CLONING

PCRS

Q5 PCR

PCR mix per Reaction	
DNA*	1
Forward primer (50 µM)	1
Reverse primer (50 µM)	1
dNTPs (1 mM)	10
GC enhancer (5X)	10
Q5 buffer (5X)	10
Q5 hotstart polymerase	0,5
SDW	16,5
	50µl

PCR cycles	Temperature /°C	Time	
Initial Denature	98	30s	
Denature	98	10s	x 30
Anneal	x**	20s	
Extension	72	y† (secs per kb)	
Final Extension	72	10mins	

- DNA* Use ~50 ng plasmid DNA; if using genomic DNA, dilute 1/10 in dH_2O
- x** = annealing temperature

  = 81.5 + 0.41%GC - 600/Length + 16.6$Log_{10}[Na^+]$
  Assuming Na^+ concentration = 50 mM
  Subtract 1°C for each 1% mismatch

- y† = extension time, 20 sec/kb for plasmid DNA, 30 sec/kb for genomic DNA,

TOUCHDOWN PCR

TD PCR cycles	Temperature /°C	Time	
Initial Denature	98	30s	
Denature	98	10s	x 10
Anneal	Low - High temp**	20s	
Extension	72	y† (secs per kb)	
Denature	98	10s	x 20
Anneal	High temp**	20s	
Extension	72	y† (secs per kb)	
Final Extension	72	10mins	

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

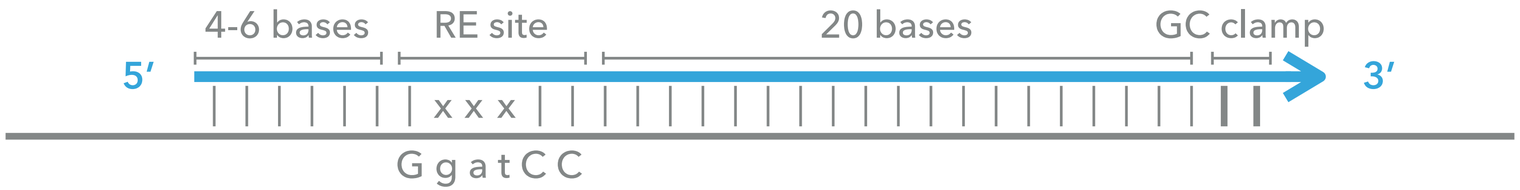
PCR mix per Reaction	
DNA	1
Forward primer	0,4
Reverse primer	0,4
dNTPs	4
GC buffer (5X)	4
OneTaq	0,1
SDW	10,1
	20µl

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

TIPS FOR PRIMER DESIGN

- PCR primers from MWG are fixed-price for 15-35 bases, don't be stingy
- Last two bases at 3′ end must be G/C = GC clamp
  - Primers should be ~65%GC to match Hvo genome, but this is not critical
- Try to limit number of mismatches to 3 or 4, or increase length of primer
  - Any mismatches should be as close as possible to 5′ end of primer
- But… restriction sites should be located ≥4 bases from 5′ end, (≥6 bases from 5′ end for NdeI), otherwise enzyme will not cut
- 5′ -[6-bases@100%homology]-[≤4-bases@mismatch]-[20-bases@100%homology] -3′



DIGESTS

DIGESTS

- Note: volume of enzyme(s) added must not exceed 10% of the total volume

	Maxiprepped DNA	Miniprepped/genomic DNA	PCR
DNA	(1-2µg)	5	~41 (eluted in 45µl)
Buffer (10x)	2	2	5
Enzyme 1	1	1	1
Enzyme 2 (if applicable)	1	1	1
SDW	x	x	x
	20µl	20µl	50µl

- Incubate at appropriate temperature for the enzymes used, for an appropriate length of time. Typically Maxi- and Mini-prepped DNA for 1-2hours, genomic and PCR DNA overnight
- Note: useful website is the NEB Double digest finder to work out appropriate buffer
- Note: nCTAGn sites are methylated in Haloferax volcanii, therefore genomic DNA will not be cut by these enzymes (e.g. XbaI).

MAGIC BOOK

Box	Name	Sequence
A	AarI	CACCTGCNNNN'NNNN.
	AatII	G.ACGT'C
	AccI	GT'MK.AC
	AciI	C'CG.C
	AclI	AA'CG.TT
	AfeI	AGC|GCT
	AflII	C'TTAA.G
	AflIII	A'CRYG.T
	AgeI	A'CCGG.T
	AleI	CACNN|NNGTG
	AluI	AG|CT
	ApaI	G.GGCC'C
	ApaLI	G'TGCA.C
	ApeKI	G'CWG.C
	AscI	GG'CGCG.CC
	AseI	AT'TA.AT
	AsiSI	GCG.AT'CGC
	Asp718I	G'GTAC.C
	AvaI	C'YCGR.G
	AvrII	C'CTAG.G
B1	BamHI	G'GATC.C
	BanI	G'GYRC.C
	BbsI	GAAGACNN'NNNN.
	BciVI	GTATCCNNNNN.N'
	BclI	T'GATC.A
	BfuAI	ACCTGCNNNN'NNNN.
	BglI	GCCN.NNN'NGGC
	BglII	A'GATC.T
	BlpI	GC'TNA.GC
	BmgBI	CAC|GTC
	BmtI	G.CTAG'C
	Bpu10I	CC'TNA.GC
B2	BsaAI	YAC|GTR
	BsaBI	GATNN|NNATC
	BsaHI	GR'CG.YC
	BsaI	GGTCTCN'NNNN.
	BsaWI	W'CCGG.W
	BseYI	C'CCAG.C
	BsiWI	C'GTAC.G
	BsmI	GAATG.CN'
	Bsp120I	G'GGCC.C
	BspEI	T'CCGG.A
	BspHI	T'CATG.A
	BspMI	ACCTGCNNNN'NNNN.
	BsrGI	T'GTAC.A
	BssHII	G'CGCG.C
	BstBI	TT'CG.AA
	BstEII	G'GTNAC.C
	BstXI	CCAN.NNNN'NTGG
	Bsu36I	CC'TNA.GG
CD	ClaI	AT'CG.AT
	DdeI	C'TNA.G
	DpnI	GA|TC
	DpnII	'GATC.
	DraI	TTT|AAA
	DraIII	CAC.NNN'GTG
EF	EagI	C'GGCC.G
	EarI	CTCTTCN'NNN.
	Eco47III	AGC|GCT
	Eco52I	C'GGCC.G
	EcoNI	CCTNN'N.NNAGG
	EcoRI	G'AATT.C
	EcoRV	GAT|ATC
	EspI	GC'TNA.GC
	FatI	'CATG.
	FspI	TGC|GCA
H	HaeII	R.GCGC'Y
	HaeIII	GG|CC
	HincII	GTY|RAC
	HindIII	A'AGCT.T
	HinfI	G'ANT.C
	HpaI	GTT|AAC
	HpaII	C'CG.G
	Hpy188I	TC.N'GA
KM	KpnI	G.GTAC'C
	MauBI	CG'CGCG.CG
	MboI	'GATC.
	MluI	A'CGCG.T
	MscI	TGG|CCA
	MseI	T'TA.A
	MspI	C'CG.G
N	NaeI	GCC|GGC
	NarI	GG'CG.CC
	NciI	CC'S.GG
	NcoI	C'CATG.G
	NdeI	CA'TA.TG
	NdeII	'GATC.
	NgoMIV	G'CCGG.C
	NheI	G'CTAG.C
	NotI	GC'GGCC.GC
	NruI	TCG|CGA
	NsiI	A.TGCA'T
	NspI	R.CATG'Y
PR	PauI	G'CGCG.C
	PciI	A'CATG.T
	PmeI	GTTT|AAAC
	PshAI	GACNN|NNGTC
	PsiI	TTA|TAA
	PspOMI	G'GGCC.C
	PstI	C.TGCA'G
	PvuI	CG.AT'CG
	PvuII	CAG|CTG
	RsrII	CG'GWC.CG
S	SacI	G.AGCT'C
	SalI	G'TCGA.C
	SapI	GCTCTTCN'NNN.
	Sau3AI	'GATC.
	SbfI	CC.TGCA'GG
	ScaI	AGT|ACT
	I-SceI	AGTTACGCTAGGG.ATAA'CAGGGTAATATAG
	SexAI	A'CCWGG.T
	SfaAI	GCG.AT'CGC
	SfiI	GGCCN.NNN'NGGCC
	SfoI	GGC|GCC
	SmaI	CCC|GGG
	SnaBI	TAC|GTA
	SpeI	A'CTAG.T
	SphI	G.CATG'C
	SspI	AAT|ATT
	SstII	CC.GC'GG
	StuI	AGG|CCT
	StyI	C'CWWG.G
	SwaI	ATTT|AAAT
TX	TaqI	T'CG.A
	TseI	G'CWG.C
	XbaI	T'CTAG.A
	XhoI	C'TCGA.G
	XmaI	C'CCGG.G
	XmnI	GAANN|NNTTC

NEW ENGLAND BioLabs Inc.

