First, electroporate/transduce plasmid into strain, keeping plates/cultures at 30°C

Making Tn exchange

Day 1: At end of day, streak freezer stock all over (i.e. not for isolated colonies) TSA+Cm plate[A] and place at 44°C.

↓

Day 2: In morning[B], check for colonies that are Cm^r. Pick several large colonies (these are likely to be your single-recombinants(SRs)) and re-streak for isolated colonies onto TSA+Cm and grow at 44°C

↓

Day 3: If streaks grow well (i.e. heavy growth in streak and isolated colonies, these are usable SRs). Pick colonies[C] to inoculate 3-ml TSB and incubate at 30°C with shaking

↓

Day 4: Subculture into a fresh tube of 3-ml TSB and incubate at 30°C with shaking

↓

Day 5: Subculture into a fresh tube of 3-ml TSB and incubate at 30°C with shaking

↓

Day 6. Subculture into a fresh tube of 3-ml TSB and incubate at 30°C with shaking. Also, Dilution plate (10^{-7} final dilution) onto TSA +ATc[D].

↓

Day 7: Replica patch large colonies on TSA, TSA+Cm, and TSA+Erm

↓

Day 8: Check for double recombinants[E] (DRs)
1. Erm^r Cm^s are back to parent DRs
2. Erm^s Cm^s are desired DRs
   - -screen these by PCR for replacement
   - -if using pKAN, pTET, or pSPC, screen for appropriate antibiotic resistance.

This should be repeated for 2 more days if needed mutant isn't immediately found.

SR = single recombinant
DR = double recombinant

[A]The frequency of recombination is dependent, in part, on the length of the homologous DNA fragment used (we have used ~500 bp). This is less than is often used for allelic exchange, therefore expect to get fewer SRs than if using longer homologous DNA fragments. We recommend streaking on several plates.

[B] If you wait too long, it will be hard to discern the large from small colonies.

[C] pick colonies from multiple SRs to increase chances of getting mutant. Also, inoculate SRs into TSB + Cm and grow at 44^{o}C to make freezer stocks of SRs to come back to if necessary

[D] anhydrotetracycline (Atc) is a counter-selection that inhibits growth of cells carrying plasmid. Do not use with pTET. Alternately, just plate on TSA at 10^{-8} and patch more colonies on next day.

[E] if you get Erm^{S} Cm^{R}, then these are DRs that have desired replacement but have not lost plasmid yet, so restreak on TSA and repatch the next day

Other considerations

1. We use erythromycin (Erm) at 5 µg ml^{-1}, chloramphenicol (Cm) at 10 µg ml^{-1}, and anhydrotetracycline (Atc) at 100 ng ml^{-1}

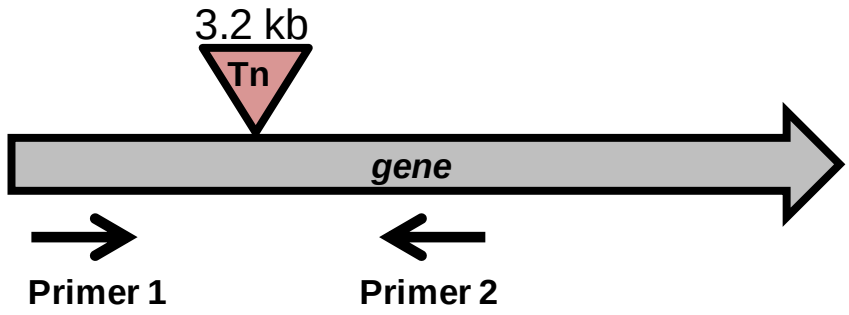
2.These plasmids are temperature-sensitive. 30^{o}C is the replicative temperature while 44^{o}C is non-permissable and therefore good growth on Cm occurs when the plasmid has incorporated into the chromosome.

3. If the desired mutant is not obtained in this time frame, start over or inoculate TSB from frozen SRs.

4. SRs have the plasmid incorporated in the chromosome next to the Tn that you are trying to replace.

5. Getting Cm^{S} DRs relies on a combination of the plasmid excising from the chromosome and then loss of plasmids during replication at 30^{o}C because of no antibiotic selection. The timing of these two events are unpredictable and therefore sometimes you will get your desired DRs right away and sometimes it may take longer. Therefore, we recommend repeating days 6-8 until DRs are identified.

