hp-Vector Expression System for *Bacillus megaterium*

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Content

An Efficient Alternative to *E. coli*: Stable Protein Production with High Yield – Suited not only for Industrial Scale

MoBiTec offers this expression system as an easy-to-handle tool with *E. coli/ B. megaterium* shuttle vectors and - to be ordered separately - *B. megaterium* protoplasts ready for transformation.

1. Introduction

1.1. General features of *Bacillus megaterium*

First described over 100 years ago, *B. megaterium* has recently been gaining more and more importance in scientific as well as industrial applications. The source of its significant name "*megaterium*" is its large size of the vegetative cells (over 10 µm) and its spores.

B. megaterium is able to grow on a wide variety of carbon sources and thus has been found in many ecological niches such as waste from meat industry or petrochemical effluents. Also, the degradation of persistent insecticides by *B. megaterium* has been documented (Saxena *et al*., 1987; Selvanayagam and Vijaya, 1989) offering potential applications as detoxifying agent. One of the genetic regulatory elements for carbon utilization is the xylose operon. It has been described by Rygus and Hillen (1991) and is used in the expression system which MoBiTec is offering.

Further, several *B. megaterium* proteins are of importance. For example, a family of P_{450} cytochrome monooxygenases is similar to eukaryotic P_{450} playing a role in many diseases. Industrial applications of enzymes excreted by *B. megaterium* are diverse, starting from amylases used in bread industry to penicillin amidase which is used for the generation of new synthetic antibiotics.

An overview about the features of this unique organism is given in review articles as "Prime time for *Bacillus megaterium*" (Vary, 1994), “A short story about a big magic bug” (Bunk *et al.*, 2010), and “*Bacillus megaterium* - from simple soil bacterium to industrial protein production host“ (Vary *et al.*, 2007).

1.2. *Bacillus megaterium* as expression host

In molecular biology, *B. megaterium* has been proven to be an excellent host for the expression of non-homologous DNA. All cloning vectors of the *B. megaterium* system (all derivatives of the original pWH1520 (Rygus and Hillen, 1991; Malten *et al.*, 2004; Barg *et al.*, 2005; Biedendieck *et al.*, 2007)) rely on the above mentioned xylose operon used as regulatory element. Remarkable improvement work was done by R. Biedendieck (Braunschweig, Germany).

In contrast to other *Bacillus* strains *B. megaterium* has the advantage that no alkaline protease is present. This fact enables excellent production and secretion of foreign proteins without degradation (Meinhardt *et al.*, 1989; Rygus and Hillen, 1991). In addition,

due to its Gram-positive character there are no endotoxins found in the cell wall. Protein yields are exceptionally good, also if inexpensive substrates are used. Recombinant plasmids are structurally and segregationally stable. For example, the *B. megaterium* glucose dehydrogenase gene (*ghd*) has been cloned back into a *B. megaterium* expression vector. The vector and the production of Ghd remained stable without selective pressure over a period of three weeks with daily subculturing (Meinhardt *et al.*, 1989).

Several proteins have successfully been overproduced in *B. megaterium* (see chapter 3). Rygus and Hillen (1991) describe cloning and expression of the genes *lacZ* from *E. coli*, *gdh* from *B. megaterium*, *mro* (mutarotase) from *Acinetobacter* and human *puk* (a urokinase-like plasminogen activator, rscuPA). Using the xylose operon the recombinant protein production was 130- to 350-fold induced without showing proteolysis. Such a system offers unique possibilities for the industrial production as well as purification of recombinant proteins (Biedendieck *et al.,* 2007). Further, it is of great interest to manufacturers in the biomedical field. In a diagnostic test for AIDS, the HIV coat protein is commercially produced by *B. megaterium* (Ginsburgh *et al.*, 1989).