Performance Chart for Restriction Enzymes

Enzyme	Supplied NEBuffer	r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity	Notes
AatII	rCutSmart	<10	50*	50	100	37°	80°	B	λ DNA	CpG	
AbaSI	rCutSmart + DTT	25	50	50	100	25°	65°	C	T4 wild-type phage DNA (fully ghmC-modified)		e
Acc65I	r3.1	10	75*	100	25	37°	65°	A	pBC4 DNA	dcm CpG	
AccI	rCutSmart	50	50	10	100	37°	80°	A	λ DNA	CpG	
AciI	rCutSmart	<10	25	100	100	37°	65°	A	λ DNA	CpG	
AclI	rCutSmart	<10	<10	<10	100	37°	No	B	λ DNA	CpG	
AcuI	rCutSmart	50	100	50	100	37°	65°	B	λ DNA		1, b, d
AfeI	rCutSmart	25	100	25	100	37°	65°	B	pXba DNA	CpG	
AflII	rCutSmart	50	100	10	100	37°	65°	A	ΦX174 RF I DNA		
AflIII	r3.1	10	50	100	50	37°	80°	B	λ DNA		
AgeI-HF	rCutSmart	100	50	10	100	37°	65°	A	λ DNA	CpG	
AhdI	rCutSmart	25	25	10	100	37°	65°	A	λ DNA	CpG	a
AleI-v2	rCutSmart	<10	<10	<10	100	37°	65°	B	λ DNA	CpG	
AluI	rCutSmart	25	100	50	100	37°	80°	B	λ DNA		b
AlwI	rCutSmart	50	50	10	100	37°	No	A	λ DNA (dam-)	dam	1, b, d
AlwNI	rCutSmart	10	100	50	100	37°	80°	A	λ DNA	dcm	
ApaI	rCutSmart	25	25	<10	100	37°	65°	A	pXba DNA	dcm CpG	
ApaLI	rCutSmart	100	100	10	100	37°	No	A	λ DNA (HindIII digest)	CpG	
ApeKI	r3.1	25	50	100	10	75°	No	B	λ DNA	CpG	
ApoI-HF	rCutSmart	10	100	10	100	37°	80°	B	λ DNA		
AscI	rCutSmart	<10	10	10	100	37°	80°	A	λ DNA	CpG	
AseI	r3.1	<10	50*	100	10	37°	65°	B	λ DNA		3
AsiSI	rCutSmart	100	100	25	100	37°	80°	B	XhoI digested pXba	CpG	2, b
AvaI	rCutSmart	<10	100	25	100	37°	80°	A	λ DNA	CpG	
AvaII	rCutSmart	50	75	10	100	37°	80°	A	λ DNA	dcm CpG	
AvrII	rCutSmart	100	50	50	100	37°	No	B	λ DNA (HindIII digest)		
BaeGI	r3.1	75	75	100	25	37°	80°	A	λ DNA		
BaeI	rCutSmart	50	100	50	100	37°	65°	A	λ DNA	CpG	e
BamHI	r3.1	75*	100*	100	100*	37°	No	A	λ DNA		3
BamHI-HF	rCutSmart	100	50	10	100	37°	No	A	λ DNA		
BanI	rCutSmart	10	25	<10	100	37°	65°	A	λ DNA	dcm CpG	1
BanII	rCutSmart	100	100	50	100	37°	80°	A	λ DNA		2
BbsI	r2.1	100	100	25	75	37°	65°	B	λ DNA		
BbsI-HF	rCutSmart	10	10	10	100	37°	65°	B	λ DNA		
BbvCI	rCutSmart	10	100	50	100	37°	No	B	λ DNA	CpG	1, a
BbvI	rCutSmart	100	100	25	100	37°	65°	B	pBR322 DNA		3
BccI	rCutSmart	100	50	10	100	37°	65°	A	pXba DNA		3, b
BceAI	r3.1	100*	100*	100	100*	37°	65°	A	pBR322 DNA	CpG	1
BcgI	r3.1	10	75*	100	50*	37°	65°	A	λ DNA	dam CpG	e
BciVI	rCutSmart	100	25	<10	100	37°	80°	C	λ DNA		b
BclI	r3.1	50	100	100	75	50°	No	A	λ DNA (dam-)	dam	
BclI-HF	rCutSmart	100	100	10	100	37°	65°	B	λ DNA (dam-)	dam	
BcoDI	rCutSmart	50	75	75	100	37°	No	B	λ DNA	CpG	
BfaI	rCutSmart	<10	10	<10	100	37°	80°	B	λ DNA		2, b
BfuAI	r3.1	<10	25	100	10	50°	65°	B	λ DNA	CpG	3
BglI	r3.1	10	25	100	10	37°	65°	B	λ DNA	CpG	
BglII	r3.1	10	10	100	<10	37°	No	A	λ DNA		
BlpI	rCutSmart	50	100	10	100	37°	No	A	λ DNA		d
BmgBI	r3.1	<10	10	100	10	37°	65°	B	λ DNA	CpG	3, b, d
BmrI	r2.1	75	100	75	100*	37°	65°	B	λ DNA (HindIII digest)		b
BmtI-HF	rCutSmart	50	100	10	100	37°	65°	B	pXba DNA		
BpmI	r3.1	75	100	100	100*	37°	65°	B	λ DNA		2
Bpu10I	r3.1	10	25	100	25	37°	80°	B	λ DNA		3, b, d
BpuEI	rCutSmart	50*	100	50*	100	37°	65°	B	λ DNA		d
BsaAI	rCutSmart	100	100	100	100	37°	No	C	λ DNA	CpG	
BsaBI	rCutSmart	50	100	75	100	60°	80°	B	λ DNA (dam-)	dam CpG	2
BsaHI	rCutSmart	50	100	100	100	37°	80°	C	λ DNA	dcm CpG	
BsaI-HFv2	rCutSmart	100	100	100	100	37°	80°	B	pXba DNA	dcm CpG	
BsaJI	rCutSmart	50	100	100	100	60°	80°	A	λ DNA		
BsaWI	rCutSmart	10	100	50	100	60°	80°	A	λ DNA		
BsaXI	rCutSmart	50*	100*	10	100	37°	No	C	λ DNA		e
BseRI	rCutSmart	100	100	75	100	37°	80°	A	λ DNA		d
BseYI	r3.1	10	50	100	50	37°	80°	B	λ DNA	CpG	d
BsgI	rCutSmart	25	50	25	100	37°	65°	B	λ DNA		d
BsiEI	rCutSmart	25	50	<10	100	60°	No	A	λ DNA	CpG	
BsiHKAI	rCutSmart	25	100	100	100	65°	No	A	λ DNA		
BsiWI	r3.1	25	50*	100	25	55°	65°	B	ΦX174 DNA	CpG	
BsiWI-HF	rCutSmart	50	100	10	100	37°	No	B	ΦX174 DNA	CpG	
BslI	rCutSmart	50	75	100	100	55°	No	A	λ DNA	dcm CpG	b
BsmAI	rCutSmart	50	100	100	100	55°	No	B	λ DNA	CpG	
BsmBI-v2	r3.1	<10	50	100	25	55°	80°	B	λ DNA	CpG	
BsmFI	rCutSmart	25	100	50	100	65°	80°	A	pBR322 DNA	dcm CpG	1
BsmI	rCutSmart	25	100	<10	100	65°	80°	A	λ DNA		
BsoBI	rCutSmart	25	100	100	100	37°	80°	A	λ DNA		
Bsp1286I	rCutSmart	25	25	25	100	37°	65°	A	λ DNA		3
BspCNI	rCutSmart	100	75	10	100	37°	80°	A	λ DNA		b
BspDI	rCutSmart	25	75	50	100	37°	80°	A	λ DNA	dam CpG	
BspEI	r3.1	<10	10	100	<10	37°	80°	B	λ DNA (dam-)	dam CpG	
BspHI	rCutSmart	10	50	25	100	37°	80°	A	λ DNA	dam	
BspMI	r3.1	10	50*	100	10	37°	65°	B	λ DNA		
BspQI	r3.1	100*	100*	100	100*	50°	80°	B	λ DNA		3
BsrBI	rCutSmart	50	100	100	100	37°	80°	A	λ DNA	CpG	d
BsrDI	r2.1	10	100	75	25	37°	80°	A	λ DNA		3, d
BsrFI-v2	rCutSmart	25	25	0	100	37°	No	C	pBR322 DNA	CpG	
BsrGI-HF	rCutSmart	10	100	100	100	37°	80°	A	λ DNA		
BsrI	r3.1	<10	50	100	10	65°	80°	B	ΦX174 DNA		b
BssHII	rCutSmart	100	100	100	100	50°	65°	B	λ DNA	CpG	
BssSI-v2	rCutSmart	10	25	<10	100	37°	No	B	λ DNA		
BstAPI	rCutSmart	50	100	25	100	60°	80°	A	λ DNA	CpG	b
BstBI	rCutSmart	75	100	10	100	65°	No	A	λ DNA	CpG	
BstEII-HF	rCutSmart	<10	10	<10	100	37°	No	A	λ DNA		
BstNI	r3.1	10	100	100	75	60°	No	A	λ DNA		a
BstUI	rCutSmart	50	100	25	100	60°	No	A	λ DNA	CpG	b
BstXI	r3.1	<10	50	100	25	37°	80°	B	λ DNA	dcm	3
BstYI	rCutSmart	25	100	75	100	60°	No	A	λ DNA		
BstZ17I-HF	rCutSmart	100	100	10	100	37°	No	A	λ DNA	CpG	
Bsu36I	rCutSmart	25	100	100	100	37°	80°	C	λ DNA (HindIII digest)		b
BtgI	rCutSmart	50	100	100	100	37°	80°	B	pBR322 DNA		
BtgZI	rCutSmart	10	25	<10	100	60°	80°	A	λ DNA	CpG	3, b, d
BtsCI	rCutSmart	10	100	25	100	50°	80°	B	λ DNA		
BtsI-v2	rCutSmart	100	100	25	100	37°	No	A	λ DNA		1
BtsIMutI	rCutSmart	100	50	10	100	55°	80°	A	pUC19 DNA		b
Cac8I	rCutSmart	50	75	100	100	37°	65°	B	λ DNA	CpG	b
ClaI	rCutSmart	10	50	50	100	37°	65°	A	λ DNA (dam-)	dam CpG	
CspCI	rCutSmart	10	100	10	100	37°	65°	A	λ DNA		e
CviAII	rCutSmart	50	50	10	100	25°	65°	C	λ DNA		
CviKI-1	rCutSmart	25	100	100	100	37°	No	A	pBR322 DNA		1, b
CviQI	r3.1	75	100*	100	75*	25°	No	C	λ DNA		b
DdeI	rCutSmart	75	100	100	100	37°	65°	B	λ DNA		
DpnI	rCutSmart	100	100	75	100	37°	80°	B	pBR322 DNA (dam methylated)	CpG	b
DpnII	U	25	25	100*	25	37°	65°	B	λ DNA (dam-)	dam	
DraI	rCutSmart	75	75	50	100	37°	65°	A	λ DNA		
DraIII-HF	rCutSmart	<10	50	10	100	37°	No	B	λ DNA	CpG	b
DrdI	rCutSmart	25	50	10	100	37°	65°	A	pUC19 DNA	CpG	3
EaeI	rCutSmart	10	50	<10	100	37°	65°	A	λ DNA	dcm CpG	b
EagI-HF	rCutSmart	25	100	100	100	37°	65°	B	pXba DNA	CpG	
EarI	rCutSmart	50	10	<10	100	37°	65°	B	λ DNA	CpG	b, d
EciI	rCutSmart	100	50	50	100	37°	65°	A	λ DNA	CpG	2
Eco53kI	rCutSmart	100	100	<10	100	37°	65°	A	pXba DNA	CpG	3, b
EcoNI	rCutSmart	50	100	75	100	37°	65°	A	λ DNA		b
EcoO109I	rCutSmart	50	100	50	100	37°	65°	A	λ DNA (HindIII digest)	dcm	3
EcoP15I	r3.1 + ATP	75	100	100	100	37°	65°	A	pUC19 DNA		e
EcoRI	U	25	100*	50	50*	37°	65°	C	λ DNA	CpG	
EcoRI-HF	rCutSmart	10	100	<10	100	37°	65°	C	λ DNA	CpG	
EcoRV	r3.1	10	50	100	10	37°	80°	A	λ DNA	CpG	
EcoRV-HF	rCutSmart	25	100	100	100	37°	65°	B	λ DNA	CpG	
Esp3I	rCutSmart	100	100	<10	100	37°	65°	B	λ DNA	CpG	
FatI	r2.1	10	100	50	50	55°	80°	A	pUC19 DNA		
FauI	rCutSmart	100	50	10	100	55°	65°	A	λ DNA	CpG	3, b, d
Fnu4HI	rCutSmart	<10	<10	<10	100	37°	No	A	λ DNA	CpG	a
FokI	rCutSmart	100	100	75	100	37°	65°	A	λ DNA	dcm CpG	3, b, d
FseI	rCutSmart	100	75	<10	100	37°	65°	B	pBC4 DNA	dcm CpG	
FspEI	rCutSmart + Enz. Activ.	<10	<10	<10	100	37°	80°	B	pBR322 (dcm+) DNA		1, e
FspI	rCutSmart	10	100	10	100	37°	No	C	λ DNA	CpG	b
HaeII	rCutSmart	25	100	10	100	37°	80°	A	λ DNA	CpG	
HaeIII	rCutSmart	50	100	25	100	37°	80°	A	λ DNA		
HgaI	r1.1	100	100	25	100*	37°	65°	A	ΦX174 DNA	CpG	1
HhaI	rCutSmart	25	100	100	100	37°	65°	A	λ DNA	CpG	
