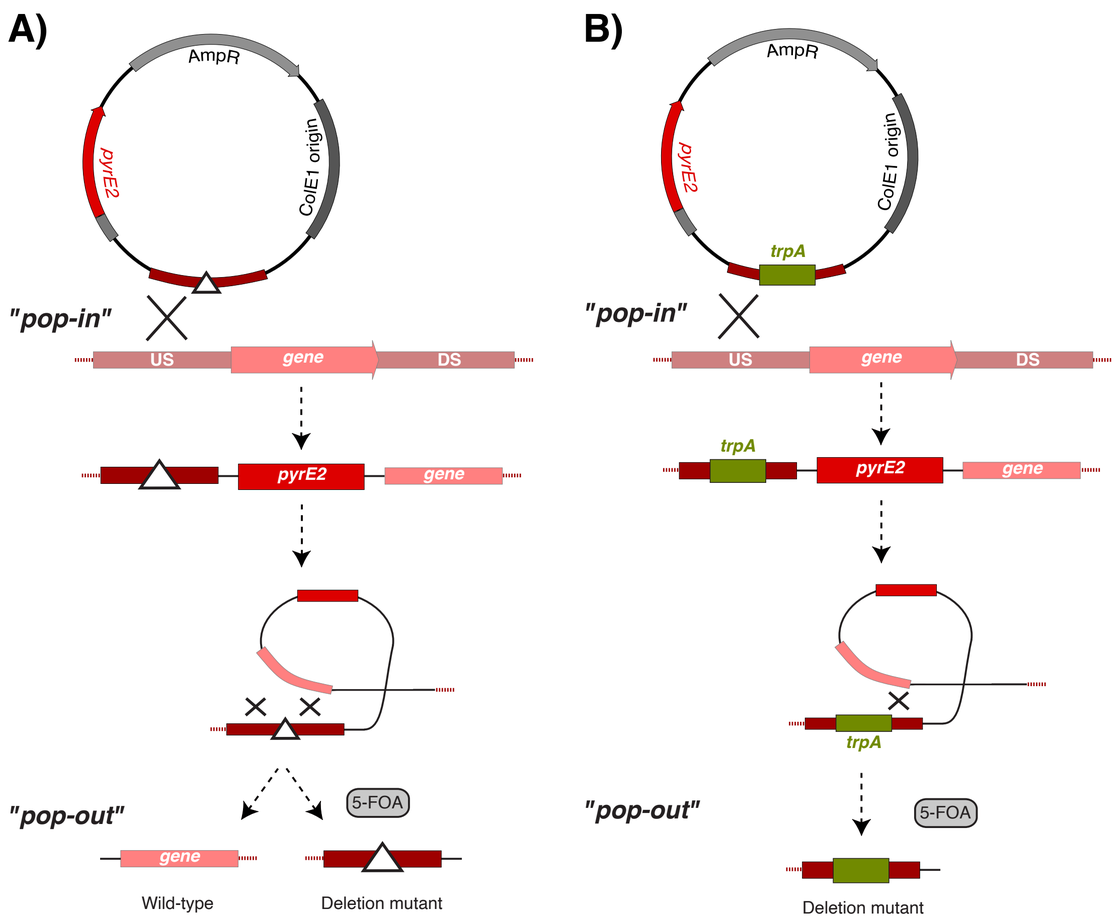
GENE DELETION POP-IN/POP-OUT

POP-IN/POP-OUT METHOD OF GENE DELETION

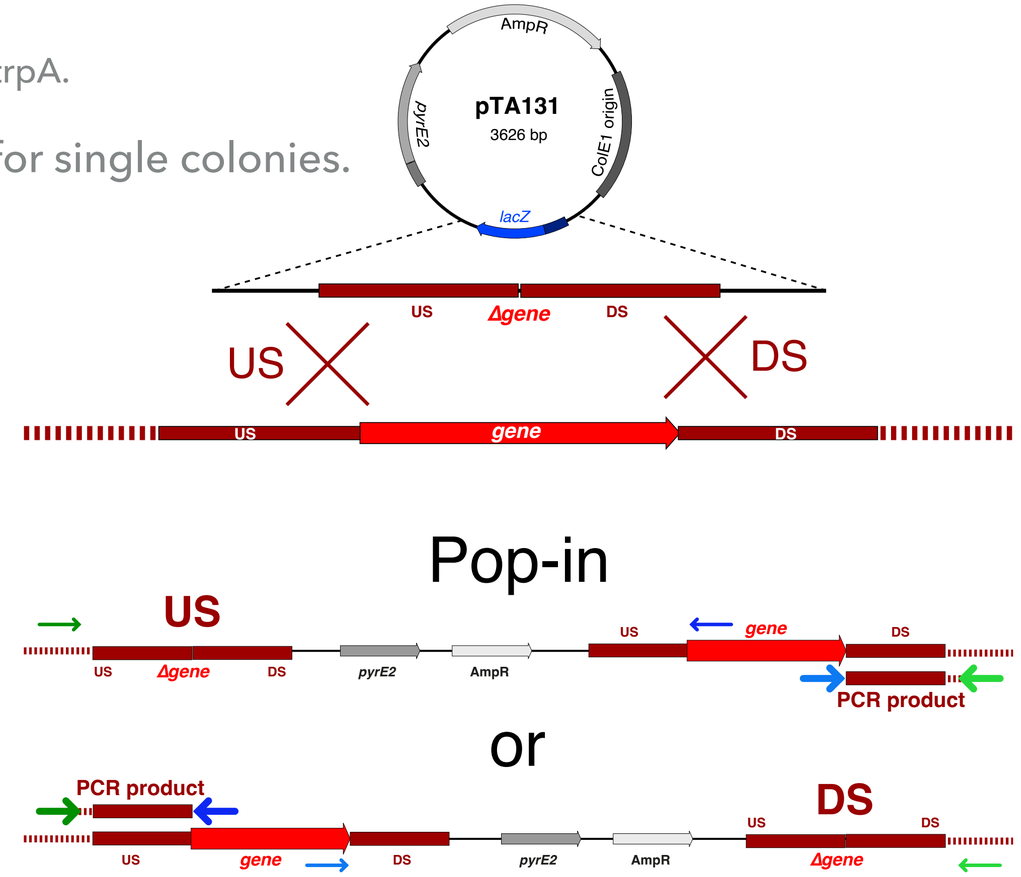
- **(a)** Transformation of Haloferax with gene deletion vector results in “pop-in” integration at chromosomal locus due to recombination between homologous flanking regions. Culturing of transformant in nonselective media and plating on agar containing 5-FOA counterselects for recombination event that has removed pyrE2 marker. This “pop- out” event may revert back to wild-type allele or result in gene deletion, depending on orientation of recombination.
- **(b)** Optional trpA marker can be used to preferentially select for pop-out events resulting in gene deletion.



Bitan-Banin et al (2003); Allers et al (2004)

POP-IN

- Transform (See Haloferax Transformation) parent strain with 1µg gene deletion vector.
  - Use dam- DNA unless parent strain is Δmrr.
  - If using trpA-marked gene deletion, ensure parent strain is ΔtrpA.
- Restreak pop-in transformants on Hv-Ca (+ trp/+thy) for single colonies.
  - Choose 4 or more pop-in transformants to restreak.
- Optional: check orientation of pop-in by colony PCR.
  - Use external primer that binds outside of flanking homology, and internal primer that distinguishes between upstream and downstream pop-in orientation.
  - To maximise the chance of gene deletion pop-out, choose the less likely pop-in orientation (because the flanking homology is shorter).