1.3. General features of the hp-Vector Expression Systems

With our improved hp-(high performance) vectors the yields of recombinant proteins can be more than tenfold enhanced in comparison to the basic plasmids carrying the native promoter system. All plasmids have an established multiple cloning site (MCS) for versatile cloning, an optimized promoter, and a ribosome binding site (RBS). We offer vectors encoding C- or N-terminal His_6- or Strep-tags for easy purification, a tag-free plasmid, and a vector carrying two ribosomal binding sites (2RBS) for simultaneous dual expression. The protein secretion with signal peptides of the lipase A (LipA) or of the YocH (putative exported cell wall-binding protein) is increased up to ninefold while the secreted protein can also be fused to a C-terminal His_6-tag. Induction of protein production of all vectors is achieved by the tightly regulated and efficiently inducible xylose operon.

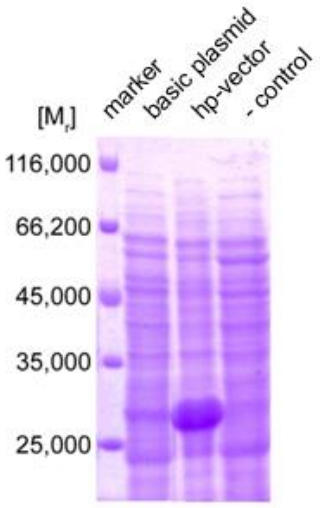


Fig. 1. Soluble protein fractions 6 h after induction of heterologous gene expression

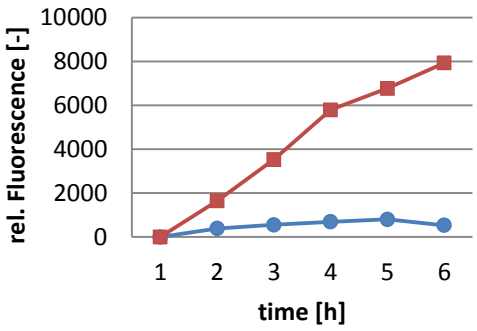


Fig. 2. Relative fluorescence mediated by the optimized hp-vector (red; square) and the basic plasmid (blue; circle), respectively, over time [h] after induction

2. Summary of Advantages

- *B. megaterium* is not pathogenic.
- No endotoxins found in the cell wall
- MoBiTec host strains have been found to be asporogenic on common media
- Tightly regulated and efficiently xylose-inducible promoter system (up to 350-fold)
- High performance vectors with optimized promoter sequence
- Stable, high yield protein production
- Protein yield up to 10 times better than protein production with basic plasmid
- Encoding C- or N-terminal His_6- or Strep-tag for versatile purification which are removable using TEV and Factor Xa sites
- 2RBS (ribosome binding site) vector for simultaneous dual expression
- Secretion with signal peptides of LipA or of YocH up to ninefold increased compared to basic plasmid
- Since alkaline proteases are not produced there is no indication of proteolytic instability even up to 5 h after induction.
- Compatible with *Bacillus subtilis* vectors
- System might be suitable also for other *Bacillus* ssp.

3. Expression Vectors and Strains

Vectors of the 1623hp series

All vectors of the 1623hp series were constructed from the basic vector pSTOP1622 (referring to the manual on *Bacillus megaterium* Protein Production System) which is used as a shuttle vector for xylose-inducible production of recombinant target proteins in *Bacillus megaterium*. The pSTOP1622 contains one stop codon downstream of the multiple cloning site (MCS). The plasmid p3STOP1623 has been created by introducing two additional stop codons downstream of the MCS into the pSTOP1622 allowing cloning in all three reading frames.
The high performance (hp)-expression vectors as indicated below were generated by optimizing the promoter P_{xylA} (optimized -35 box) and ribosomal binding site (optimized RBS) of the basic vector p3STOP1623.

p3STOP1623hp
This high performance plasmid contains two additional stop codons downstream of the MSC, an optimized -35 region of P_{xylA} and an optimized RBS. All following plasmids are based on this plasmid.

pC-HIS1623hp
This plasmid allows a C-terminal 6xHis-tag (3' of MCS) fusion including a stop codon downstream of the tag.

pN-HIS-TEV1623hp
The plasmid encodes an N-terminal 6xHis-tag fusion including a TEV (tobacco etch virus) recognition site that is cleavable with TEV protease treatment.

