Bacillus subtilis
pBacTag Tagging Vectors

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Contents

1 Introduction

Gram-positive bacteria are well known for their contributions to agricultural, medical and food biotechnology and for the production of recombinant proteins. Among them, *Bacillus subtilis* has been developed as an attractive host and model organism, because of several reasons:

- *Bacillus subtilis* is non-pathogenic and awarded GRAS (generally regarded as safe) status from the US Food and Drug Administration.
- There is no significant bias in codon usage.
- It is capable of secreting high levels of functional proteins directly into the culture medium. At present, about 60% of the commercially available enzymes are produced by *Bacillus* species.
- A large body of information on *B. subtilis* is already available, greatly facilitating fundamental research experiments or the construction of improved protein production strains. The complete *B. subtilis* genome information is available in addition to many data on transcription, translation, protein folding, secretion mechanisms and gene manipulation results.

However, the functions of the about 4,100 *B. subtilis* genes identified, are still incompletely clarified. The pBacTag Tagging system has been developed to disburden further functional studies. On this, the pBacTag Tagging Vectors enable the directed functional analysis of genes by two different modes of action:

- Specific inactivation of genes of interest within the chromosome (followed by phenotypical analysis)
- Chromosomal expression of the gene of interest as translational fusion with an epitope or localization tag fused to the 3'-end (for selective protein purification, detection by commercially available antibodies or for localization studies).

The tagging or inactivation of the target gene is achieved by chromosomal integration of the pBacTag Tagging Vector into the *B. subtilis* chromosome by homologous recombination.

2 The pBacTag Tagging Vectors

The pBacTag Tagging Vectors enable the directed functional analysis of genes. Tagging or inactivation of target genes is achieved by chromosomal integration of a pBacTag Vector via homologous recombination. All pBacTag Tagging Vectors are derivatives of pMutin vectors (Vagner et al., 1998; Kaltwasser et al., 2002) with the following properties:

- pBacTag Tagging Vectors are able to replicate in *E. coli*, but unable to propagate in *B. subtilis*. The latter enables chromosomal integration with *B. subtilis* (and other bacterial species, in which pBR322 based plasmids are not able to replicate), by homologous recombination, using the erythromycin-resistance gene as selection marker. For propagation in *E. coli*, the β-lactamase gene can be used for selection purpose, causing resistance against ampicillin.
- The IPTG inducible P*spac* promoter allows, after chromosomal integration, the controlled expression of genes that are located downstream of the target gene. This

is important, because most of *B. subtilis* genes are organized in multicistronic units, and downstream genes within the same operon may be separated from their native promoter by the integration event. The P*spac* is therefore an indispensable tool to avoid polar effects from expression changes of downstream genes.

- For proper cloning, the vectors contain a multiple cloning site downstream of the P*spac* with the following unique restriction sites: KpnI, Eco47III, ClaI and EagI.
- To ensure efficient termination of transcription of the hybrid gene, the vectors contain the *trpA* terminator of the *E. coli* tryptophan operon downstream of the tag.
- The terminators $t_1t_2t_0$ (t_1 and t_2 of the *E. coli rnb* operon and the lambda terminator λt_0 downstream of the erythromycin resistance gene take care for proper RNA polymerase termination at this place and prevent any "read through" to genes downstream of the P*spac*.
- Three of the tagging vectors - pBacTag-DYKDDDK (also known as FLAG®), pBacTag-cMyc and pBacTag-HA – allow the expression of epitope tagged fusion proteins. These proteins can be detected in immunoblotting experiments by using commercially available antibodies against the respective tag. The fusion proteins can also easily being purified using the tag in affinity chromatography. Since the tags are very short (FLAG®: 7 aa, cMyc: 10 aa, HA (hemagglutinin): 9 aa) protein function is usually not disturbed.
- Localization tags can be fused to the protein of interest, using pBacTag-GFP+, pBacTag-YFP and pBacTag-CFP. The fusion proteins containing a fluorescing tag can be analyzed for their cell compartmental localization. The GFP+ tag (pBacTag-GFP+) is an improved variant of the common GFP, which produces enhanced fluorescence.