To confirm proper exchange of the *bursa aurealis* Tn with the cassettes indicated in the table below, design and use gene-specific primers that flank the *bursa aurealis* insertion site using the guide below:

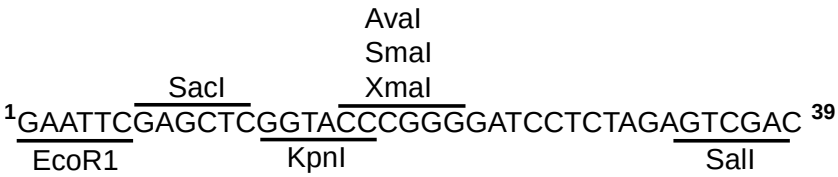


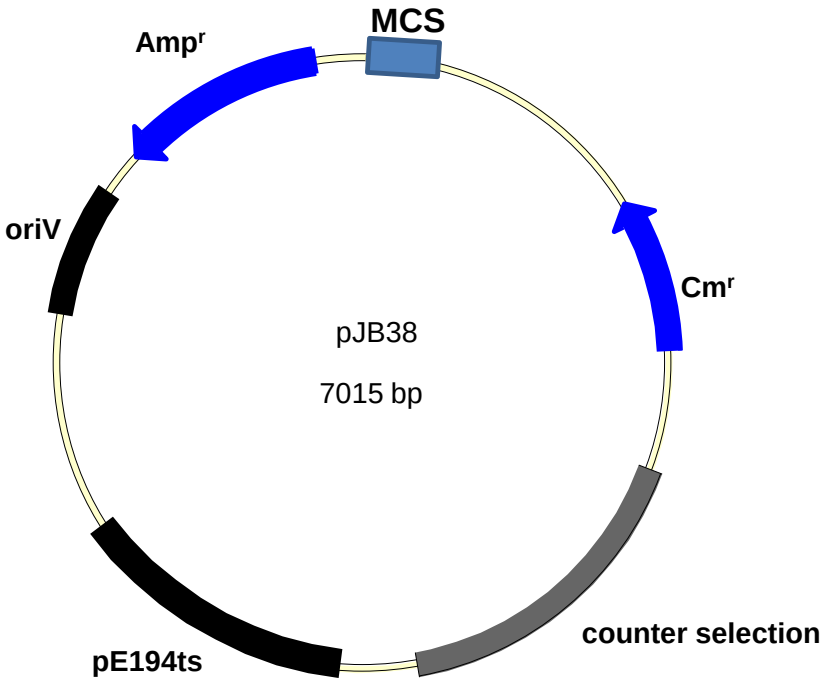
For example, if gene-specific primers are positioned 250 bp away from the insertion, then a PCR product of 3.7 kb will be generated (use of WT genomic DNA will yield a 500 bp product).

The table below provides the expected sizes of the PCR products that would be generated with each of the plasmids used and with the primers positioned 250 bp away from the Tn insertion.

Plasmid used	Difference from *bursa aurealis** (~3.2 kb)	Expected size* (if WT=0.5 kb)
pTnT	-2.1 kb	1.6 kb
pKAN	-0.9 kb	2.8 kb
pSPC	-1.0 kb	2.7 kb
pTET	+0.25 kb	3.9 kb
pBFP	-1.4 kb	2.3 kb
pFP650	-1.4 kb	2.3 kb
pGFP	-1.4 kb	2.3 kb
pRFP	-1.4 kb	2.3 kb
pYFP	-1.4 kb	2.3 kb

*sizes are approximate

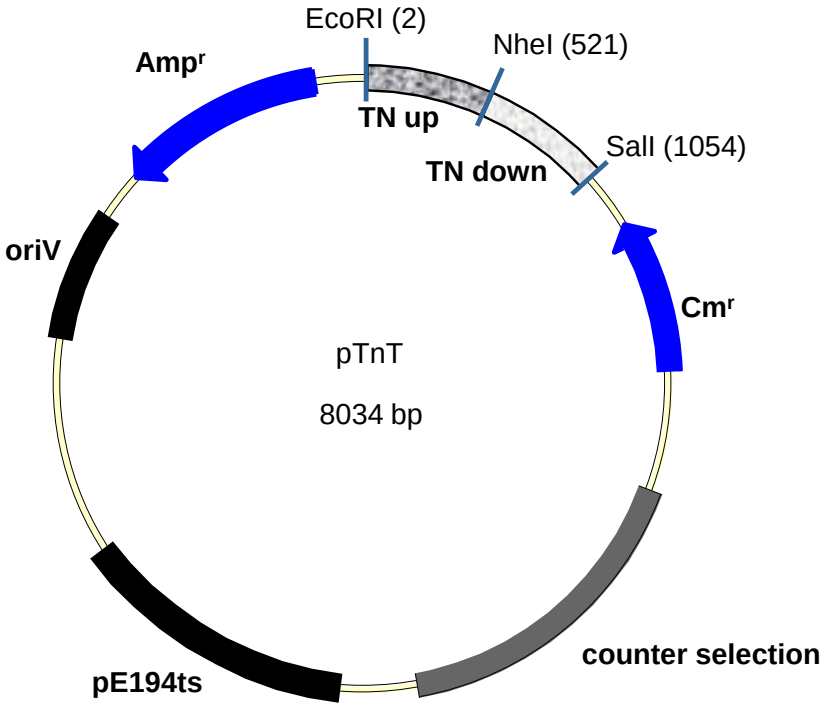




E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})
-MCS: shown multi-cloning site