pSP_{LipA}-hp
This vector contains a His_6-tag for N-terminal fusion of recombinant gene including the coding sequence of the signal peptide of *B. megaterium* protein LipA for secretion.

pSP_{YocH}-hp
The plasmid encodes the signal peptide of *B. megaterium* protein YocH which enables secretion of proteins fused to an N-terminal His_6-tag.

p3STOP1623-2RBShp
This carries an additional optimized ribosomal binding site. It is suitable for simultaneous dual expression.

pC-STREP1623hp
This plasmid allows a C-terminal StrepII-tag fusion to the protein of interest.

pN-STREP-Xa1623hp
pN-StrepXa1623hp encodes an N-terminal StrepII-tag fusion followed by a Factor Xa recognition site which is cleavable with Factor Xa protease.

pN-STREP-TEV1623hp
This plasmid carries the coding region of an N-terminal StrepII-tag fusion with TEV (tobacco etch virus) recognition site which is cleavable with TEV protease.

Vectors for special requirements

pMGBm19

pMGBm19 is an *E.coli*/ *Bacillus* shuttle vector with xylose-inducible P_{xylA} promoter that is designed for co-expression studies. It can be used in combination with any other vector of the 1520, 1622, and 1623hp series, since it contains an origin of replication of a different compatibility group (pMB100 replicon).

pMMEc4

Since the xylose-inducible P_{xylA} promoter is not tightly controlled in *E. coli*, cloning the toxic genes into vectors of the 1520, 1622, and 1623hp series, respectively, using *E. coli* as host may be difficult. In such cases, we recommend using the pMMEc4 helper plasmid. This *E. coli* vector (not replicating in *Bacillus*!) encodes for the xylose repressor XylR and is designed for blocking any expression starting from the P_{xylA} promoter while cloning gene of interest within *E. coli* (Jordan et al., 2007).
In pMMEc4 the expression of *xylR* is controlled by the arabinose-dependent promoter P_{BAD} and the AraC protein. In the presence of 0.2% arabinose, the AraC protein binds to the operator sequence that activates the expression of the *xylR* gene, and additionally upregulates its own expression. The vector pMMEc4 carries the p15A origin of replication that is compatible with vectors from other incompatibility groups, like ColE1.

pGFP1624hp

pGFP1624hp is a positive control vector expressing *gfp* under control of xylose-inducible P_{xylA} promoter. This vector belongs to the high performance expression vectors series of *B. megaterium* (1623 series) with an optimized promoter region that leads to improved protein yield.
pGFP1624hp is an *E.coli*/ *B. megaterium* shuttle vector. The vector can be propagated within *E. coli* by selection with 100 µg/ml ampicillin. For maintenance of the vector in *B. megaterium* use 10 µg/ml of tetracycline.

Bacillus megaterium strains

1) The Strain WH320 (#BMEG02) is a chemical mutant of strain DSM319 which is deficient in the production of β-galactosidase (Δ*lacZ*). It was described by Rygus and Hillen (Rygus and Hillen, 1991).

2) The Strain YYBm1 (#BMEG04) carries the *nprM* deletion and an additional deletion of the xylose isomerase gene *xylA*. It is thus unable to metabolize xylose which is used as inducer for gene activation (Yang *et al*. 2006).

3) The Strain MS941 (#BMEG50) was generated from the wild-type strain DSM319 by deletion of major extracellular protease gene *nprM* (Wittchen and Meinhardt 1995). Because of reduced extracellular protease activity this strain is well suited for extracellular protein production.

4. Protocols

4.1. Cloning the DNA fragment of interest

The *E. coli*/ *B. megaterium* shuttle vectors are supplied as lyophilized DNA. Green and Sambrook (2012) describe all standard protocols for propagation of the plasmid in *E. coli*, plasmid DNA preparation, restriction endonuclease cleavages, and ligation of the DNA fragment of interest into the vector. After ligation of the insert the vectors should be propagated in *E. coli* (amp^R) before transforming into *Bacillus* protoplasts (tet^R). For all cloning purposes and propagation of plasmids we recommend to use *E. coli* strain DH10B.