2.1 Mechanism of pBacTag Tagging Vectors

The mechanism of pBacTag Tagging Vectors is illustrated with pBacTag-GFP+ as example. This vector can be used for creating a GFP+ fusion protein from any chromosomally located gene of interest, by fusing a gfp+ tag to the chosen gene. In this example the gene of interest is named *orf2*. It is part of an operon, including three genes in total (*orf1*, *orf2* and *orf3*). For getting the gfp+ tag fused to the *orf2*, the 3' part of the gene (*orf2*') has to be inserted into the multiple cloning site of the pBacTag-GFP+ vector. After transforming *B. subtilis* cells with this construct, chromosomal integration of the vector is achieved by selecting for cells with resistance against erythromycin. The integration is facilitated by homologous recombination of both *orf2*' copies (one copy being within the plasmid, the other one within the chromosomal DNA). The mechanism the pBacTag-GFP+ vector (with *orf2*') is integrated into the genome, is displayed in Figure 1.

After vector integration, the complete *orf2* is fused to the *gfp*+ gene and can be transcribed from the native promoter located upstream of *orf1*. Transcription of the tagged gene is terminated at the *trpA* terminator downstream of *gfp*+. *orf3* (formerly within the operon) is no more transcribed from its native promoter. Instead, its transcription is ensured by the IPTG inducible promoter P*spac*.

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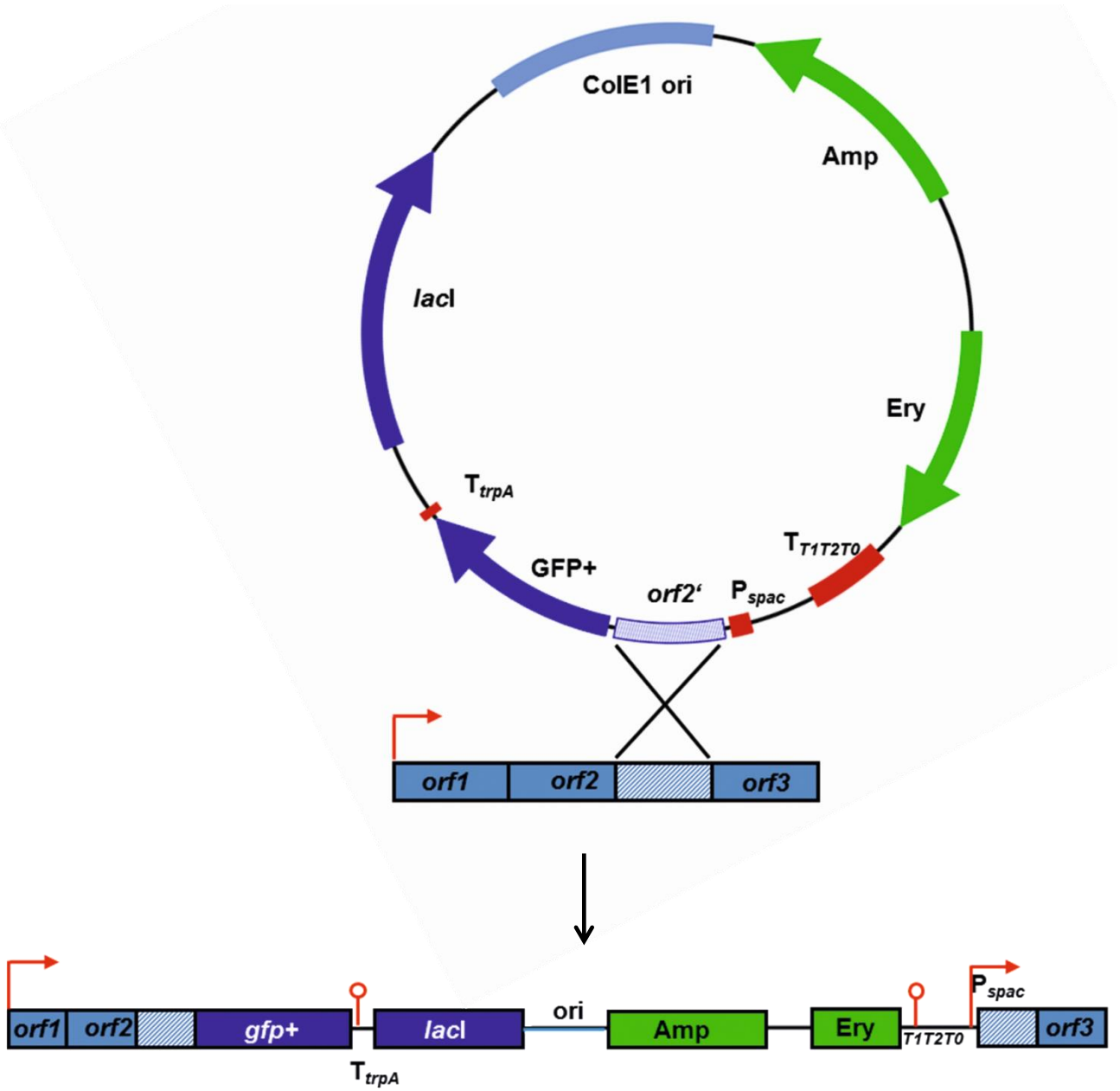


