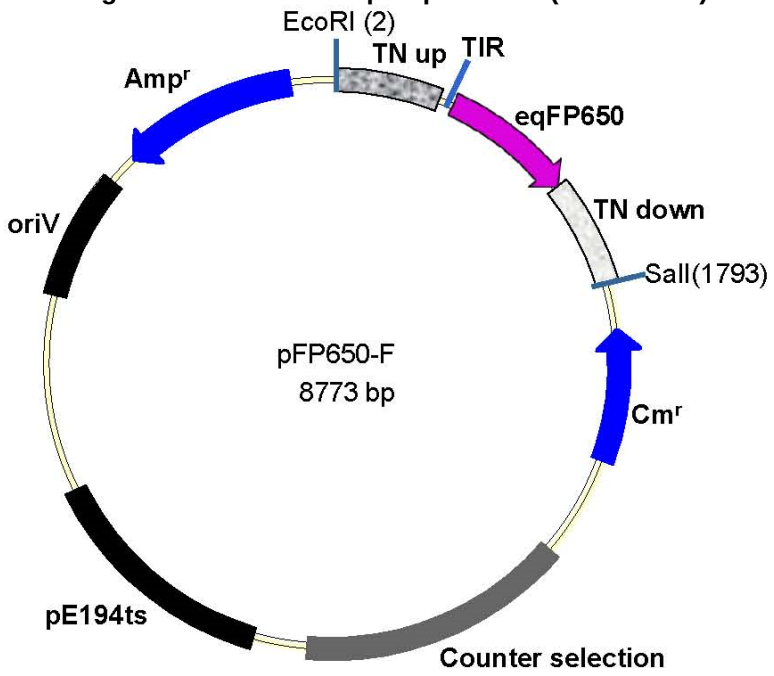


E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-dsRed: encodes DsRed.T3(DNT)
-TN up/down: homologous DNA to *bursa aurealis*

DsRed.T3 spectra can be found in:
Bevis,B.J., and B.S. Glick. 2002. Rapidly maturing variants of *Discosoma* red fluorescent protein (DsRed). Nature Biotechnology. 20:83-87.

Figure 15: Plasmid Map of pFP650-F (NRC-49945)

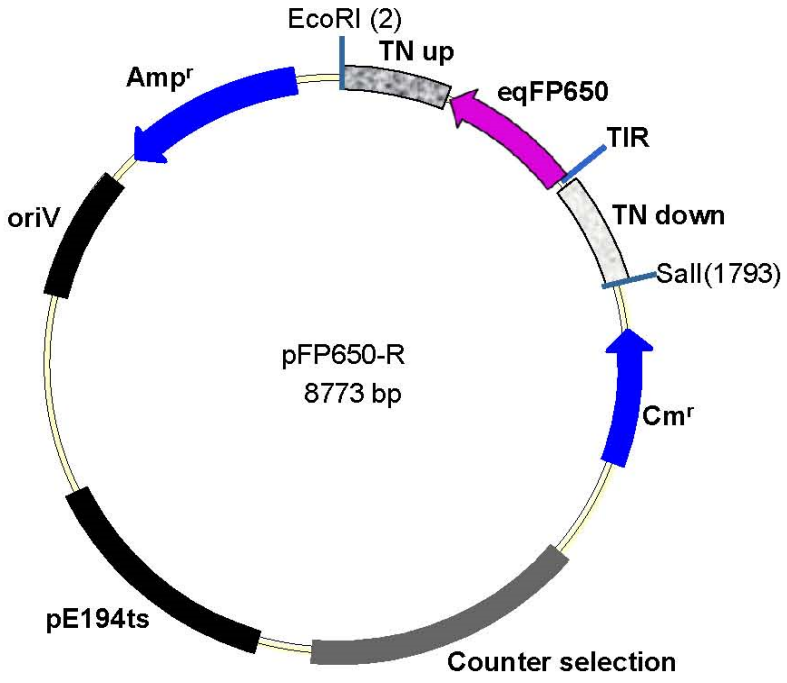


E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-eqFP650: encodes eqFP650
-TN up/down: homologous DNA to *bursa aurealis*

eqFP650 spectra can be found in:
Shcherbo D, et al.. 2010. Near-infrared fluorescent proteins. Nat. Methods 7:827-829.

Figure 16: Plasmid Map of pFP650-R (NRC-49946)



E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-eqFP650: encodes eqFP650
-TN up/down: homologous DNA to *bursa aurealis*

eqFP650 spectra can be found in:
Shcherbo D, et al.. 2010. Near-infrared fluorescent proteins. Nat. Methods 7:827-829.