4.2. General remarks on the handling of *B. megaterium*

B. megaterium strains grow well on rich media such as Luria-Bertani (LB) broth at 30 °C and 37 °C. Make sure to aerate liquid cultures well in baffled flasks by vigorous agitation.

We found MS941, WH320, YYBm1, and derived strains to be asporogenic on common growth media - they will die on the plates stored at 4 °C within two weeks, so prepare glycerol stocks (30% w/v) as a backup and streak the working cultures on fresh plates every 7 to 10 days.

Positive clones carrying the plasmid with a gene of interest can be selected by adding 10 µg/ml of tetracycline to the growth medium.

To prove successful overexpression of the target gene harvest small samples of the culture just before, and at certain time intervals after induction of recombinant gene expression with xylose. To obtain crude extracts for protein analysis, cells have to be lysed using lysozyme. Simple boiling of cells in sample buffer (Laemmli, 1970), which is quite convenient for *E. coli*, does not lyse *B. megaterium* cells.

4.3. Transformation of *B. megaterium* protoplasts

For recombinant protein production *B. megaterium* protoplasts are transformed with the plasmids coding for the protein of interest. After transformation it is advisable to screen at least three different clones for protein production as the yield can vary among different clones.

Since intact *B. megaterium* cells cannot be transformed, MoBiTec conveniently provides protoplasts of *B. megaterium* cells, which are ready for transformation (strains MS941, WH320, and YYBm1). They can be used at least 2 months after date of arrival and have to be stored at -80 °C. The protoplast suspension is supplied in 5 aliquots of 500 µl each to prevent multiple freezing and thawing of protoplasts that are not used immediately. One aliquot is provided per transformation. It is advisable to use two of the vials for the control experiments as described below.

Control experiments:

1. Negative control: protoplasts without DNA

Note: *Each lot of protoplasts undergoes this test during our quality control as well.*

This is a test reassuring that the protoplasts are not only fully viable but also free of contaminations before using them for transformation. Perform this test according to the transformation protocol as demonstrated below. After incubation at 30 °C, apply CR5-top agar to the protoplasts and split the sample in two portions. You may plate one sample on a LB plate with antibiotic such as tetracycline, and another one on a plate without any drug. In this case, bacterial colonies will grow only on a solid medium without antibiotic.

2. Positive control: protoplasts transformed with an empty plasmid (not included)

This is a test control for a successful transformation and should yield lots of colonies on the plates supplemented with an antibiotic (here: tetracycline). If this transformation works well, but you have problems with the plasmid containing your target gene, the problem is most likely associated with your construct.

Essential buffers (listed in chapter 5) should be kept at room temperature (RT) at least 30 minutes before using them.

Transformation procedure:

1. Combine 500 µl of protoplast suspension and 3-5 µg of plasmid DNA in a 15 ml tube, one for each transformation. DNA should be purified using a commercial preparation kit. Elute the DNA from the column using water.
2. Add 1.5 ml of PEG-P, mix gently, and incubate for 2 minutes at RT.
3. Add 5 ml SMMP and mix carefully by rolling the tube.
4. Harvest cells by gentle centrifugation (1,300 x g for 10 minutes at RT), discard the supernatant immediately after centrifugation. Supernatant does not have to be removed completely.
   (*Note: do not check for a cell pellet - most of the time it will be invisible*)
5. Add 500 µl of SMMP to remaining supernatant (containing bacterial cells) and transfer to a 1.5 ml microcentrifuge tube.
6. Incubate at 30 °C for 90 minutes with gentle shaking or rolling of tubes (max. 100 rpm) or incubate for 45 min without shaking followed by another 45 min while shaking at 300 rpm.
7. Prepare 2.5 ml aliquots of CR5-top agar in sterile tubes.
8. After incubation at 30 °C add all cells to 2.5 ml top agar, mix gently by rolling the tube between both hands (do not vortex!), and pour onto a pre-warmed plate of LB containing desired antibiotic.
9. Incubate overnight at 30 °C - expect colonies of varying diameter because some will be covered with agar and others have easier access to air.
   (*Note: bacterial colonies on the top of the agar surface will be shiny*)
10. Streak several single colonies (at least 3) on fresh plates within two days.