Fig.1: Chromosomal integration of pBacTag-GFP+:
The 3' end of *orf2* (*orf2*') was ligated into the multiple cloning site of the pBacTag-GFP vector. The *orf2* belongs to an operon containing a total of three genes (*orf1*, *orf2* and *orf3*). The pBacTag-GFP+ vector (with *orf2*') is integrated into the chromosome of *B. subtilis* via homologous recombination of both *orf2*' sites, by a single crossing over event. Now, the complete *orf2* is fused to the *gfp*+ gene and can be transcribed from the native promoter localized upstream of *orf1*. Transcription of the tagged gene is terminated at the *trpA* terminator downstream of *gfp*+. Now, *orf3* is no more transcribed from the native promoter of the operon. Instead, its transcription can be induced from the Pspac promoter by adding IPTG. Broken arrows denote the promoters of the operon and Pspac. Promoters of *lacI* and the resistance genes are not depicted. The lollipop strands denote the *trpA* and the three lambda terminators (T1,T2,T0).

3 *B. subtilis* and *E. coli* Host Strains

The following bacterial strains are available from MoBiTec:

- *B. subtilis* 1012 wild type: *leuA8 metB5 trpC2 hsdRM1*
- *B. subtilis* 168 Marburg: *trpC2* (Trp^-)
- *B.subtilis* AS1: 1012 *hrcA*::*neo* (producing strain for enhancing solubility of intracellular protein Schulz and Schumann, 1996, and Phan *et al.*, 2006)
- *B. subtilis* WB800N: *nprE aprE epr bpr mpr::ble nprB::bsr Δvpr wprA::hyg cm::neo;* NeoR (eight fold protease deficient strain for heterologous protein secretion)
- *E. coli* QuickCells F- (chemical competent): recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 ø80lacZΔM15 Δ(lacZYA-argF)U169
- *E. coli* RichCells F- (chemical competent): recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Ø 80lacZ Δ M15 Δ (lacZYA - argF)U169

Note: The neomycin marker of *B. subtilis AS1* and *WB800N* is usually not required. For ordering details see “8. Related Products”, p. 10.

4 Storage and Handling Instructions

Storage and handling of plasmids

Plasmids are supplied lyophilized. Upon receipt, add 50 µl distilled water (final concentration 0.1 µg/µl) and incubate at 50 °C for 5 minutes. Vortex for 1 minute and store at -20 °C.

All plasmids of this system can be propagated in *E. coli*.

Storage and handling of *B. subtilis* and *E. coli* strains

Detailed protocols for *E. coli* and *B. subtilis* molecular genetic handling (growth, transformation, etc.) can be found in the relevant laboratory manuals such as Sambrook and Russell (2001).

B. subtilis and *E. coli* can be grown aerobically at 37 °C in 2xYT medium (Bagyan *et al.*, 1998). Under optimal conditions the doubling time of *E. coli* is 20 min, of *B. subtilis* 30 min.

2xYT medium:	16 g	tryptone
	10 g	yeast extract
	5 g	sodium chloride (NaCl)
	add distilled water to 1000 ml, autoclave at 121 °C for 15 min	

Antibiotics:	*B. subtilis*	erythromycin (0.3 µg/ml)
	E. coli	ampicillin (50 µg/ml)

5 Chromosomal Integration of pBacTag

Detailed protocols for *E. coli* and *Bacillus* molecular genetic handling (growth, transformation, etc.) can be found in the relevant laboratory manuals such as Sambrook and Russell (2001).