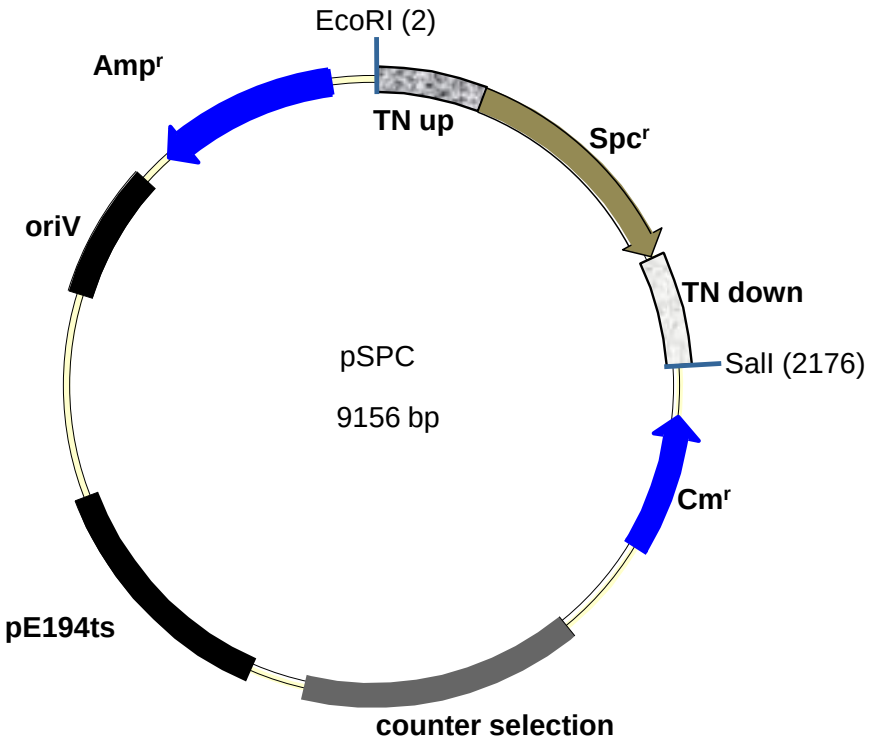
S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})

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



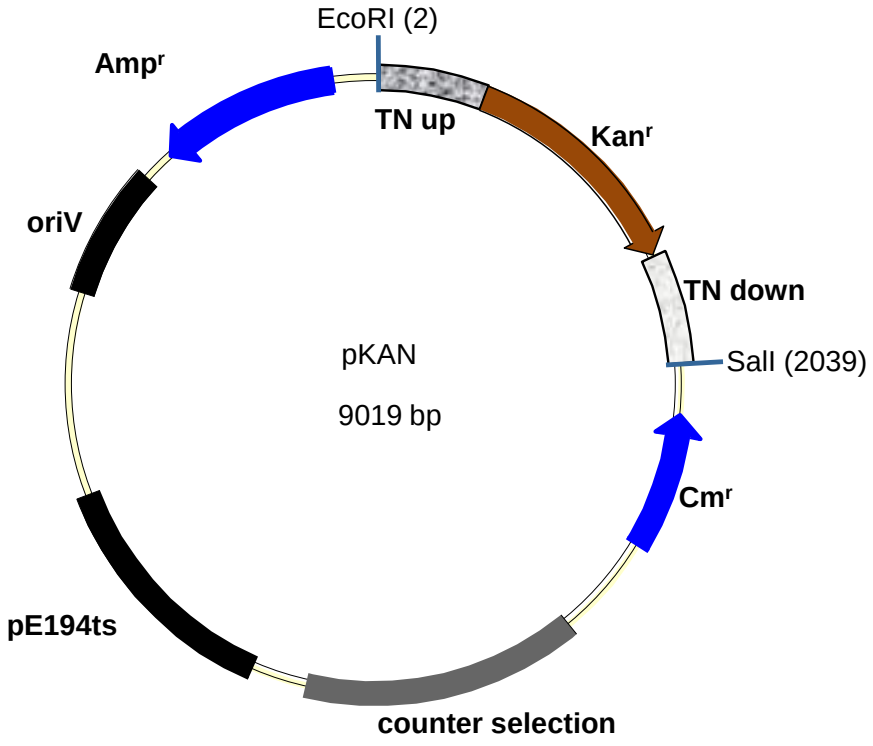
E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