Note*: protein production may vary among the single colonies due to yet unknown reasons.*

4.4. Recombinant protein production and secretion

I. Test protein production

1. Grow the recombinant *B. megaterium* cells in suited medium (e.g., LB medium) including antibiotic (here: tetracycline) in baffled flasks to an optical density (OD_{578nm}) of 0.3-0.4 at 37 °C under vigorous shaking at 250 rpm.
   The main culture should be set up with an overnight culture in a dilution of 1:100.
2. Take a sample as control before induction.
3. Induce the xylose-inducible promoter by addition of 0.5% (w/v) of D-xylose.
4. Incubate at 37 °C and shake vigorously (250 rpm).
5. Withdraw samples every 30 to 60 minutes until an OD_{578nm} of around 4 to 8 (depending on the growth medium and volume) is reached (now, cells have entered the stationary phase). Take samples both for the OD_{578nm} measurement and protein analysis. For extracellular protein analysis take 2 ml of cell culture. Intracellular protein analysis requires a higher volume than 2 ml of sample.
6. Centrifuge each sample to harvest cells and cell-free supernatant.
7. For extracellular protein analysis remove the supernatant and store at 4 °C, for intracellular protein analysis completely discard the supernatant and store the cells at -20 °C.

II. Analysis of intracellular proteins

1. Resuspend cells in 30 µl of lysis buffer.
2. Incubate at 37 °C for 30 min with vigorous shaking at 1,000 rpm in a thermomixer. An effective cell lysis can be obtained by whirling the samples every 10 minutes.
3. Centrifuge for 30 min at 4 °C and 13,000 rpm to separate the insoluble fraction (pellet) from the soluble fraction (supernatant).
4. Mix 27 µl of supernatant containing soluble proteins with 13 µl of SDS sample buffer.
5. Completely remove the supernatant. Resuspend the pelleted fraction in 30 ml of 8M urea. Centrifuge for 30 min at 4 °C and 13,000 rpm.
6. Mix 27 µl of the supernatant with 13 µl of SDS sample buffer.
7. Heat each sample for 5 min at 95 °C.
8. Load 7.5 µl of each sample onto an SDS-PAGE gel.

III. Ammonium sulfate precipitation of proteins in the cell-free supernatant

1. Add 600 mg of ground ammonium sulfate to 1.5 ml of cell-free supernatant and incubate for two hours at 4 °C while shaking.
2. Centrifuge at 13,000 rpm and 4 °C for 30 minutes.
3. Completely remove the supernatant, centrifuge again for 1 min, and make sure the protein pellet is dry.
4. Add 10 µl of 8 M urea (in 50 mM Tris-HCl, pH 7.5) and 5 µl SDS sample buffer to dissolve the proteins again.
5. Spin briefly at 13,000 rpm, heat to 99 °C for 5 minutes, and load onto an SDS polyacrylamide gel for analysis.
6. Determine enzymatic activities with the appropriate assays (not included in the kit).
7. Perform Western blot using appropriate antibodies (not included in the kit).

IV. Scale up protein production

1. Grow larger culture and induce as indicated above.
2. Harvest cells at the time point of maximal protein overproduction, as determined by the test experiments.