5.1 Transformation protocol A

The following transformation protocol is adopted from Klein et al., 1992. Please note that immediate usage may result in higher transformation rates.

Preparation of competent *Bacillus subtilis* cells

- Culture appropriate recipient cells in 5 ml HS medium at 37 °C overnight
- Inoculate 50 ml HS medium with 0.5 ml of the overnight culture
- Incubate under vigorous shaking at 37 °C
- Record the growth curve
- Take samples of 10 ml each when cells reach the stationary phase at 15 min intervals
- Add 1 ml of sterile glycerol (87%), mix and leave for 15 min on ice
- Fractionate into 1 ml aliquots, freeze in liquid nitrogen and store at -80 °C
- Check one aliquot from each time point with a reference plasmid DNA to identify the time point(s) yielding high level competent cells
- Discard the non- or low competent aliquots

Transformation of competent *Bacillus subtilis* cells and selection for chromosomal pBacTag integration

- Thaw one aliquot at 37 °C
- Use these cells to inoculate 20 ml LS medium
- Shake cells slowly in a 30 °C water bath to obtain maximal competence (about 2 h)
- Take 1 ml aliquots into glass or 2 ml plastic reaction tubes, add 10 µl of 0.1 M EGTA (CB-0732-10GAM), and incubate for 5 min at room temperature
- Add pBacTag Vector DNA (5-40 ng) and incubate for 2 h at 37 °C while well shaking (well mixing is important when using plastic reaction tubes)
- If glass tubes were used, transfer cell suspension into an plastic reaction tube
- Centrifuge, discard supernatant carefully and resuspend the cells into the final supernatant remaining on the pellet
- Plate on selective 2xYT or LB medium (0.3µg /ml erythromycin)
- Incubate at 37°C overnight
- Successful integration of the pBacTag vector as single copy might be controlled by Southern blot hybridization

5.2 Transformation protocol B

Electroporation of *B. subtilis* (modified from Zhang *et al.*, 2011)

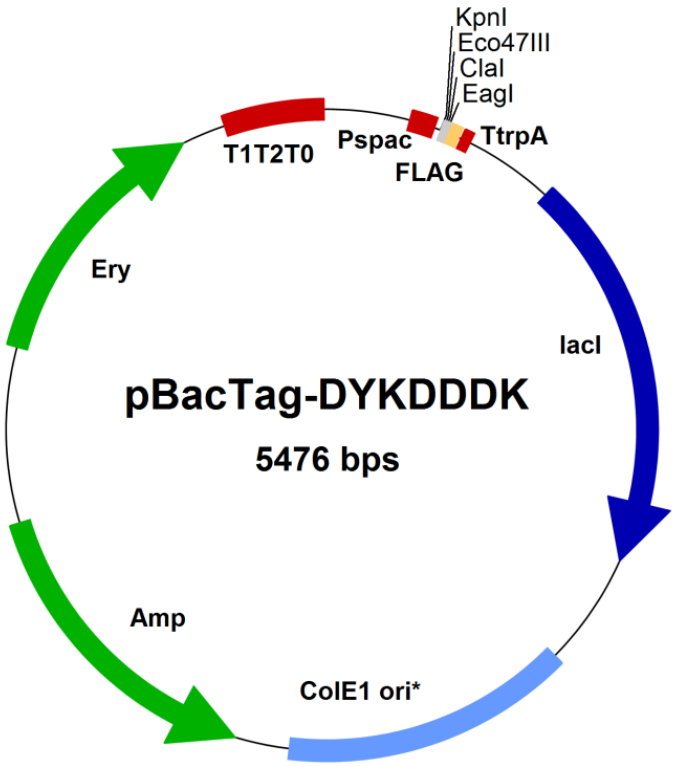
- Culture *B. subtilis* in 2xYT medium overnight
- Dilute 100-fold with 2xYT medium
- Grow culture to an OD600 of 0.2
- Then supplement culture with 1% DL-threonine, 2% glycine, 0.1% tryptophan and 0.03% Tween 80
- Grow while shaking for 1 h
- Cool on ice for 20 min
- Spin at 5000 x g for 10 min at 4 °C
- Wash twice with electroporation buffer
- Resuspend in electroporation buffer at 1/100 of the original culture volume
- Add 100 µl cell to an ice-cold 2 mm cuvette
- Add 2 µl pBacTag vector DNA (25 ng/ml)
- Shock by a single 12.5 kV/cm pulse (Gene Pulser; Bio-Rad), resistance 200 Ω, capacitance 25 µF
- Immediately add 1 ml 2xYT broth containing 0.5 M sorbitol and 0.38 M mannitol
- Incubate at 37 °C for 3 h
- Plate on selective 2xYT (0.3 µg/ml erythromycin)
- Incubate at 37°C overnight
- Successful integration of the pBacTag vector as single copy might be controlled by Southern blot hybridization