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*



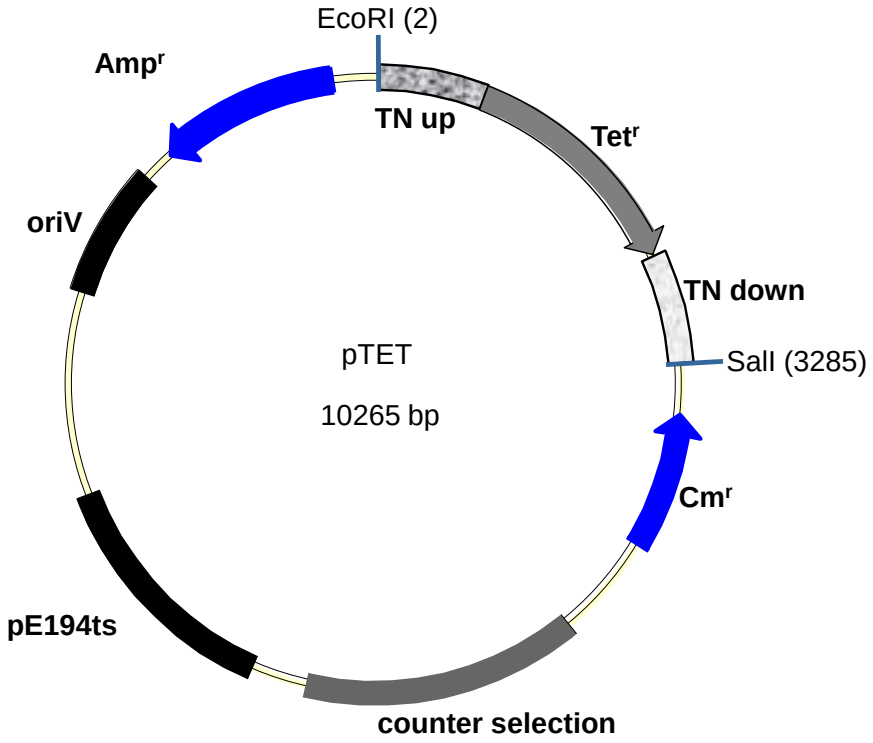
E. coli Features
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

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*



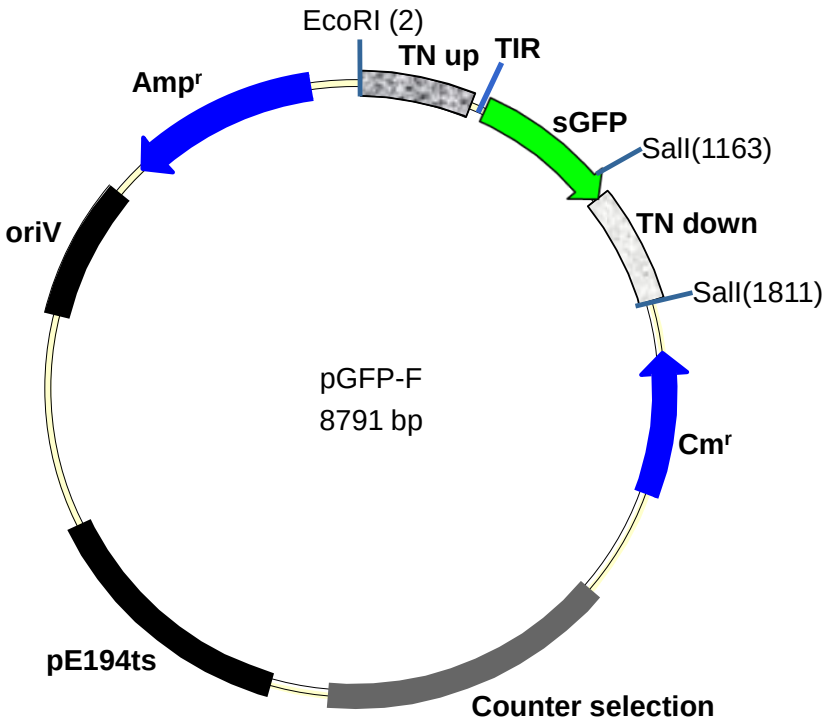
E. coli Features
-oriV: high copy origin
-AmpR: Ampicillin resistance (100 µg ml^{-1})