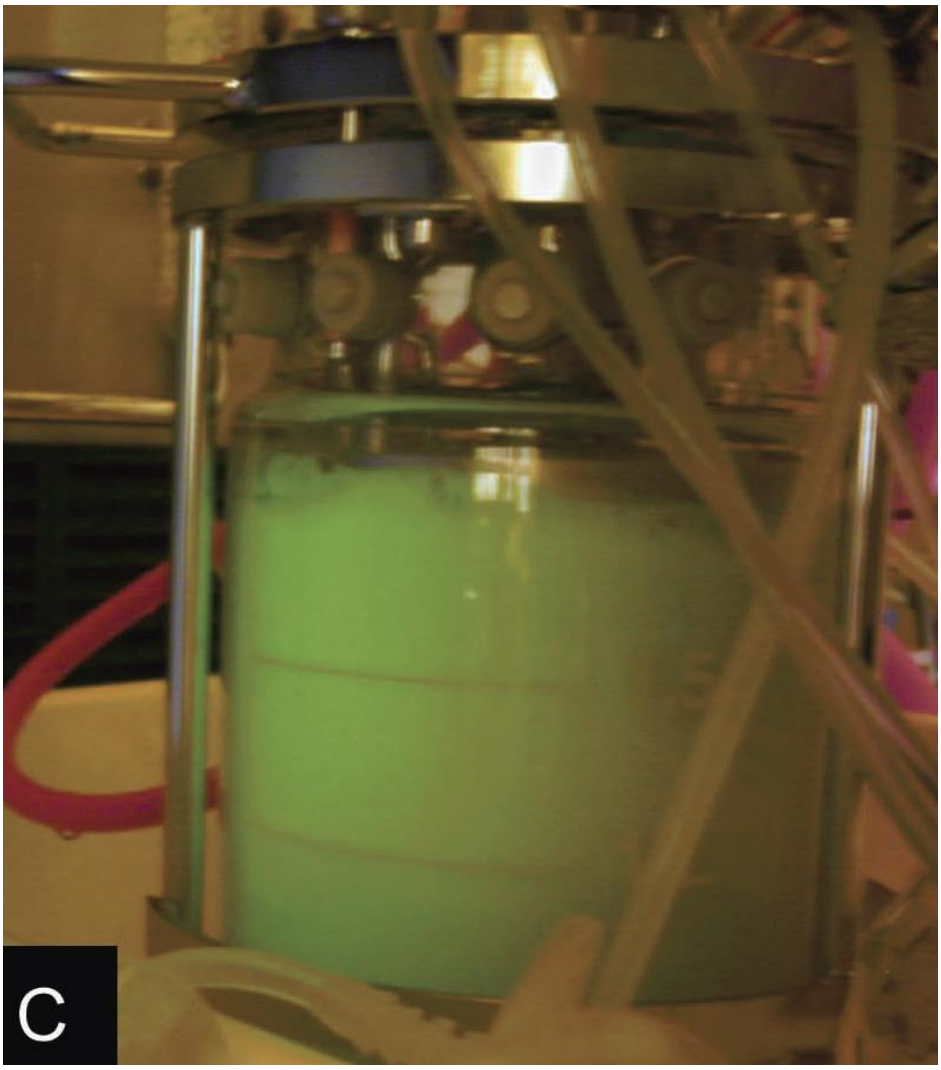


Fig. 3. *B. megaterium* carrying a plasmid coding for GFP-Strep fusion protein was grown in semi-defined minimal medium at 37 °C initially in a batch phase with 4 g/L glucose. At the end of the batch phase an exponential feeding profile was started. GFP was visualized by a lamp emitting blue light and a yellow filter using a digital camera.

5. Materials

2 × AB3 (Antibiotic Medium No. 3, DIFCO)
- 7 g AB3 (Difco) in 200 ml deionized water
- autoclave for 15 min

2 × SMM **Solubilize in the given order!**
- 1.16 g maleic acid (40 mM)
- 800 mg NaOH (80 mM)
- 2.03 g $MgCl_2$ x $6H_2O$ (40 mM)
- 85.58 g sucrose (1 M)
- solubilize each component in deion. water
- mix and fill with deion. water to 250 ml
- sterilize by filtration

SMMP
- 2 × AB3 and 2 × SMM 1:1 (freshly prepared!)