HinP1I	rCutSmart	100	100	100	100	37°	65°	A	λ DNA	CpG	
HincII	rCutSmart	25	100	100	100	37°	65°	B	λ DNA	CpG	
HindIII	r2.1	25	100	50	50	37°	80°	B	λ DNA		2
HindIII-HF	rCutSmart	10	100	10	100	37°	80°	B	λ DNA		
HinfI	rCutSmart	50	100	100	100	37°	80°	A	λ DNA	CpG	
HpaI	rCutSmart	<10	75*	25	100	37°	No	A	λ DNA	CpG	1
HpaII	rCutSmart	100	50	<10	100	37°	80°	A	λ DNA	CpG	
HphI	rCutSmart	50	50	<10	100	37°	65°	B	λ DNA	dcm	1, b, d
Hpy166II	rCutSmart	100	100	50	100	37°	65°	C	pBR322 DNA	CpG	
Hpy188I	rCutSmart	25	100	50	100	37°	65°	A	pBR322 DNA	dam	1, b
Hpy188III	rCutSmart	100	100	10	100	37°	65°	B	pUC19 DNA	dam CpG	3, b
Hpy99I	rCutSmart	50	10	<10	100	37°	65°	A	λ DNA	CpG	
HpyAV	rCutSmart	100	100	25	100	37°	65°		λ DNA	CpG	3, b, d
HpyCH4III	rCutSmart	100	25	<10	100	37°	65°	A	λ DNA		b
HpyCH4IV	rCutSmart	100	50	25	100	37°	65°	A	pUC19 DNA	CpG	
HpyCH4V	rCutSmart	50	50	25	100	37°	65°	A	λ DNA		
I-CeuI	rCutSmart	10	10	10	100	37°	65°	B	pBHS ScaI-linearized Control Plasmid		
I-SceI	rCutSmart	10	50	25	100	37°	65°	B	pGPS2 NotI-linearized Control Plasmid		
KasI	rCutSmart	50	100	50	100	37°	65°	B	pBR322 DNA	CpG	3
KpnI-HF	rCutSmart	100	25	<10	100	37°	No	A	pXba DNA		
LpnPI	rCutSmart + Enz. Activ.	<10	<10	<10	100	37°	65°	B	pBR322 (dcm+) DNA		1, e
MboI	rCutSmart	75	100	100	100	37°	65°	A	λ DNA (dam-)	dam CpG	
MboII	rCutSmart	100*	100	50	100	37°	65°	C	λ DNA (dam-)	dam	b
MfeI-HF	rCutSmart	75	25	<10	100	37°	No	A	λ DNA		
MluCI	rCutSmart	100	10	10	100	37°	No	A	λ DNA		
MluI-HF	rCutSmart	25	100	100	100	37°	No	A	λ DNA	CpG	
MlyI	rCutSmart	50	50	10	100	37°	65°	A	λ DNA		b, d
MmeI	rCutSmart	50	100	50	100	37°	65°	B	ΦX174 RF I DNA	CpG	b, c
MnlI	rCutSmart	75	100	50	100	37°	65°	B	λ DNA		b
MscI	rCutSmart	25	100	100	100	37°	80°	C	λ DNA	dcm	
MseI	rCutSmart	75	100	75	100	37°	65°	A	λ DNA		
MslI	rCutSmart	50	50	<10	100	37°	80°	A	λ DNA		
MspA1I	rCutSmart	10	50	10	100	37°	65°	B	λ DNA	CpG	
MspI	rCutSmart	75	100	50	100	37°	No	A	λ DNA		
MspJI	rCutSmart + Enz. Activ.	<10	<10	<10	100	37°	65°	B	pBR322 (dcm+) DNA		1, e
MwoI	rCutSmart	<10	100	100	100	60°	No	B	λ DNA	CpG	
NaeI	rCutSmart	25	25	<10	100	37°	No	A	pXba DNA	CpG	b
NarI	rCutSmart	100	100	10	100	37°	65°	A	pXba DNA	CpG	
Nb.BbvCI	rCutSmart	25	100	100	100	37°	80°	A	supercoiled plasmid DNA		e
Nb.BsmI	r3.1	<10	50	100	10	65°	80°	A	supercoiled plasmid pBR322 DNA		e
Nb.BsrDI	rCutSmart	25	100	100	100	65°	80°	A	supercoiled pUC19 DNA		e
Nb.BssSI	r3.1	10	100	100	25	37°	No	B	supercoiled pUC19 DNA		e
Nb.BtsI	rCutSmart	75	100	75	100	37°	80°	A	supercoiled pUC101 DNA (dam-/dcm-)		e
NciI	rCutSmart	100	25	10	100	37°	No	A	λ DNA	CpG	b
NcoI	r3.1	100	100	100	100	37°	80°	A	λ DNA		
NcoI-HF	rCutSmart	50	100	10	100	37°	80°	B	λ DNA		
NdeI	rCutSmart	75	100	100	100	37°	65°	A	λ DNA		
NgoMIV	rCutSmart	100	50	10	100	37°	No	A	pXba DNA	CpG	1
NheI-HF	rCutSmart	100	25	10	100	37°	80°	C	λ DNA (HindIII digest)	CpG	
NlaIII	rCutSmart	<10	<10	<10	100	37°	65°	B	ΦX174 RF I DNA		
NlaIV	rCutSmart	10	10	10	100	37°	65°	B	pBR322 DNA	dcm CpG	
NmeAIII	rCutSmart	10	10	<10	100	37°	65°	B	ΦX174 RF I DNA		c
NotI	r3.1	<10	50	100	25	37°	65°	C	pBC4 DNA	CpG	
NotI-HF	rCutSmart	25	100	25	100	37°	65°	A	pBC4 DNA	CpG	
NruI-HF	rCutSmart	0	25	50	100	37°	No	A	λ DNA	dam CpG	
NsiI	r3.1	10	75	100	25	37°	65°	B	λ DNA		
NsiI-HF	rCutSmart	<10	20	<10	100	37°	80°	B	λ DNA		
NspI	rCutSmart	100	100	<10	100	37°	65°	A	λ DNA		
Nt.AlwI	rCutSmart	10	100	100	100	37°	80°	A	pUC101 DNA (dam-/dcm-)	dam	e
Nt.BbvCI	rCutSmart	50	100	10	100	37°	80°	A	supercoiled plasmid DNA	CpG	e
Nt.BsmAI	rCutSmart	100	50	10	100	37°	65°	A	supercoiled plasmid DNA	CpG	e
Nt.BspQI	r3.1	<10	25	100	10	50°	80°	B	supercoiled pUC19 DNA		e
Nt.BstNBI	r3.1	0	10	100	10	55°	80°	A	T7 DNA		e
Nt.CviPII	rCutSmart	10	100	25	100	37°	65°	A	pUC19 DNA	CpG	e
PI-PspI	U + rAlbumin	10	10	10	10	65°	No	B	pAKR7 XmnI-linearized Control Plasmid		
PI-SceI	U + rAlbumin	10	10	10	10	37°	65°	B	pBSvdeX XmnI-linearized Control Plasmid		
PacI	rCutSmart	100	75	10	100	37°	65°	A	pNEB193 DNA		
PaeR7I	rCutSmart	25	100	10	100	37°	No	A	λ DNA (HindIII digest)	CpG	
PaqCI	rCutSmart PaqCI Activator	10	100	10	100	37°	65°	B	λ DNA	CpG	1
PciI	r3.1	50	75	100	50*	37°	80°	B	pXba DNA		
PflFI	rCutSmart	25	100	25	100	37°	65°	A	pBC4 DNA		b
PflMI	r3.1	0	100	100	50	37°	65°	A	λ DNA	dcm	3, b, d
PleI	rCutSmart	25	50	25	100	37°	65°	A	λ DNA	CpG	b, d
PluTI	rCutSmart	100	25	<10	100	37°	65°	A	pXba DNA	CpG	b
PmeI	rCutSmart	<10	50	10	100	37°	65°	A	λ DNA	CpG	
PmlI	rCutSmart	100	50	<10	100	37°	65°	A	λ DNA (HindIII digest) DNA	CpG	
PpuMI	rCutSmart	<10	<10	<10	100	37°	No	B	λ DNA (HindIII digest)	dcm	
PshAI	rCutSmart	25	50	10	100	37°	65°	A	λ DNA	CpG	
PsiI-v2	rCutSmart	25	50	10	100	37°	65°	B	λ DNA		3
PspGI	rCutSmart	25	100	50	100	75°	No	A	T7 DNA	dcm	3
PspOMI	rCutSmart	10	10	<10	100	37°	65°	B	pXba DNA	dcm CpG	
PspXI	rCutSmart	<10	100	25	100	37°	No	B	λ DNA (HindIII digest)	CpG	
PstI	r3.1	75	75	100	50*	37°	80°	C	λ DNA		
PstI-HF	rCutSmart	10	75	50	100	37°	No	C	λ DNA		
PvuI-HF	rCutSmart	25	100	100	100	37°	No	B	pXba DNA	CpG	
PvuII	r3.1	50	100	100	100*	37°	No	B	λ DNA		
PvuII-HF	rCutSmart	<10	<10	<10	100	37°	No	B	λ DNA		
RsaI	rCutSmart	25	50	<10	100	37°	No	A	λ DNA	CpG	
RsrII	rCutSmart	25	75	10	100	37°	65°	C	λ DNA	CpG	
SacI-HF	rCutSmart	10	50	<10	100	37°	65°	A	λ DNA (HindIII digest)	CpG	
SacII	rCutSmart	10	100	10	100	37°	65°	A	pXba DNA	CpG	
SalI	r3.1	<10	<10	100	<10	37°	65°	A	λ DNA (HindIII digest)	CpG	
SalI-HF	rCutSmart	10	100	100	100	37°	65°	A	λ DNA (HindIII digest)	CpG	
SapI	rCutSmart	75	50	<10	100	37°	65°	B	λ DNA		
Sau3AI	r1.1	100	50	10	100	37°	65°	A	λ DNA	CpG	b
Sau96I	rCutSmart	50	100	100	100	37°	65°	A	λ DNA	dcm CpG	
SbfI-HF	rCutSmart	50	25	<10	100	37°	80°	B	λ DNA		
ScaI-HF	rCutSmart	100	100	10	100	37°	80°	B	λ DNA		
ScrFI	rCutSmart	100	100	100	100	37°	65°	C	λ DNA	dcm CpG	2, a
SexAI	rCutSmart	100	75	50	100	37°	65°	A	pBC4 DNA (dcm-)	dcm	3, b, d
SfaNI	r3.1	<10	75	100	25	37°	65°	B	ΦX174 RF I DNA	CpG	3, b
SfcI	rCutSmart	75	50	25	100	37°	65°	B	λ DNA		3
SfiI	rCutSmart	25	100	50	100	50°	No	C	pXba DNA	dcm CpG	
SfoI	rCutSmart	50	100	100	100	37°	No	B	λ DNA (HindIII digest)	dcm CpG	
SgrAI	rCutSmart	100	100	10	100	37°	65°	A	λ DNA	CpG	1
SmaI	rCutSmart	<10	<10	<10	100	37°	65°	B	λ DNA (HindIII digest)	CpG	b
SmlI	rCutSmart	25	75	25	100	55°	No	A	λ DNA		b
SnaBI	rCutSmart	50*	50	10	100	37°	80°	A	T7 DNA	CpG	1
SpeI-HF	rCutSmart	25	50	10	100	37°	80°	C	pXba-XbaI DNA		
SphI	r2.1	100	100	50	100	37°	65°	B	λ DNA		2
SphI-HF	rCutSmart	50	25	10	100	37°	65°	B	λ DNA		
SrfI	rCutSmart	10	50	0	100	37°	65°	B	pNEB193-SrfI DNA	CpG	
SspI-HF	rCutSmart	25	100	<10	100	37°	65°	B	λ DNA		
StuI	rCutSmart	50	100	50	100	37°	No	A	λ DNA	dcm	
StyD4I	rCutSmart	10	100	100	100	37°	65°	B	λ DNA	dcm CpG	
StyI-HF	rCutSmart	25	100	25	100	37°	65°	A	λ DNA		
SwaI	r3.1	10	10	100	10	25°	65°	B	pXba DNA		b, d
TaqI-v2	rCutSmart	50	100	50	100	65°	No	B	λ DNA	dam	
TfiI	rCutSmart	50	100	100	100	65°	No	C	λ DNA	CpG	
TseI	rCutSmart	75	100	100	100	65°	No	B	λ DNA	CpG	3
Tsp45I	rCutSmart	100	50	<10	100	65°	No	A	λ DNA		
TspMI	rCutSmart	50*	75*	50*	100	75°	No	B	pBC4 DNA	CpG	d
TspRI	rCutSmart	25	50	25	100	65°	No	B	λ DNA		
Tth111I	rCutSmart	25	100	25	100	65°	No	B	pBC4 DNA		b
WarmStart Nt.BstNBI	r3.1	0	10	100	25	55°	80°	A	T7 DNA		
XbaI	rCutSmart	<10	100	75	100	37°	65°	A	λ DNA (dam-/Hind III digest)	dam	
XcmI	r2.1	10	100	25	100*	37°	65°	C	λ DNA		2
XhoI	rCutSmart	75	100	100	100	37°	65°	A	λ DNA (HindIII digest)	CpG	b
XmaI	rCutSmart	25	50	<10	100	37°	65°	A	pXba DNA	CpG	3
XmnI	rCutSmart	50	75	<10	100	37°	65°	A	λ DNA		b
ZraI	rCutSmart	100	25	10	100	37°	80°	B	λ DNA	CpG	