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*



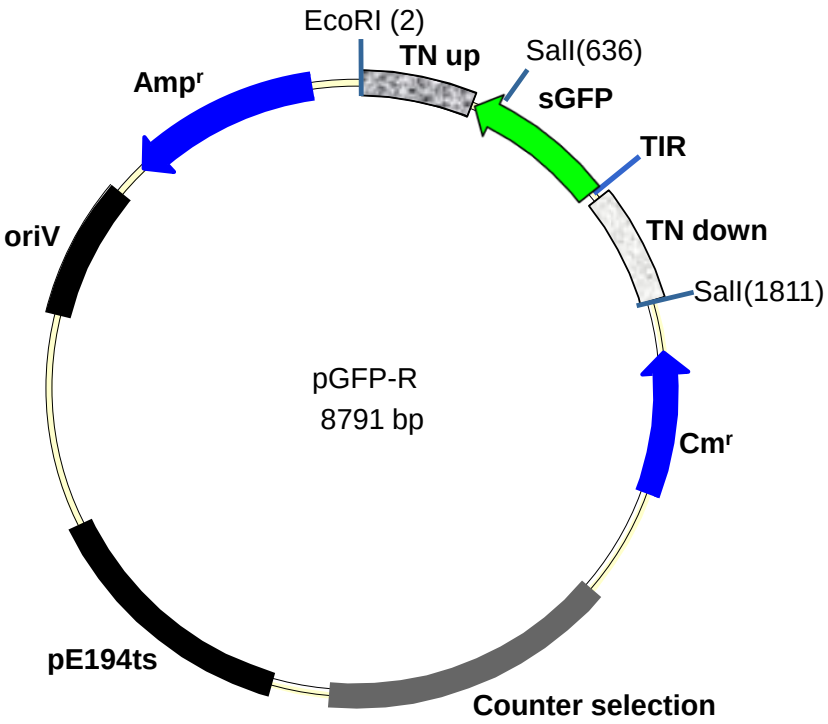
<u>*E. coli* Features</u>
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

<u>*S. aureus* Features</u>
-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*



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

<u>*S. aureus* Features</u>
-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*



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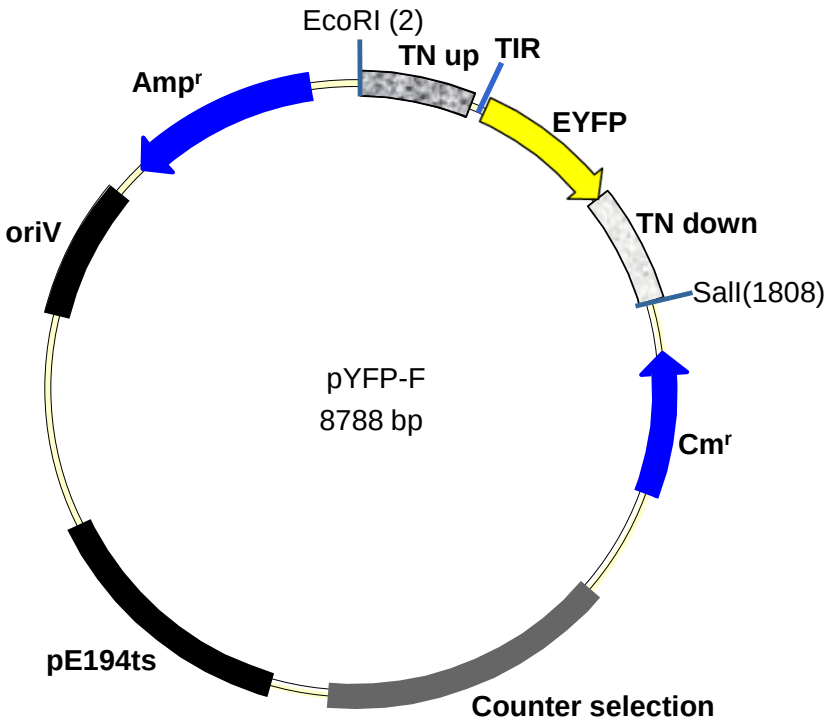
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-sGFP : encodes superfolder GFP
-TN up/down: homologous DNA to *bursa aurealis*