PEG-P
- solubilize 20 g PEG-6000 with 1 × SMM and fill to 50 ml
- autoclave for 15 min

CR5-top agar
prepare separately for 500 ml:

Solution A
- 51.5 g sucrose
- 3.25 g MOPS
- 300 mg NaOH
- add to deionized water to 250 ml
- adjust pH to 7.3 with NaOH
- sterilize by filtration

Solution B
- 2 g agar
- 100 mg casamino acids
- 5 g yeast extract
- add deionized water to 142.5 ml
- autoclave for 15 min

8 × CR5-salts
- 1.25 g K_2SO_4
- 50 g $MgCl_2 \times 6\ H_2O$
- 250 mg KH_2PO_4
- 11 g $CaCl_2$
- solubilize in 625 ml deion. water
- autoclave for 15 min

12% proline
- 3 g L-proline
- add deionized water to 25 ml
- sterilize by filtration

20% glucose
- 5 g glucose
- add deionized water to 25 ml
- sterilize by filtration or autoclave

for a 2.5 ml portion of CR5-top agar add the following (in the given order!):
- 1.25 ml **solution A**
- 288 µl CR5-salts
- 125 µl 12% proline
- 125 µl 20% glucose

<u>90 minutes after transformation:</u>
- boil **solution B**
- add 713 µl to the provided CR5-top agar
- immediately add the regenerated protoplasts and put onto prewarmed agar plates containing the corresponding antibiotic (here: tetracycline)

Lysis buffer
- 100 mM Na_3PO_4 (pH 6,5 – see instruction below)
- 5 mg/ml lysozyme
- add 1 µl of a 1 M $MgSO_4$ and 2 µl HS-Nuclease (5 U/µl, cat.# GE-NUC10700-01) per ml lysis buffer shortly before use

For Na_3PO_4 buffer at pH 6.5 prepare a stock solution of NaH_2PO_4 and Na_2HPO_4 and mix both solutions in the ratio given below:

- Prepare 200 mM NaH_2PO_4	Take 68,5 ml
- <u>Prepare 200 mM Na_2HPO_4</u>	<u>Take 31,5 ml</u>
200 mM Na_3PO_4	100,0 ml

For 100 mM Na_3PO_4 add an equal amount of deionized water (e.g.,100 ml Na_3PO_4 + 100 ml H_2O). Finally, if necessary the pH can be adjusted with H_3PO_4 or NaOH.

6. References

Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Dijl, J. M. & Hecker, M. (2001). A proteomic view on genome-based signal peptide predictions. Genome Res. *11*, 1484-1502.

Biedendieck, R., Beine, R., Gamer, M., Jordan, E., Buchholz, K., Seibel, J., Dijkhuizen, L., Malten, M. & Jahn, D. (2007). Export, purification, and activities of affinity tagged *Lactobacillus reuteri* levansucrase produced by *Bacillus megaterium*. Appl. Microbiol. Biotechnol *74*, 1062-1073.

Biedendieck, R., Borgmeier, C., Bunk, B., Stammen, S., Scherling, C., Meinhardt, F., Wittmann, C. & Jahn, D. (2011) Systems biology of recombinant protein production using *Bacillus megaterium*. Methods Enzymol. *500,* 165-195.

Biedendieck, R., Bunk, B., Fürch, T., Franco-Lara, E., Jahn, M. & Jahn, D. (2010) Systems biology of recombinant protein production in *Bacillus megaterium* in "Advances in Biochemical Engineering / Biotechnology" (Scheper, T., Ed.); Biosystems Engineering (Wittmann, C., Ed.) Springer, Berlin, Heidelberg *120,* 133-161

Biedendieck, R., Yang, Y., Deckwer, W., Malten, M. & Jahn, D. (2007). Plasmid system for the intracellular production and purification of affinity-tagged proteins in *Bacillus megaterium*. Biotechnol. Bioeng. *96*, 525-537.

Borgmeier, C., Biedendieck, R., Hoffmann, K., Jahn, D. & Meinhardt, F. (2011) Transcriptome profiling of *degU* expression reveals unexpected regulatory patterns in *Bacillus megaterium* and discloses new targets for optimizing expression. Appl. Microbiol. Biotechnol. *92*, 583-596.