5.3 Media and solutions

S-base (Spizizen's salt):	2 g $(NH_4)_2SO_4$ 14 g K_2HPO_4 6 g KH_2PO_4 1 g sodium citrate add distilled water to 100 ml and autoclave add 0.1 ml 1M $MgSO_4$ after autoclaving
HS medium:	66.5 ml distilled water 10 ml 10x S-base 2.5 ml 20% (w/v) glucose 5 ml 0.1% (w/v) L-tryptophan 1 ml 2% (w/v) casein 5 ml 10% (w/v) yeast extract (Difco) 10 ml 8% (w/v) arginine, 0.4% histidine autoclave all components separately tryptophan solution: sterile filtration
LS medium	80 ml distilled water 10 ml 10x S-base 2.5 ml 20% (w/v) glucose 0.5 ml 0.1% (w/v) L-tryptophan 0.5 ml 2% (w/v) casein 5 ml 2% (w/v) yeast extract (Difco) 0.25 ml 1 M $MgCl_2$ 0.05 ml 1 M $CaCl_2$ autoclave all components separately tryptophan solution: sterile filtration
0.1 M EGTA	dissolve 3.8 g EGTA in 50 ml distilled water adjust the pH to 7.2 using 10 N NaOH add distilled water to 100 ml autoclave
Electroporation buffer	0.5 M trehalose 0.5 M sorbitol 0.5 M mannitol 0.5 mM MgCl2 0.5 mM K2HPO4 0.5 mM KH2PO4 pH 7.4 filter-sterilize and store frozen

7 Vector Maps

7.1 pBacTag Vectors with epitope tag



	Type	Start	End	Name	Description
	Promoter	228	298	Pspac	*spac* promoter
	Region	316	338	MCS	Multiple Cloning Site
	Tag	340	369	FLAG®	FLAG® tag
	Terminator	376	403	TtrpA	Termination sequence of *trpA* gene
	Gene	653	1735	lacI	*lacI* repressor gene
	Replication/ori	2049	2840	ColE1 ori*	Origin, belonging to the ColE1 incompatibility group
	Selectable Genetic Marker	3851	2991	Amp	Ampicillin resistance
	Selectable Genetic Marker	4335	5072	Ery	Erythromycin resistance
	Terminator	5193	5468	T1T2T0	Terminators t_1, t_2 of *rnb* operon of *E. coli* and λ terminator t_0