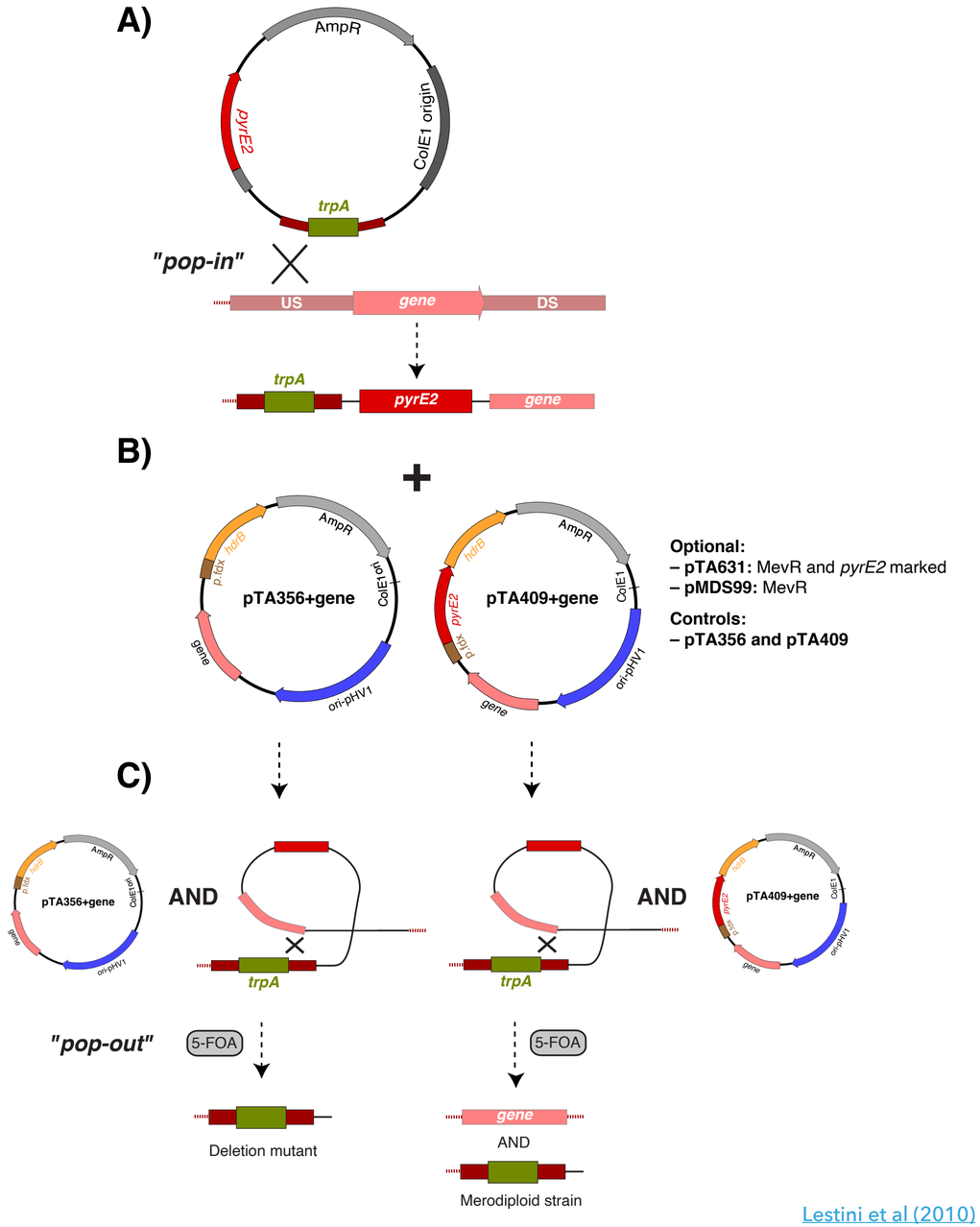


POP-OUT

- Set up 5 ml overnight culture in Hv-YPC (+Thy) with single colony. Next day, dilute 1/500 into fresh 5 ml Hv-YPC (+Thy) for non-selective growth. To rest of 5 ml culture, add 1.5 ml 80% glycerol 6% SW, and mix. Divide into 2 aliquots of 2 ml and store at –80°C.
- Repeat 1/500 dilution 2x more times (total 3 x overnight growths). Plate 100 µl of 10^{-2}, 10^{-3} and 10^{-4} dilution (in 18% SW) on Hv-Ca +5-FOA. Around 0.1 – 1% of cells should be 5-FOA^{R}.
  - Optional: If using trpA-marked deletion, also plate on Hv-Ca +5-FOA +Trp; gene deletion will be 5-FOA^{R} trp^{+}, whereas reversion to wild type is 5-FOA^{R} trp^{-}.
- Patch out 40-120 of 5-FOA^{R} colonies on Hv-YPC (+Thy), grow for 2 days and take a colony lift. Use a probe corresponding to the deleted portion of gene, and choose colonies that do NOT hybridise with this probe. Restreak these on Hv-YPC (+Thy).
- Set up 5 ml overnight cultures in Hv-YPC (+Thy) for genomic DNA minipreps, then check for gene deletion by restriction digest and Southern blot (see Haloferax Blotting: Southern Blot). Use a probe to the flanking upstream/downstream regions (not the probe used for the colony lift).
- Set up 5 ml overnight culture in Hv-YPC (+Thy) of confirmed gene deletion, add 1.5 ml 80% glycerol 6% SW, and mix. Divide into 2 aliquots of 2 ml and store at –80°C.
  - If gene is not essential, reintroduce wild-type gene into deletion strain to verify that phenotype is due to intended gene deletion and not secondary mutation. Carry out pop-in/pop-out protocol, using wild-type gene in pTA131 to replace trpA-marked gene deletion, select trp- 5-FOA^{R} cells, and check gene restoration by Southern.

FOR 'ESSENTIAL' GENES

- **(a)** Use trpA-marked gene deletion construct and check pop-in orientation by colony PCR. If deletion still impossible, use wild-type gene on episomal plasmid for *in trans* complementation.
  - Requires *ΔhdrB* strain for Thy$^+$ selection.
- **(b)** Clone wild-type gene plus promoter in both pTA356 (hdrB marker) and pTA409 (pyrE2 and hdrB marker). Both plasmids use ori-pHV1 low copy-number origin.