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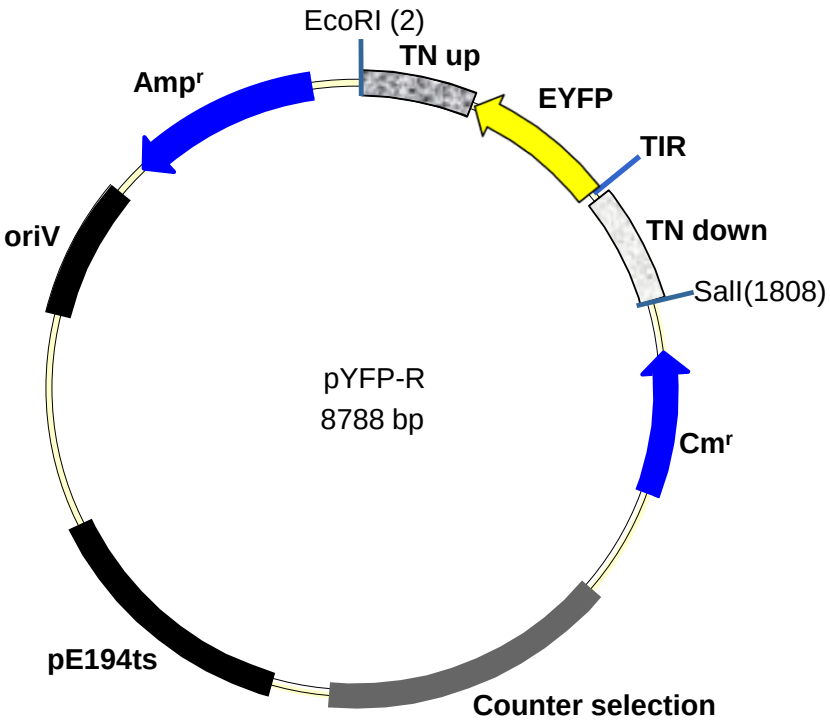
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

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*



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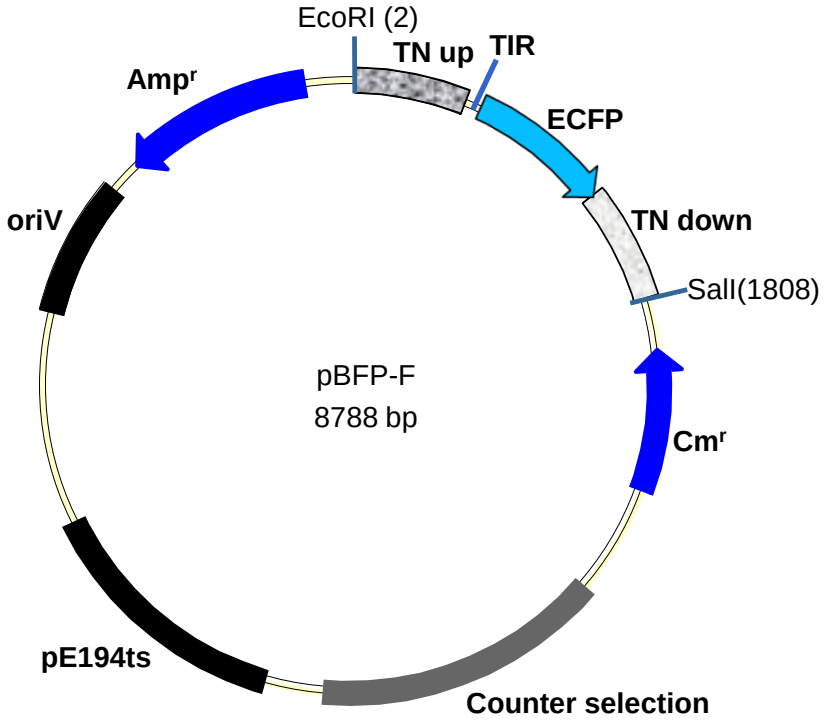
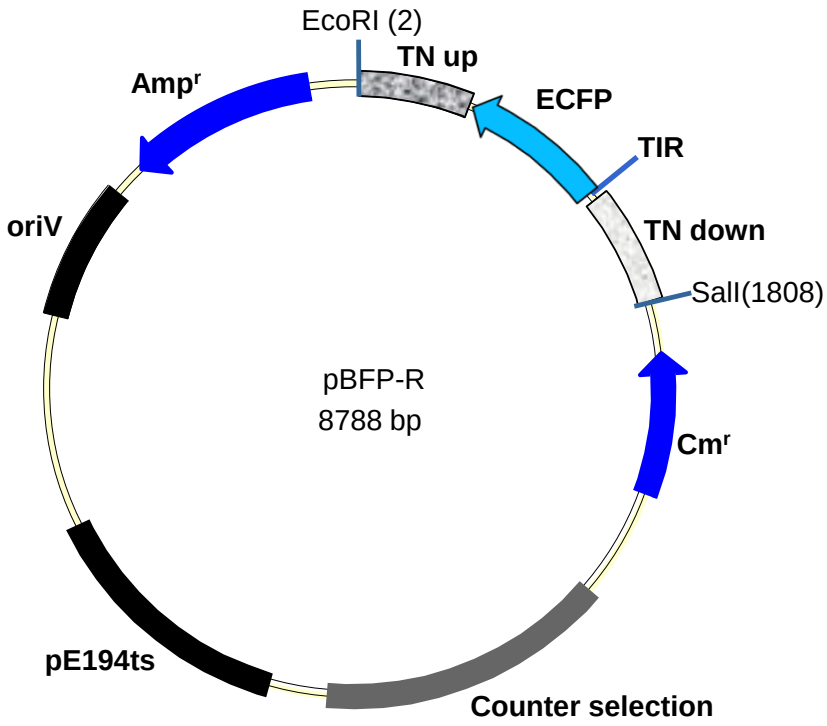
S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-EYFP: encodes EYFP
-TN up/down: homologous DNA to *bursa aurealis*