Single Letter Codes:
B = C or G or T; D = A or G or T; H = A or C or T; K = G or T; M = A or C; N = A or C or G or T; R = A or G; S = C or G; V = A or C or G; W = A or T; Y = C or T

Chart Legend and Notes:

- Recombinant
- Time-Saver qualified (cleaves substrate in 5 – 15 min under recommended conditions)
- Engineered for maximum performance
- dam methylation sensitivity
- dcm methylation sensitivity
- CpG methylation sensitivity (applies to eukaryotic genomic DNA only)
- Indicates that the restriction enzyme requires two or more sites for cleavage
- U Supplied with its own unique reaction buffer that is different from the four standard NEBuffers. Compatibility with the four standard NEBuffers is indicated by the chart.
- SAM/ATP Supplied with a separate vial of S-adenosylmethionine (SAM) or Adenosine 5´-Triphosphate (ATP). To obtain 100% activity, SAM or ATP should be added to the 1X reaction mix as specified on the product data card.

The following notes appear with any enzymes having ligation efficiencies lower than 100% as assessed by ligation and recutting:

a. Ligation is < 10%
b. Ligation is 25% – 75%
c. Recutting after ligation is < 5%
d. Recutting after ligation is 50% – 75%
e. Ligation and recutting after ligation is not applicable since the enzyme is either a nicking enzyme, is affected by methylation, or if the enzyme cleaves outside its recognition sequence.

The following notes appear with any enzymes when star activity is a concern:

1. Star activity may result from extended digestion, high enzyme concentration or a glycerol concentration of > 5%.
2. Star activity may result from extended digestion.
3. Star activity may result from a glycerol concentration of > 5%.
* May exhibit star activity in this buffer

+For added flexibility, NEB offers an isoschizomer or HF enzyme, supplied with rCutSmart Buffer.

To address the need for BSA-free reagents, NEB has switched our BSA-containing reaction buffers to Recombinant Albumin (rAlbumin)-containing buffers. We are also in the process of transitioning our enzyme formulations to contain rAlbumin. NEB has rigorously tested these changes and has not seen a difference in performance with these changes. Learn more at **www.neb.com/BSA-free**.

NEBuffer Compositions (1X)

- NEBuffer r1.1: 10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 100 µg/ml Recombinant Albumin (pH 7.0 @ 25°C)
- NEBuffer r2.1: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 µg/ml Recombinant Albumin (pH 7.9 @ 25°C)
- NEBuffer r3.1: 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 µg/ml Recombinant Albumin (pH 7.9 @ 25°C)
- rCutSmart Buffer: 50 mM Potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium acetate, 100 µg/ml Recombinant Albumin (pH 7.9 @ 25°C)

Double Digest Finder · NEBcutter® · NEBioCalculator® · NEBcloner® · Enzyme Finder · REBASE® and more...