  - Optional: where Thy$^+$ selection is not possible/ desirable, use Mevinolin selection. pMDS99 has Mev^R marker and pTA631 has pyrE2 and Mev^R.
- **(c)** Use *in trans* complementation plasmids (plus pTA356 and pTA409 empty vector controls) to transform trpA-marked deletion pop-in strain, select and restreak on Hv-Ca.



Lestini et al (2010)

FOR 'ESSENTIAL' GENES CONT…

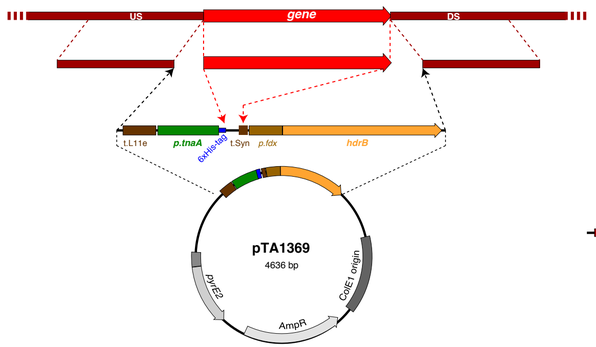
- Carry out pop-out as usual but grow cells with pTA356±gene in Hv-YPC, and cells with pTA409±gene in Hv-YPC+Thy. The former selection maintains episomal plasmid, the latter allows plasmid loss.
- Plate on Hv-Ca +5-FOA without Trp (to select for deletion; for optional control, use +Trp). Use +Thy for cells with pTA409±gene (will be cured by 5-FOA) but without Thy for cells with pTA356±gene.
- Select colonies growing on Hv-Ca +5-FOA (+Thy) plates without Trp, and restreak on Hv-Ca (+Thy). Set up 5 ml cultures in Hv-YPC (+Thy) for genomic DNA minipreps, check deletion by digest and Southern. Restriction digest must distinguish between chromosomal gene (or deletion) and episomal pTA356+gene.
  - If gene is essential, successful deletion from chromosomal locus is only be observed in strain containing pTA356+gene (maintained on media without thymidine), since episomal gene will complement chromosomal deletion. In all other cases, gene is not present on plasmid (empty vector), or plasmid has been lost by curing with 5-FOA (pTA409+gene). In these cases wild-type gene will still be present at chromosomal locus, alongside the trpA-marked deletion (i.e. merodiploid strain).
- To confirm gene is essential, set up culture in Hv-Ca +Thy of strain with deletion of chromosomal gene, which still contains complementing pTA356+gene (growth with thymidine will relieve selection for pTA356+gene). Plate on Hv-Ca +Thy (at 10^{-6} dilution), then patch onto Hv-Ca +/- Thy.
  - If gene is really essential, it will be impossible to lose *in trans* complementing pTA356+gene, and no thy– colonies will be obtained.