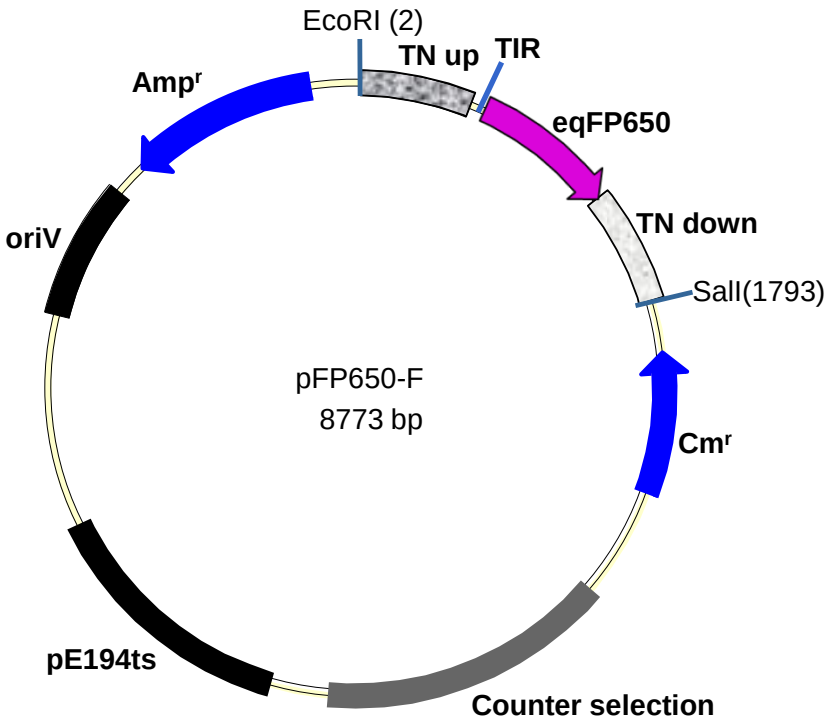
<u>*E. coli* Features</u>
-oriV: high copy origin
-Amp^r: Ampicillin resistance (100 µg ml^{-1})

<u>*S. aureus* Features</u>
-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*



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

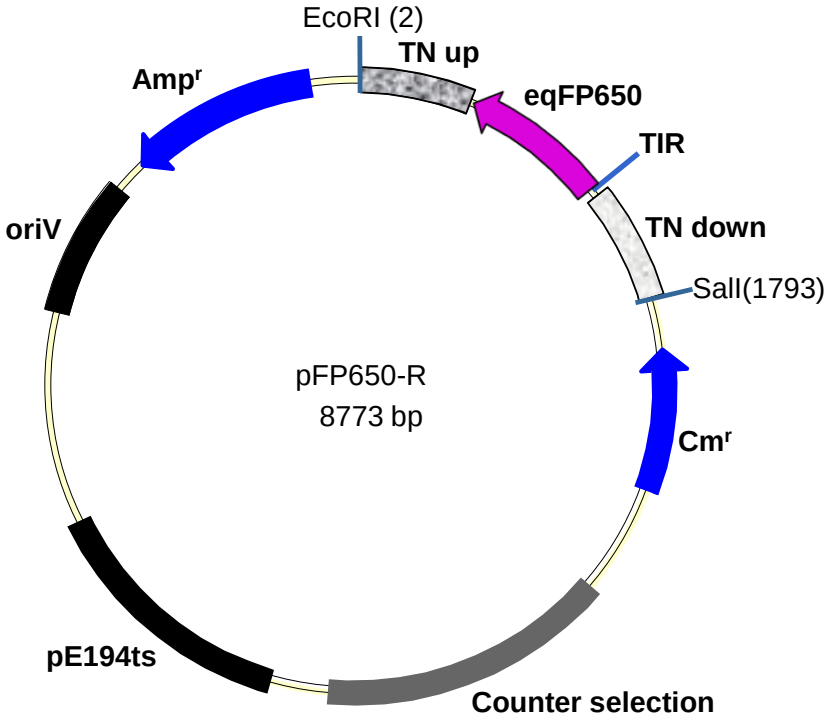
S. aureus Features
-pE194ts: temperature-sensitive origin
-counterselection: anhydrotetracycline-inducible (100 ng ml^{-1})
-Cm^r: chloramphenicol resistance (10 µg ml^{-1})
-TIR: optimized RBS
-ECFP: encodes ECFP
-TN up/down: homologous DNA to *bursa aurealis*