To benefit from our interactive NEB online tools & resources, please visit **www.neb.com/nebtools**

Activity of DNA Modifying Enzymes in rCutSmart Buffer

A selection of DNA modifying enzymes were assayed in rCutSmart Buffer and functional activity was compared to the activity in their supplied buffers. Reactions were set up according to the recommended reaction conditions, with rCutSmart Buffer (plus required supplement) replacing the supplied buffer.

Tech Tip: When supplements are required, one can simply add the supplied buffer of the respective modifying enzyme at 1X concentration to the rCutSmart Buffer to achieve appropriate activity for most applications – no change of buffers needed.

Enzyme	Activity in rCutSmart	Required Supplements
Phosphatases:		
Antarctic Phosphatase	+++	Requires Zn^{2+}
Shrimp Alkaline Phosphatase (rSAP)	+++	
Quick CIP	+++	
Ligases:		
T4 DNA Ligase	+++	Requires ATP
Hi-T4™ DNA Ligase	+++	Requires ATP
Salt-T4® DNA Ligase	+	Requires ATP
E. coli DNA Ligase	+++	Requires NAD
T3 DNA Ligase	+++	Requires ATP + PEG
T7 DNA Ligase	+++	Requires ATP + PEG
Polymerases:		
T4 DNA Polymerase	+++	
DNA Polymerase I, Large (Klenow) Fragment	+++	
DNA Polymerase I	+++	
DNA Polymerase Klenow Exo⁻	+++	
Bst DNA Polymerase	+++	
phi29 DNA Polymerase	+++	Requires DTT
T7 DNA Polymerase (unmodified)	+++	
Transferases/Kinases:		
T4 Polynucleotide Kinase	+++	Requires ATP + DTT
T4 PNK (3´ phosphatase minus)	+++	Requires ATP + DTT
CpG Methyltransferase (M. SssI)	+++	
GpC Methyltransferase (M. CviPI)	+	Requires DTT
T4 Phage β-glucosyltransferase (T4-BGT)	+++	

Enzyme	Activity in rCutSmart	Required Supplements
Nucleases, other:		
DNase I (RNase-free)	+++	Requires Ca^{2+}
DNase I-XT	+++	Requires Ca^{2+}
Endonuclease III (Nth)	+++	
Endonuclease VIII	+++	
Exonuclease VII	+++	
Exonuclease V (Rec BCD)	+++	Requires ATP
Exonuclease III	+++	
Exonuclease I	+++	
FPG	+++	
Lambda Exonuclease	++	
McrBC	+++	
Micrococcal Nuclease	+++	Requires Ca^{2+}
RecJf	+++	
T5 Exonuclease	+++	
T7 Exonuclease	+++	
Thermolabile Exo I	+++	
Thermolabile USER II	+++	
Thermostable USER III	++	
Thermostable OGG	+++	
USER™ Enzyme	+++	

+++ full functional activity
++ 50–100% functional activity
+ 0–50% functional activity

Updates to this chart can be found at **NEBCutSmart.com**

DEPHOSPHORYLATION

rSAP PHOSPHATASE

- No clean-up
- Add 1µl to digest, works in CutSmart buffer
- Incubate at 37°C for 30 mins
- Heat inactivate at 65°C for 10 mins

ANTARCTIC PHOSPHATASE

- After clean-up elute in 50µl

	Reaction Mix
DNA	~45
Phosphatase buffer	5
Antarctic phosphatase	1
	50µl

- Incubate at 37°C for 45 mins
- Heat inactivate at 65°C for 10 mins

BLUNT ENDING

BLUNT ENDING

- After clean up elute in 45µl

	Reaction Mix
DNA	~40
Buffer 2	5
dNTPs	5
BSA	1
Klenow	1
	~50µl

- Incubate at room temperature for 30mins MAX
- Heat inactivate at 75°C for 20mins

LIGATIONS

T4 LIGASE: INSERT INTO VECTOR

- Note: Use ligation tubes

	+Insert	Vector only control
SDW	-	35
Vector	18	9
Insert	27	-
T4 buffer	5	5
T4 ligase	1	1
	50µl	50µl

- Incubate in 16˚C water-bath in cold room overnight

T4 LIGASE: LINKER INTO INVERSE PCR (FOR GENE DELETION)

- Note: vector only control in this case the inverse PCR product with no linker
- Note: Use ligation tubes

	+Linker	Vector only control
SDW	-	9
Vector	16	8
Linker (1/10 in TE)	1	-
T4 buffer	2	2
T4 ligase	1	1
	20µl	20µl

- Incubate in 15°C water-bath in cold room overnight

ELECTROLIGASE

- Note: Use ligation tubes

	+Insert	Vector only control
SDW	-	4
Vector	1	1
Insert	4	-
Buffer	5	5
ElectroLigase	1	1
	11µl	11µl

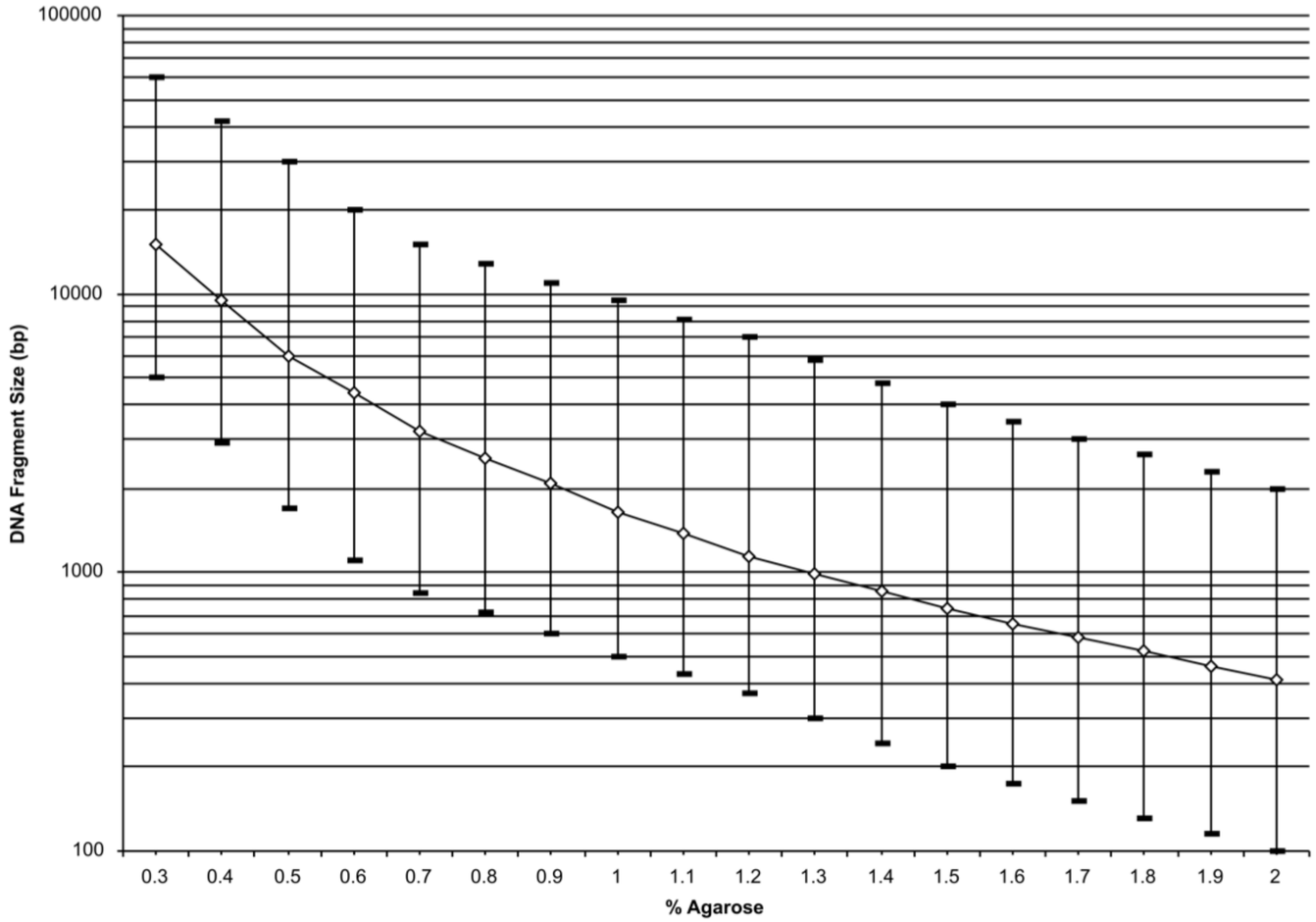
- Incubate at 25°C (on bench) for 1hour
- Heat-inactive ligase at 65°C for 15 mins

SEQUENCING (INTERNAL)

- 70-150 ng/µl of plasmid DNA (preferably 100 ng/µl)
- 5µl of template per reaction
- 3-5µl of primer per reaction
- Print and complete sequencing form
- Drop samples (in a sealable plastic bag) and form to D106

AGAROSE GELS

WHAT % AGAROSE GEL TO I NEED?