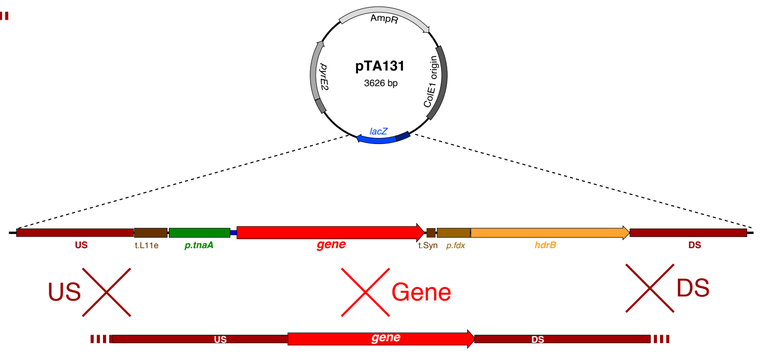
TRYPTOPHAN-INDUCIBLE 'ESSENTIAL' GENES

- To replace native gene at chromosomal locus with tryptophan-inducible allele, clone gene under control of tryptophan-inducible p.tnaA promoter in pTA1369 (or p.tnaM3 reduced-activity version in pTA1451)
  - The hdrB marker in pTA1369 (or pTA1451) is used to select for replacement of native gene
  - For genes in operons, use ectopic integration at pyrE2 locus with pGB68
- Carry out pop-in as usual, parent strain must be ΔpyrE2 ΔhdrB trpA+ (e.g. H98), otherwise growth without tryptophan will be impossible
  - For ectopic integration at pyrE2 use ΔhdrB pyrE2+ strain e.g. H729. Plate on Hv-Ca (+Nov for pGB68)
- Check for orientation of pop-in integration by colony PCR
  - The pop-in orientation must be upstream; only this orientation ensures that Thy+ pop-outs are p.tnaA::gene hdrB+
- Carry out pop-out as usual, but maintain selection for hdrB by growth in Hv-YPC
  Plate on Hv-Ca +5-FOA +Trp plates, to ensure Trp-inducible gene is expressed
  - Optional: Usual concentration of tryptophan is 50 µg/ml (~0.25 mM) but it might be necessary to increase or decrease this, depending on expression of native gene.
- Select 5-FOA^R colonies, patch onto Hv-Ca and Hv-Ca +Trp plates (include Ura for ectopic integration at pyrE2)
  - Then select colonies that grow on Hv-Ca+Trp but not without Trp
  - Then set up 5 ml cultures in Hv-YPC for genomic DNA minipreps, check gene replacement by digest and Southern

Large et al (2007)

TRYPTOPHAN-INDUCIBLE 'ESSENTIAL' GENES CONT...