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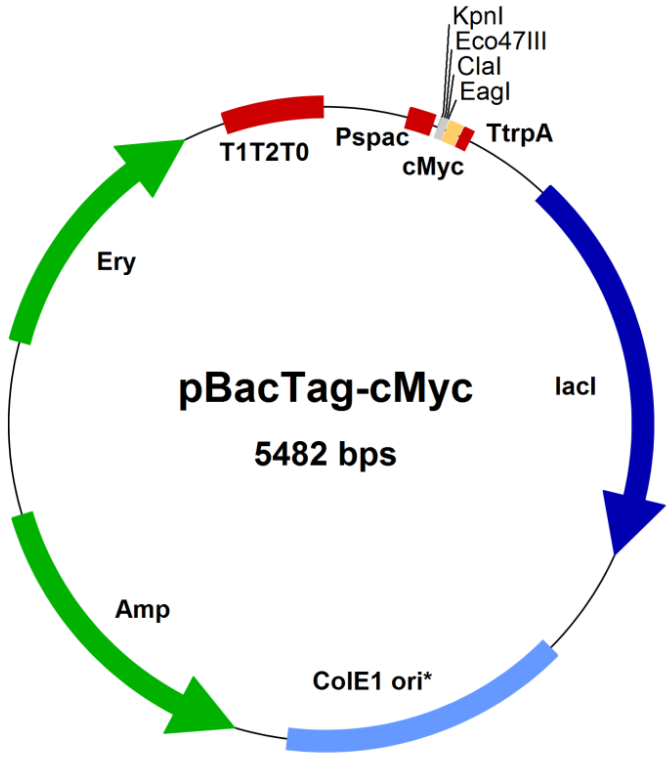
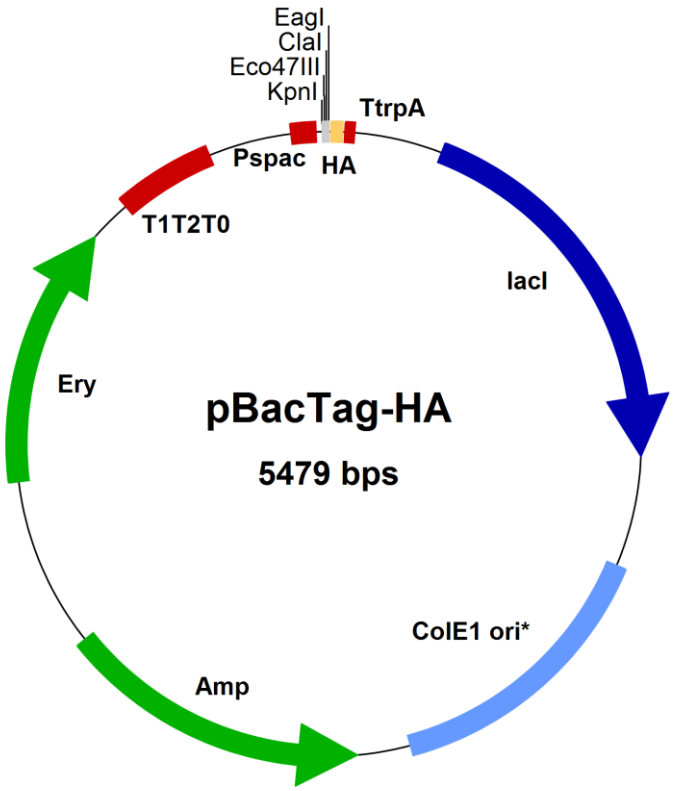
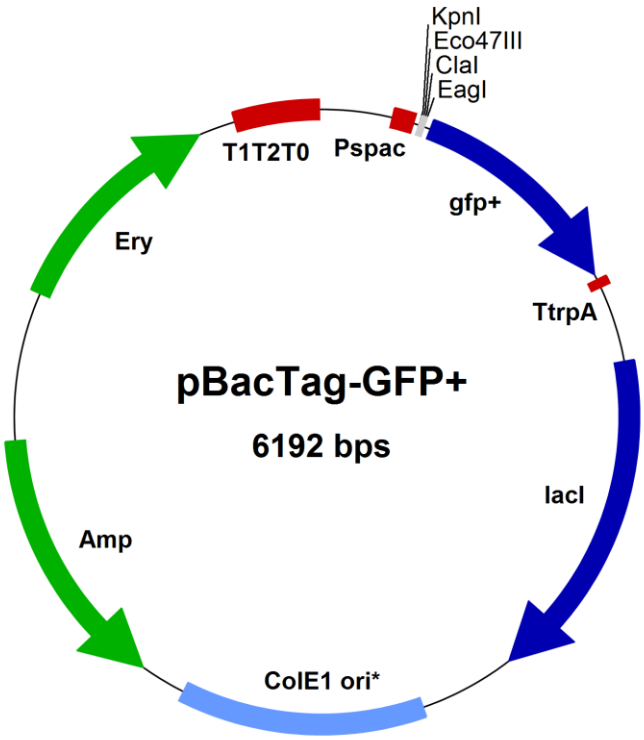


	Type	Start	End	Name	Description
	Promoter	228	298	Pspac	*spac* promoter
	Region	316	338	MCS	Multiple Cloning Site
	Tag	340	375	cMyc	cMyc tag
	Terminator	382	409	TtrpA	Termination sequence of *trpA* gene
	Gene	659	1741	lacI	*lacI* repressor gene
	Replication/ori	2055	2846	ColE1 ori*	Origin belonging to the ColE1 incompatibility group
	Selectable Genetic Marker	3857	2997	Amp	Ampicillin resistance
	Selectable Genetic Marker	4341	5078	Ery	Erythromycin resistance
	Terminator	5199	5474	T1T2T0	Terminators t_1, t_2 of *rnb* operon of *E. coli* and λ terminator t_0