- Typically use 0.75% for large fragments, 1% for medium fragments, and 1.5% for small fragments

TBE GELS

- ‘Short-run’ gels to resolve min-prep, maxi-prep, and PCR digests
- 50ml or 100ml size gels
- Run at 100V for ~1hour
- Stain with either SYBR Safe (4µl per 100ml) or EtBr (8µl per 100ml)

TAE GELS

- ‘Long-run’ gels to resolve genomic digests
- 200ml gel size
- Typically run at 50V for 16hours
- Post-stain with EtBr

PLASMID CLONING

ISOLATE GENOMIC CLONE

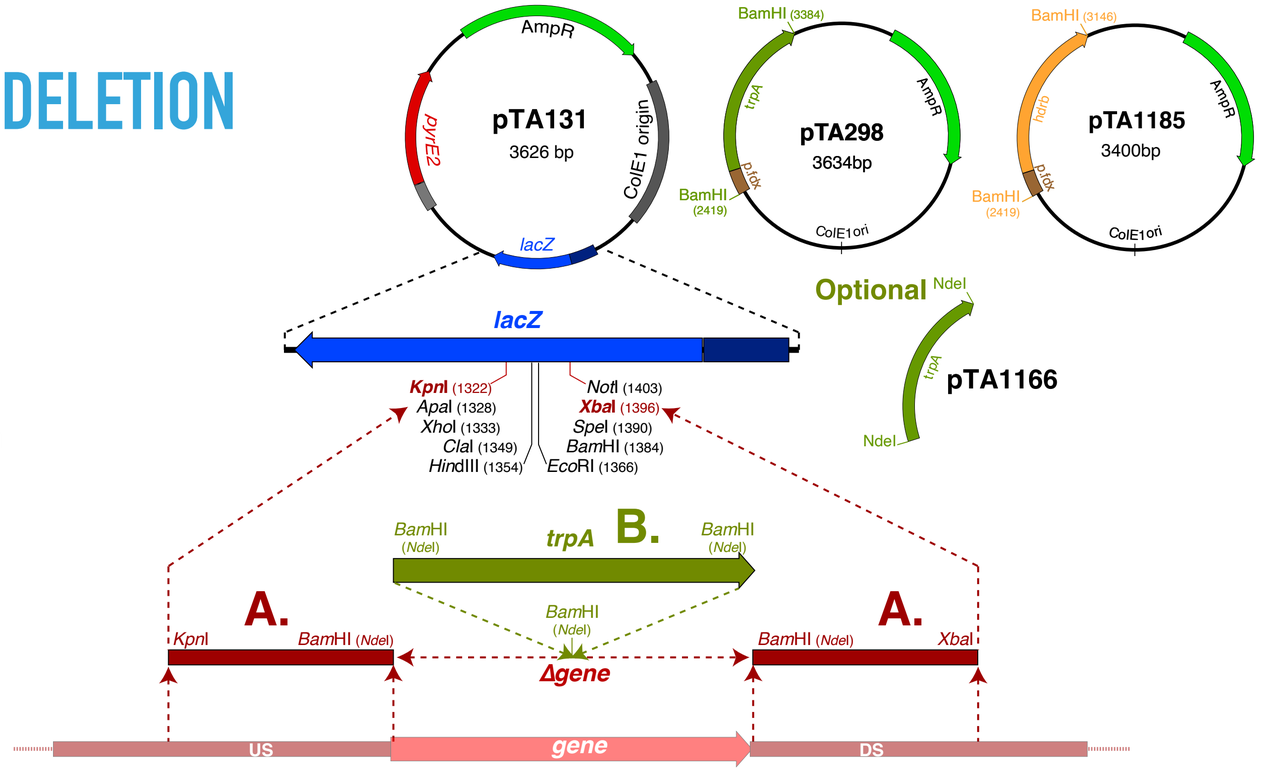
- Use PCR (or restriction digest of genomic DNA) to isolate genomic clone of gene plus flanking upstream and downstream regions.
  - Find flanking restriction enzyme(s) compatible with pBluescript (or pTA131) cloning sites, that cut ≥1 kb upstream and downstream of gene.
- For PCR, use primers just outside of flanking restriction sites, use high-fidelity polymerase, and ligate with compatible sites in pBluescript or pTA131.
- For restriction digest, excise band of expected size (±0.5 kb) from gel and ligate with compatible sites in pBluescript (or pTA131) to generate size-selected genomic library. Screen for gene by colony hybridisation with gene-specific probe made by PCR.
  - Note: nCTAGn sites are methylated in Haloferax volcanii, therefore genomic DNA will not be cut by these enzymes (e.g. XbaI).

Allers et al (2004)

PLASMIDS FOR GENE DELETION

TRADITIONAL METHOD

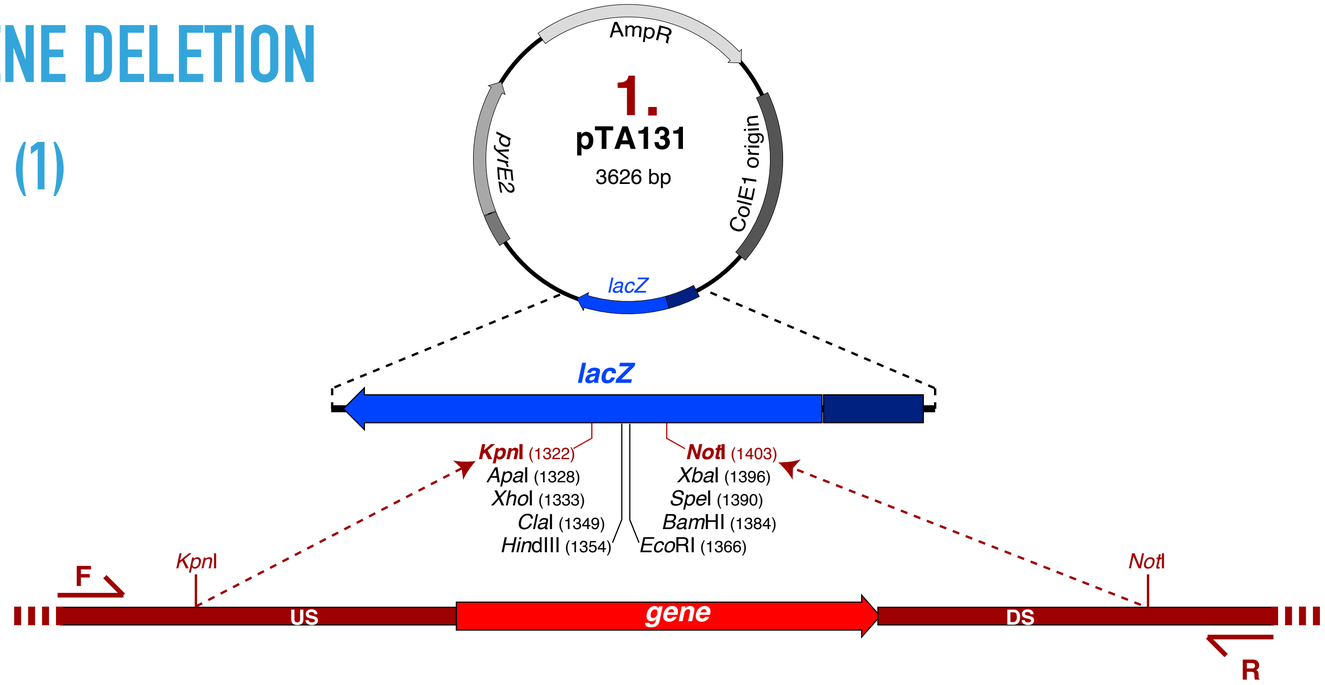
- Make Δgene deletion construct: PCR amplify upstream and downstream flanking regions using primers with KpnI/BamHI sites and BamHI/XbaI sites



- 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 (1)



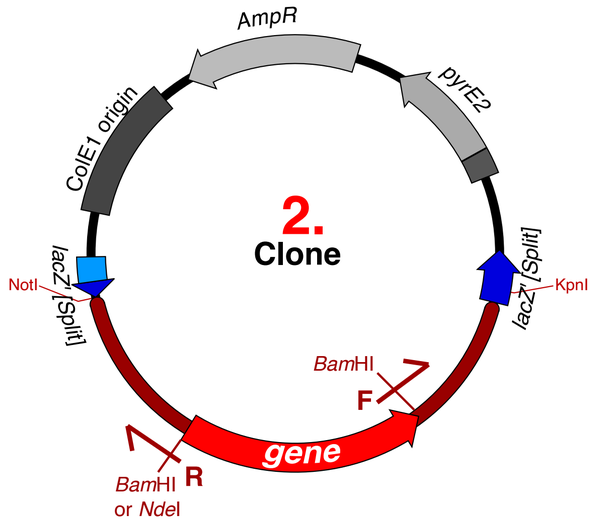
1. MAKE GENOMIC CLONE OF GENE

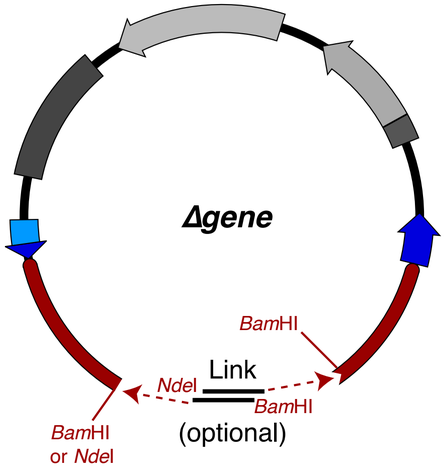
- PCR amplify gene plus ~1 kb each of US and DS flanking regions. Use high-fidelity polymerase.
- Use 100%-matched primers that bind just outside sites that are compatible with polylinker of pTA131 or pTA1431 (has no BamHI site).
- Clone in pTA131 or 1431 at compatible sites and check by sequencing.