Pop-in

Pop-out	US	Gene	DS
US	Wild-type	*p.tnaA::gene*	*p.tnaA::gene hdrB+*
Gene	*p.tnaA::gene*	Wild-type	*hdrB+*
DS	*p.tnaA::gene hdrB+*	*hdrB+*	Wild-type

COLONY PCR: ONETAQ

DNA prep:

- Touch DNA with yellow tip (the less cells the better)
- Pipette cells up and down in 100µl SDW
- Boil at 100°C for 10mins
- Leave on ice for 10mins

PCR mix per Reaction	
DNA	1
Forward primer	0,4
Reverse primer	0,4
dNTPs	4
GC buffer (5X)	4
OneTaq	0,1
SDW	10,1
	20µl

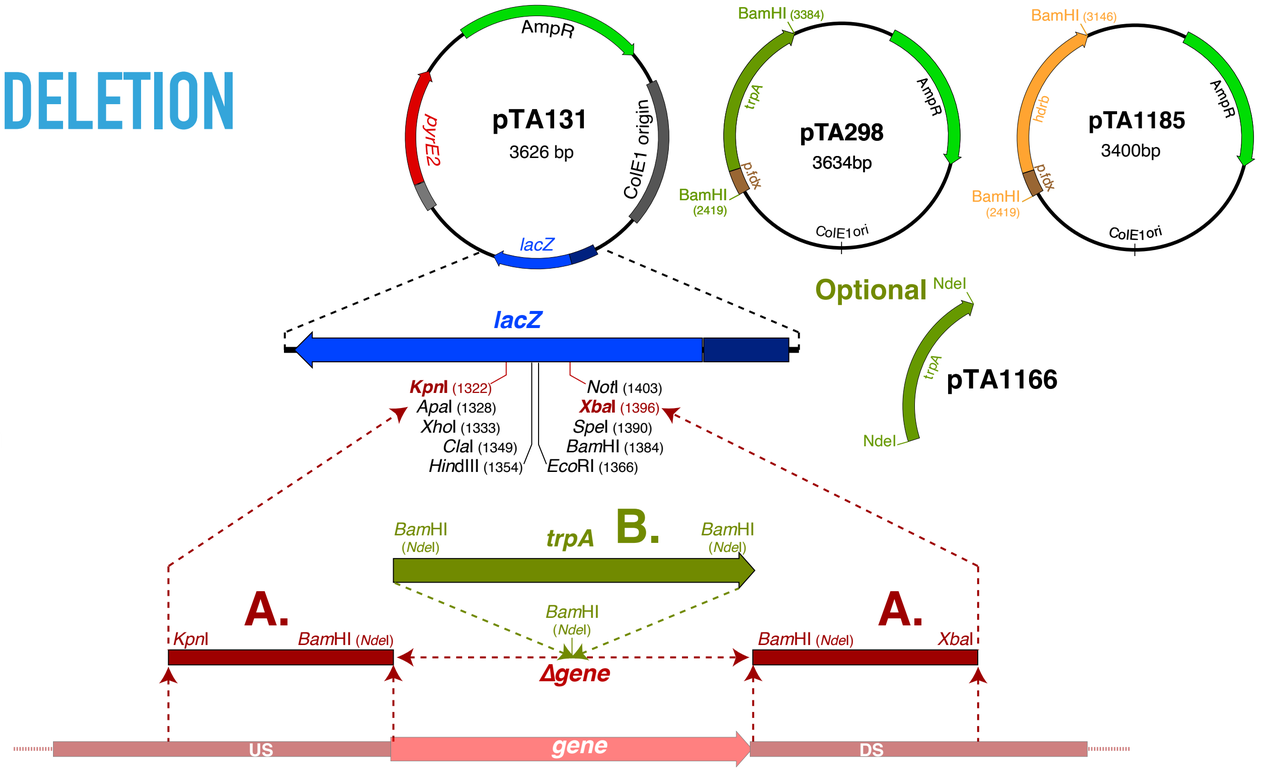
PCR cycles	Temperature /°C	Time	
Initial D	94	30s	
D	94	30s	x 30
A	x	30s	
E	68	y (1min per kb)	
Final E	68	5mins	