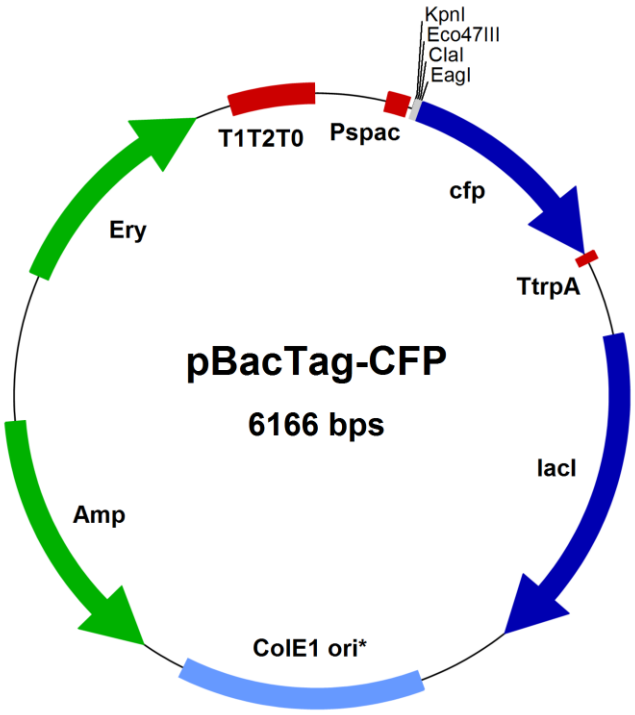


	Type	Start	End	Name	Description
	Tag	6	38	HA	Hemmagglutinin tag
	Terminator	45	72	TtrpA	Termination sequence of *trpA* gene
	Gene	322	1404	lacI	*lacI* repressor gene
	Replication/ori	1718	2509	ColE1 ori*	Origin belonging to the ColE1 incompatibility group
	Selectable Genetic Marker	3520	2660	Amp	Ampicillin resistance
	Selectable Genetic Marker	4004	4741	Ery	Erythromycin resistance
	Terminator	4862	5137	T1T2T0	Terminators t_1, t_2 of *rnb* operon of *Ecoli* and λ terminator t_0
	Promoter	5373	5443	Pspac	*spac* promoter
	Region	5461	5483	MCS	Multiple Cloning Site

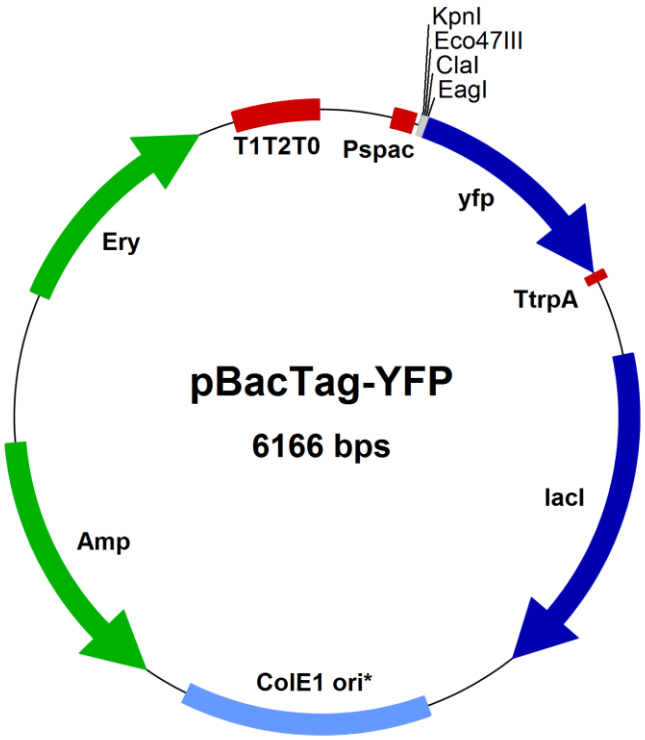
7.2 pBacTag Vectors with localization tag