Allers et al (2004)

PLASMIDS FOR GENE DELETION

ALTERNATIVE METHOD (2)



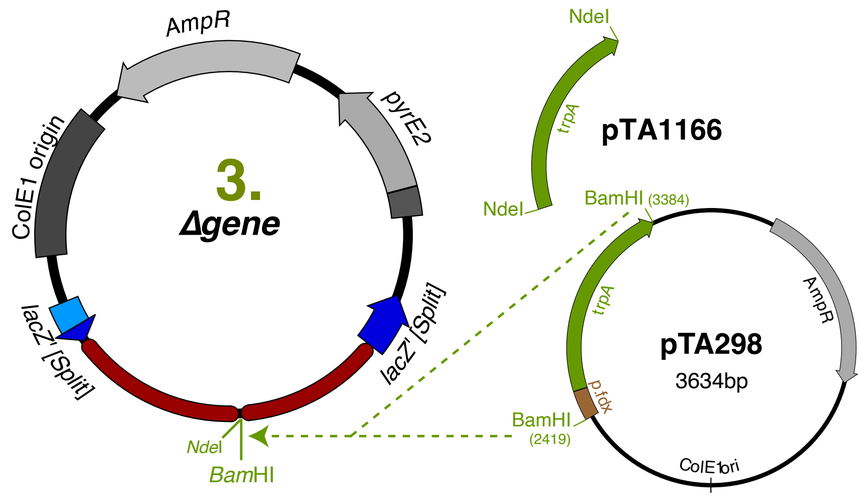


2. MAKE ΔGENE CONSTRUCT

- PCR amplify all of plasmid apart from gene using outward-facing primers:
  - F primer: binds just downstream of stop codon, incorporates novel BamHI, BglII or BclI site;
  - R primer: binds at start codon, incorporates novel BamHI site, or NdeI site at ATG start codon.
- Cut PCR product with NdeI and/or BamHI (or BglII or BclI).
- Gel purify large fragment and self-ligate.
  - If R primer has NdeI site (e.g. for for His-tagged gene) use NdeLinkBam (or NdeLinkBgl or NdeLinkBcl) linker to join NdeI and BamHI (or BglII or BclI) ends.
- Check by sequencing.

PLASMIDS FOR GENE DELETION

ALTERNATIVE METHOD (3)



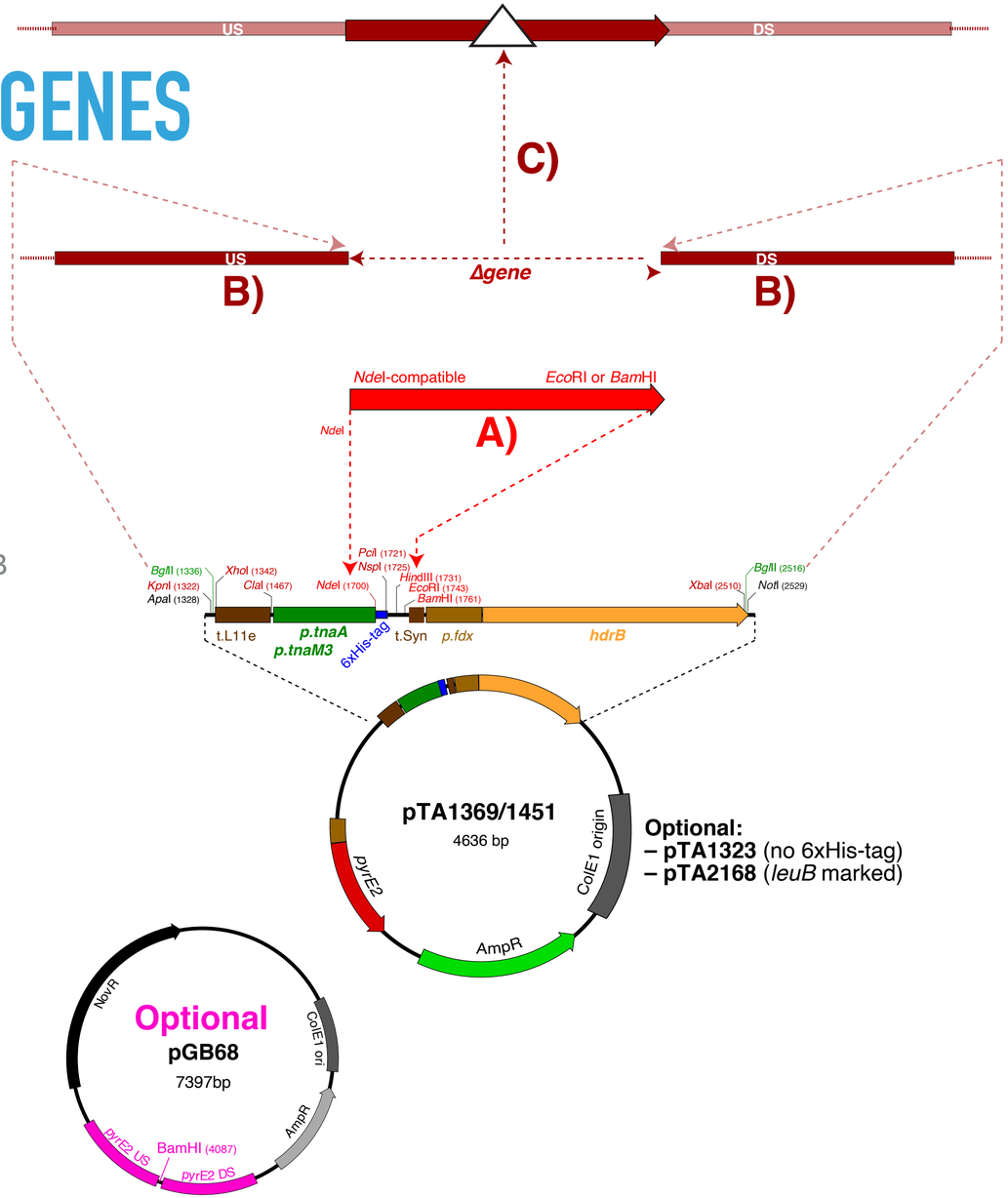
3. MAKE ΔGENE::TRPA+ CONSTRUCT

- 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

PLASMIDS FOR INDUCIBLE GENES
CHROMOSOMAL ALLELE

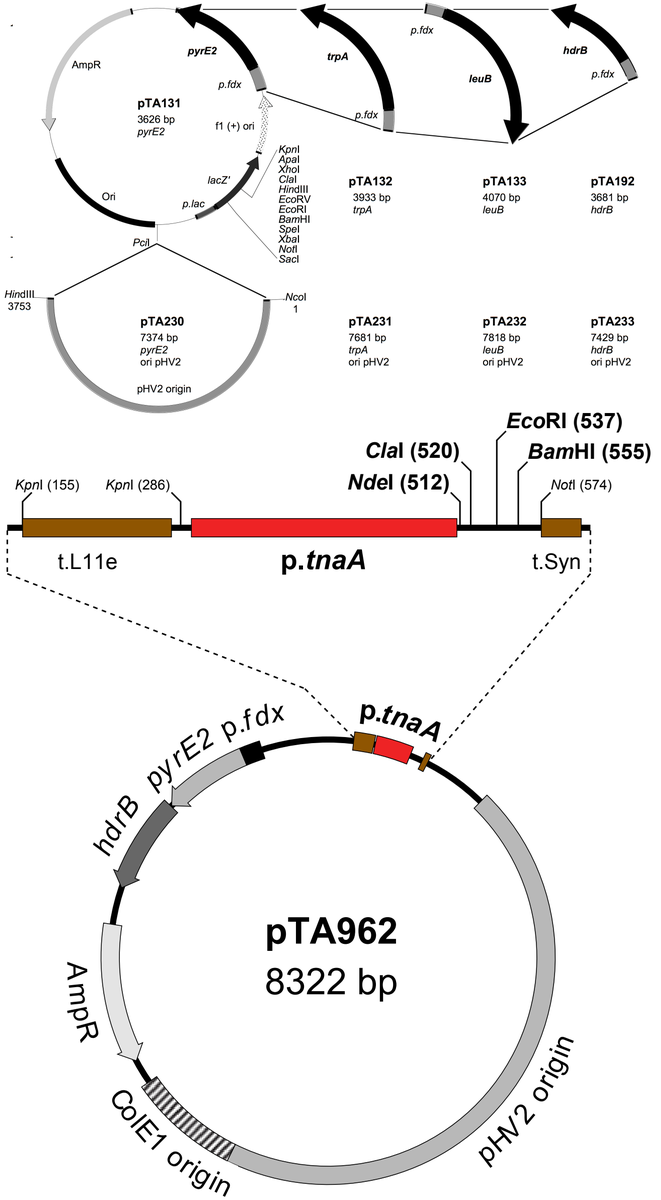
- PCR amplify gene with NdeI-compatible site at 5′ end, and EcoRI or BamHI site at 3′ end. Insert into pTA1369 or pTA1451 at NdeI site, and either EcoRI or BamHI site.
  - Optional: For no hdrB marker, add XbaI site at 3′ end of gene and insert in pTA1369 at XbaI to replace hdrB
  - To retain native promoter, PCR amplify upstream flanking region using primers with XhoI site at 5′ end and NdeI site at 3′ end (at ATG start codon).
- Digest pTA1369(1451)+gene at flanking BglII sites and insert gene expression cassette at BamHI site of Δgene plasmid, replacing trpA marker (if present).
  - Optional: for ectopic integration at pyrE2 locus, use flanking BglII sites and insert at BamHI site in pGB68 (instead of Δgene plasmid).
- Transform vector into a strain containing gene deletion, to replace with p.tnaA::gene allele



Allers et al (2004); Large et al (2007)

PLASMIDS FOR INDUCIBLE GENES
EPISOMAL ALLELE

- pTA962 is derived from pTA230
  - pTA962 as tryptophan inducible p.tnaA promoter
- PCR amplify gene with:
  - NdeI-compatible or ClaI-compatible site at 5′ end
  - EcoRI or BamHI-compatible site at 3′ end (or NotI)
- Insert into pTA962 between NdeI / ClaI site and EcoRI / BamHI site
  - Optional: For no hdrB marker (pyrE2 only), use pTA927 instead
  - Note: pTA963 is like pTA962 with 6xHis tag (but no ClaI site), it has been replaced by pTA1228 etc.
- Transform vector into a ΔhdrB trpA⁺ strain containing gene deletion
  - For tryptophan-inducible complementation, need trpA⁺ strain