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*

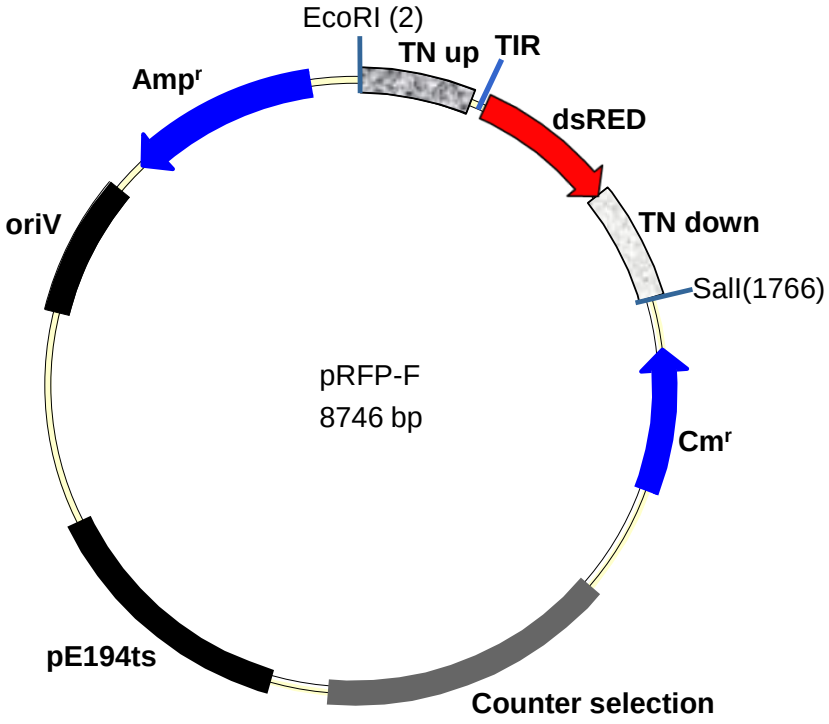
eqFP650 spectra can be found in:
Shcherbo D, et al.. 2010. Near-infrared fluorescent proteins. Nat. Methods 7:827-829.



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*

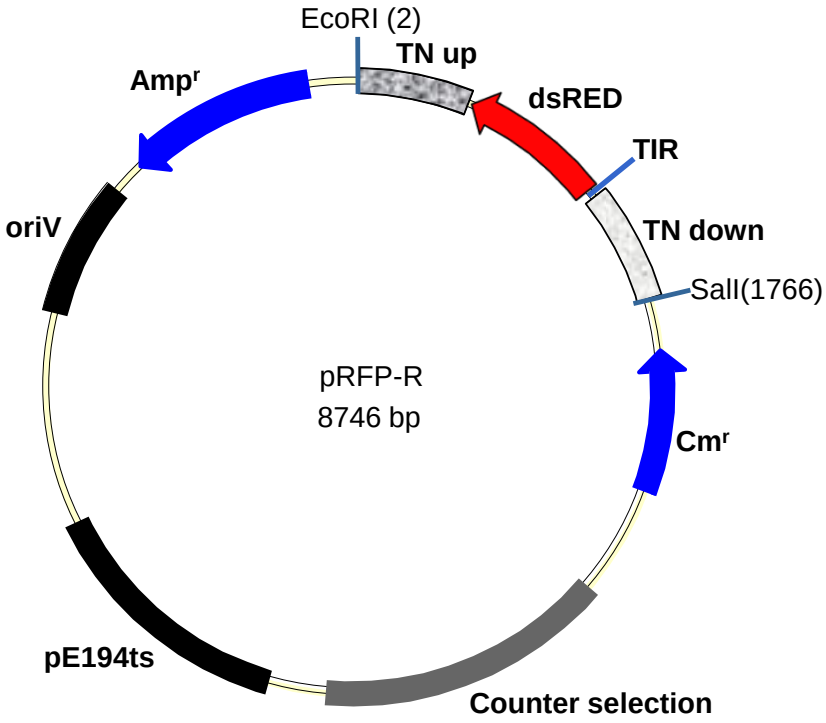
eqFP650 spectra can be found in:
Shcherbo D, et al.. 2010. Near-infrared fluorescent proteins. Nat. Methods 7:827-829.



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.



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.