Can use Q5 hotstart polymerase instead, but costs more

PLASMIDS FOR GENE DELETION

TRADITIONAL METHOD

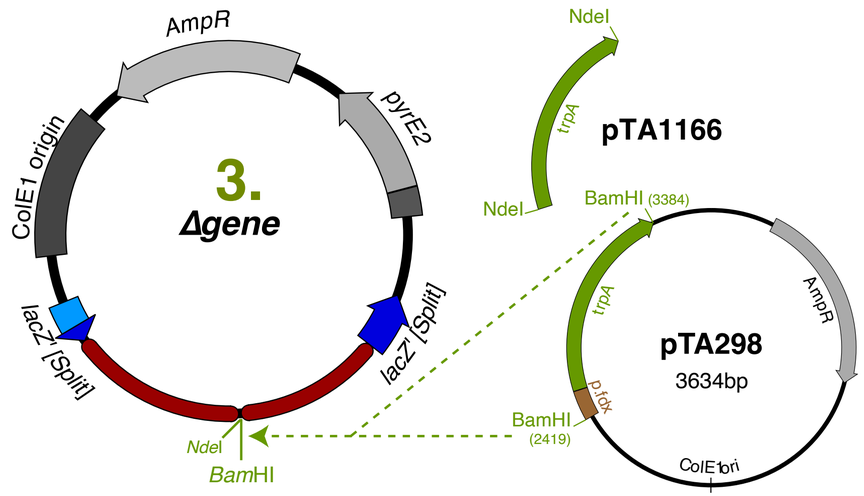
- Make Δgene deletion construct: PCR amplify upstream and downstream flanking regions using primers with KpnI/BamHI sites and BamHI/XbaI sites



- Join upstream and downstream flanking regions at internal BamHI sites, ligate into pTA131 at KpnI and XbaI sites.
  - Optional: for operonic genes, use primers with internal NdeI sites instead of BamHI to remove the high-expression p.fdx promoter.
- Make Δgene::trpA+ construct: excise trpA marker from pTA298 using BamHI, insert between upstream and downstream flanking regions at internal BamHI site.
- Similarly, a Δgene::hdrB+ construct can be created by excision of the hdrB marker from pTA1185 using BamHI and subsequent insertion into the internal BamHI site between the upstream and downstream flanking regions.
  - Optional: for operonic genes, insert promoter-less trpA gene flanked by NdeI sites, excised from pTA1166

PLASMIDS FOR GENE DELETION

ALTERNATIVE METHOD (3)



3. MAKE ΔGENE::TRPA+ CONSTRUCT

- Cut trpA gene out of pTA298 using BamHI, insert between US and DS regions at internal BamHI (or BglII or BclI) site
- For operonic genes, insert promoter-less trpA NdeI fragment from pTA1166.

Plasmid	Relevant Properties
pTA131	Integrative vector based on pBluescript, with pyrE2 marker (pTA1431 has no BamHI site)
pTA298	Integrative vector based on pBluescript, with pyrE2 and trpA markers
pTA356	Shuttle vector with hdrB marker and pHV1/4 replication origin
pTA409	Shuttle vector with pyrE2 and hdrB markers and pHV1/4 replication origin
pTA631	Shuttle vector with mevinolin-resistance and pyrE2 markers and pHV2 replication origin
pTA1185	Integrative vector based on pBluescript, with pyrE2 and hdrB markers
pTA1166	Vector based on pBluescript, with pyrE2 and promoter-less trpA markers
pTA1369	Vector with pyrE2 marker and p.tnaA promoter
pTA1451	Vector with pyrE2 marker and p.tnaM3 promoter
pTA2168	Vector with leuB marker and p.tnaA promoter
pTA2169	Vector with leuB marker and p.tnaM3 promoter
pTAMDS99	Shuttle vector with mevinolin-resistance marker and pHV2 replication origin
pGB68	Integrative vector with flanking ΔpyrE2 sequences and NovR marker

80% GLYCEROL 6% SW

- 80ml glycerol
- 20ml 30% SW
- Autoclave for 1 minutes at 121°C (program 1)
- When cool add 200µl 0.5 M CaCl2

18% SW

- 200ml 30% SW
- 133ml dH_2O
- Autoclave for 1 minutes at 121°C (program 1)
- When cool add 2ml 0.5M CaCl2