Allers et al (2004); Allers et al (2010); Large et al (2007)

PLASMIDS FOR PROTEIN OVER-EXPRESSION (1)

N-TERMINAL HIS TAG

- NdeI site in plasmid is directly upstream of 6xHis tag, for cloning without His-tag use PCR to incorporate NdeI site at 5′ end of gene
- For cloning with 6xHis-tag, insert gene at PciI or NspI site in plasmid, in-frame with 3' end of His tag:
  - If second codon starts with T, G, or A, cut plasmid with PciI and incorporate PciI (A/CATGT), NcoI (C/CATGG) or BspHI (T/CATGA) site at 5′ end of gene, respectively
  - If second codon starts with C, cut plasmid with NspI site and incorporate SphI (GCATG/C) site at 5′ end of gene
- Incorporate EcoRI/BamHI/NotI site at 3′ end of gene

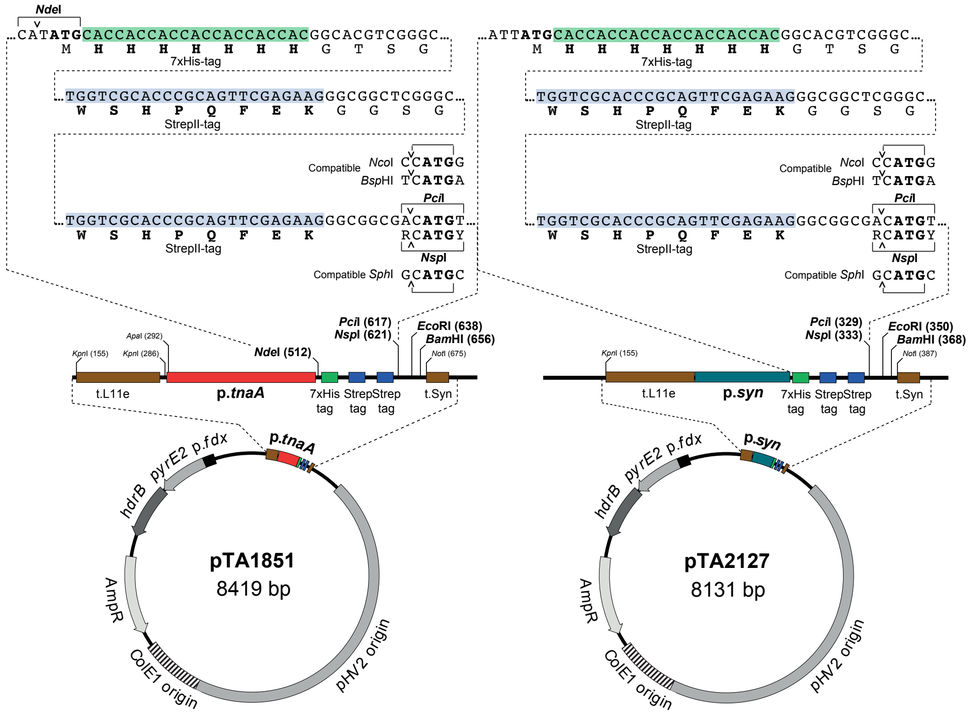
C-TERMINAL STREPII TAG

- EcoRI and BamHI sites are downstream of StrepII tag, for cloning without Strep-tag use EcoRI or BamHI site at 3′ end of gene, after stop codon
- For cloning with StrepII tag, replace stop codon of gene with NheI site (G/CTAGC), insert at NheI site of plasmid

PLASMIDS FOR PROTEIN OVER-EXPRESSION (2)

N-TERMINAL TANDEM 7XHIS-2XSTREPII TAG

- NdeI site in plasmid is upstream of N-terminal tandem 7xHis-2xStrepII tag, for cloning without any tag incorporate NdeI site at 5′ end of gene.
- For cloning with 7xHis-2xStrepII tag, insert gene at PciI or NspI site in plasmid, in-frame with 3' end of His tag:
  - If second codon starts with T, G, or A, cut plasmid with PciI and incorporate PciI (A/CATGT), NcoI (C/CATGG) or BspHI (T/CATGA) site at 5′ end of gene, respectively.
  - If second codon starts with C, cut plasmid with NspI site and incorporate SphI (GCATG/C) site at 5′ end of gene.
- Incorporate EcoRI/BamHI/NotI (or compatible) site at 3′ end of gene.



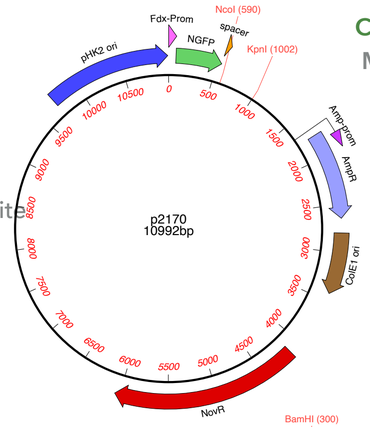
Plasmid	Relevant properties
pTA1228	pTA963 (not shown) and pTA1228 have N-terminal His tag only. Both have p.tnaA tryptophan-inducible promoter. pTA1228 has better cloning sites (NspI)
pTA1392	pTA1392 has an N-terminal His tag and a C-terminal StrepII tag, which works reasonably well. It also has the p.tnaA tryptophan-inducible promoter
pTA1403	pTA1403 (not shown) is like pTA1392 but the tags are the other way round: N-terminal StrepII tag and C-terminal His tag. This doesn't seem to work well
pTA1926	pTA1926 is like pTA1392 but has 50% strength p.tnaM3 promoter, which has 50% basal and induced activity for "toxic" proteins. The tags are like pTA1392
pTA1992	pTA1992 is like pTA1392 but has the strong constitutive p.syn promoter for "harmless" proteins. The tags are like pTA1392
pTA1851	pTA1851 has N-terminal tandem tag: 7xHis 2x StrepII, separated by linkers. This normally works best. It has the p.tnaA tryptophan-inducible promoter
pTA2127	pTA2127 is like pTA1851 but has the strong constitutive p.syn promoter for "harmless" proteins. The tandem 7xHis tag 2x StrepII tag is like pTA1851

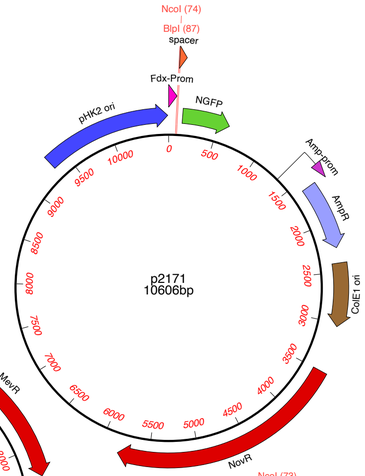
Allers et al (2010); Haque et al (2020); Karan et al (2024)

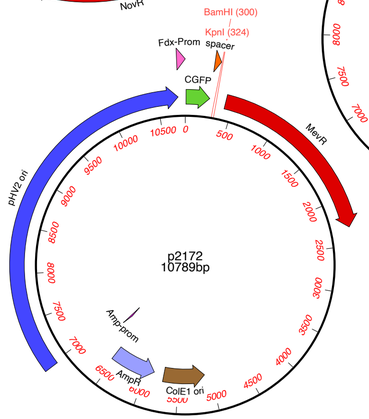
PLASMIDS FOR SPLIT GFP

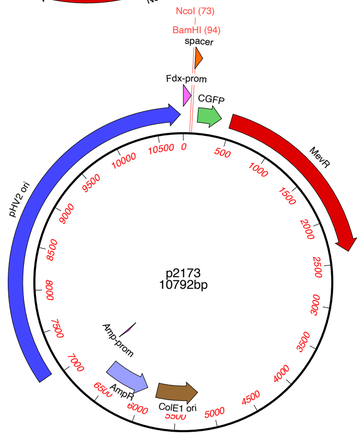
- Clone desired gene into the four split GFP episomes:
  - pTA2170 (pJAS-NGFP-Nterm): N-GFP-*gene* NovR
    - Incorporate gene of interest at NcoI/KpnI sites
      - Forward primer: ensure ATG start codon is within NcoI-compatible site (C | CATGG), will be in-frame with GFP
      - Reverse primer: KpnI site directly after Stop codon
  - pTA2171 (pJAS-NGFP-Cterm): *gene*-N-GFP NovR
    - Incorporate gene of interest at NcoI/BlpI sites
      - Forward primer as for pTA2170
      - Reverse primer: replace Stop codon with BlpI-compatible site (GC | TNAGC), ensure in-frame with GFP
  - pTA2172 (pWL-CGFP-Nterm): C-GFP-*gene* MevR
    - Incorporate gene of interest at BamHI/KpnI sites
      - Forward primer: ensure ATG start codon is directly after BamHI site (G | GATCGC), will be in-frame with GFP
      - Reverse primer as for pTA2170
  - pTA2173 (pWL-CGFP-Cterm): *gene*-C-GFP MevR
    - Incorporate gene of interest at NcoI/BamHI sites
      - Forward primer as for pTA2170
      - Reverse primer: replace Stop codon with BamHI-compatible site (G | GATCGC), ensure in-frame with GFP

	N-GFP Nov^R	
C-GFP Mev^R	pTA2170	pTA2171
pTA2172	N-GFP-*gene* C-GFP-*gene*	*gene*-N-GFP C-GFP-*gene*
pTA2173	N-GFP-*gene* *gene*-C-GFP	*gene*-N-GFP *gene*-C-GFP









Winter et al (2018)