	Type	Start	End	Name	Description
	Promoter	228	298	Pspac	*spac* promoter
	Region	316	338	MCS	Multiple Cloning Site
	Gene	355	1074	gfp+	*gfp+* gene (localization tag)
	Terminator	1092	1119	TtrpA	Termination sequence of *trpA* gene
	Gene	1369	2451	lacI	*lacI* repressor gene
	Replication/ori	2765	3556	ColE1 ori*	Origin belonging to the ColE1 incompatibility group
	Selectable Genetic Marker	4567	3707	Amp	Ampicillin resistance
	Selectable Genetic Marker	5051	5788	Ery	Erythromycin resistance
	Terminator	5909	6184	T1T2T0	Terminators t_1, t_2 of *rnb* operon of *E. coli* and λ terminator t_0



	Type	Start	End	Name	Description
	Promoter	228	298	Pspac	*spac* promoter
	Region	316	338	MCS	Multiple Cloning Site
	Gene	340	1059	cfp	*cfp* gene (localization tag)
	Terminator	1066	1093	TtrpA	Termination sequence of *trpA* gene
	Gene	1343	2425	lacI	*lacI* repressor gene
	Replication/ori	2739	3530	ColE1 ori*	Origin belonging to the ColE1 incompatibility group
	Selectable Genetic Marker	4541	3681	Amp	Ampicillin resistance
	Selectable Genetic Marker	5025	5762	Ery	Erythromycin resistance
	Terminator	5883	6158	T1T2T0	Terminators t_1, t_2 of *rnb* operon of *E. coli* and λ terminator t_0



	Type	Start	End	Name	Description
	Promoter	228	298	Pspac	*spac* promoter
	Region	316	338	MCS	Multiple Cloning Site
	Gene	340	1059	yfp	*yfp* gene (localization tag)
	Terminator	1066	1093	TtrpA	Termination sequence of *trpA* gene
	Gene	1343	2425	lacI	*lacI* repressor gene
	Replication/ori	2739	3530	ColE1 ori*	Origin belonging to the ColE1 incompatibility group
	Selectable Genetic Marker	4541	3681	Amp	Ampicillin resistance
	Selectable Genetic Marker	5025	5762	Ery	Erythromycin resistance
	Terminator	5883	6158	T1T2T0	Terminators t_1, t_2 of *rnb* operon of *E. coli* and λ terminator t_0

8 References

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Zhang,G., Bao,P., Zhang,Y., Deng,A., Chen,N. and Wen,T. (2011) Enhancing electro-transformation competency of recalcitrant *Bacillus amyloliquefaciens* by combining cell-wall weakening and cell-membrane fluidity disturbing. Anal. Biochem., 409, 130–137.

9 Order Information, Shipping and Storage

Order#	Product	Quantity
PBT001	pBacTag-DYKDDDDK vector DNA	5 µg
PBT002	pBacTag-cMyc vector DNA	5 µg
PBT003	pBacTag-HA vector DNA	5 µg
PBT004	pBacTag-GFP+ vector DNA	5 µg
PBT005	pBacTag-CFP vector DNA	5 µg
PBT006	pBacTag-YFP vector DNA	5 µg
Lyophilized from water, shipped at RT. Lyophilized plasmid DNA can be stored at 4 °C. Once the DNA has been dissolved in sterile water or buffer we recommend storage at -20 °C.		

10 Related Products

Order#	Product	Quantity
PBS020	*Bacillus subtilis* strain 1012wt	1 ml
PBS021	*Bacillus subtilis* strain 168 Marburg	1 ml
PBS022	*Bacillus subtilis* strain WB800N (for secretion vectors)	1 ml
PBS026	*Bacillus subtilis* strain AS1	1 ml
PBS020	*Bacillus subtilis* strain 1012wt	1 ml
PBS021	*Bacillus subtilis* strain 168 Marburg	1 ml
Shipped on dry ice; store at -80 °C		
CB-J902-100GAM	2xYT medium broth	100 g
CB-0339-25GAM	ampicillin sodium salt	25 g
CB-J859-100GAM	tryptone	100 g
CB-J851-100GAM	casamino acids	100 g
CB-0241-1KGAM	sodium chloride (NaCl)	1 kg
Shipped at RT		

11 Contact and Support

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