Bunk, B., Biedendieck, R., Jahn, D. & Vary, P.S. (2010) *Bacillus megaterium* and other Bacilli: Industrial Applications. In Flickinger M. C. (Ed.) Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology. Volume 1 pp 429-443. John Wiley & Sons. Inc., Hoboken, NJ.

Bunk, B., Schulz, A., Stammen, S., Münch, R., Warren, M. J., Rohde, M., Jahn, D. & Biedendieck, R. (2010). A short story about a big magic bug. Bioeng. Bugs *1:2*, 1-7.

Eppinger, M., Bunk, B., Johns, M.A., Edirisinghe, J.N., Kutumbaka, K.K., Koenig, S.S.K., Creasy, H.H., Rosovitz, M.J., Riley, D.R., Daugherty, S., Martin, M., Elbourne, L.D.H., Paulsen, I., Biedendieck, R., Braun, C., Grayburn, S., Dhingra, S., Lukyanchuk, V., Ball, B., ul-Qamar, R., Seibel, J., Bremer, E., Jahn, D., Ravel, J. & Vary, P.S. (2011) Genome sequences of the biotechnologically important *B. megaterium* strains QM B1551 and DSM319. J. Bacteriol. *193*, 4199-4213.

Green, M. R. & Sambrook, J. (2012). Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press. 4. Edition, 3 vol. (1890 pages).

Hueck, C. J., Kraus, A., Schmiedel, D & Hillen, W. (1995). Cloning, expression and functional analyses of the catabolite control protein CcpA from *Bacillus megaterium*. Mol. Microbiol. *16*, 855-864.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lee, J., Sohn, H., Shin, K., Jo, M., Kim, J., Lee, S., Shin, J., Kum, E. & Kwon, G. (2006). Isolation of a soil bacterium capable of biodegradation and detoxification of endosulfan and endosulfan sulfate. J. Agric. Food Chem. *54*, 8824-8828.

Malten, M., Biedendieck, R., Gamer, M., Drews, A., Stammen, S., Buchholz, K., Dijkhuizen, L. & Jahn, D. (2006). A *Bacillus megaterium* plasmid system for the production, export, and one-step purification of affinity-tagged heterologous levansucrase from growth medium. Appl. Environ. Microbiol. *72*, 1677-1679.

Meinhardt, F., Stahl, U. & Ebeling, W. (1989). Highly efficient expression of homologous and heterologous genes in *Bacillus megaterium*. Appl. Microbiol. Biotechnol *30*, 343-350.

Puyet, A., Sandoval, H., López, P., Aguilar, A., Martin, J. & Espinosa, M. (1987). A simple medium for rapid regeneration of *Bacillus subtilis* protoplasts transformed with plasmid DNA. FEMS Microbiol. Lett. *40*, 1-5.

Rygus, T. & Hillen, W. (1991). Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. Appl. Microbiol. Biotechnol. *35*, 594-599.

Rygus, T., Scheler, A., Allmansberger, R. & Hillen, W. (1991). Molecular cloning, structure, promoters and regulatory elements for transcription of the *Bacillus megaterium* encoded regulon for xylose utilization. Arch. Microbiol. *155*, 535-542.

Sambrook, J. & Russell, D.W. (2001). Molecular cloning: a laboratory manual. (New York: Cold Spring Harbor Laboratory Press).

Saxena, A., Zhang, R. W. & Bollag, J. M. (1987). Microorganisms capable of metabolizing the herbicide metolachlor. Appl. Environ. Microbiol. *53*, 390-396.

Stammen, S., Müller, B.K., Korneli, C., Biedendieck, R., Gamer, M., Franco-Lara, E. & Jahn, D. (2010) High yield intra- and extracellular protein production using *Bacillus megaterium*. Appl. Environ. Microbiol. *76*, 4037-4046.

Vary, P. (1992). Development of genetic engineering in *Bacillus megaterium*. Biotechnology *22*, 251-310.

Vary, P. S. (1994). Prime time for *Bacillus megaterium*. Microbiology (Reading, Engl.) *140 (Pt 5)*, 1001-1013.

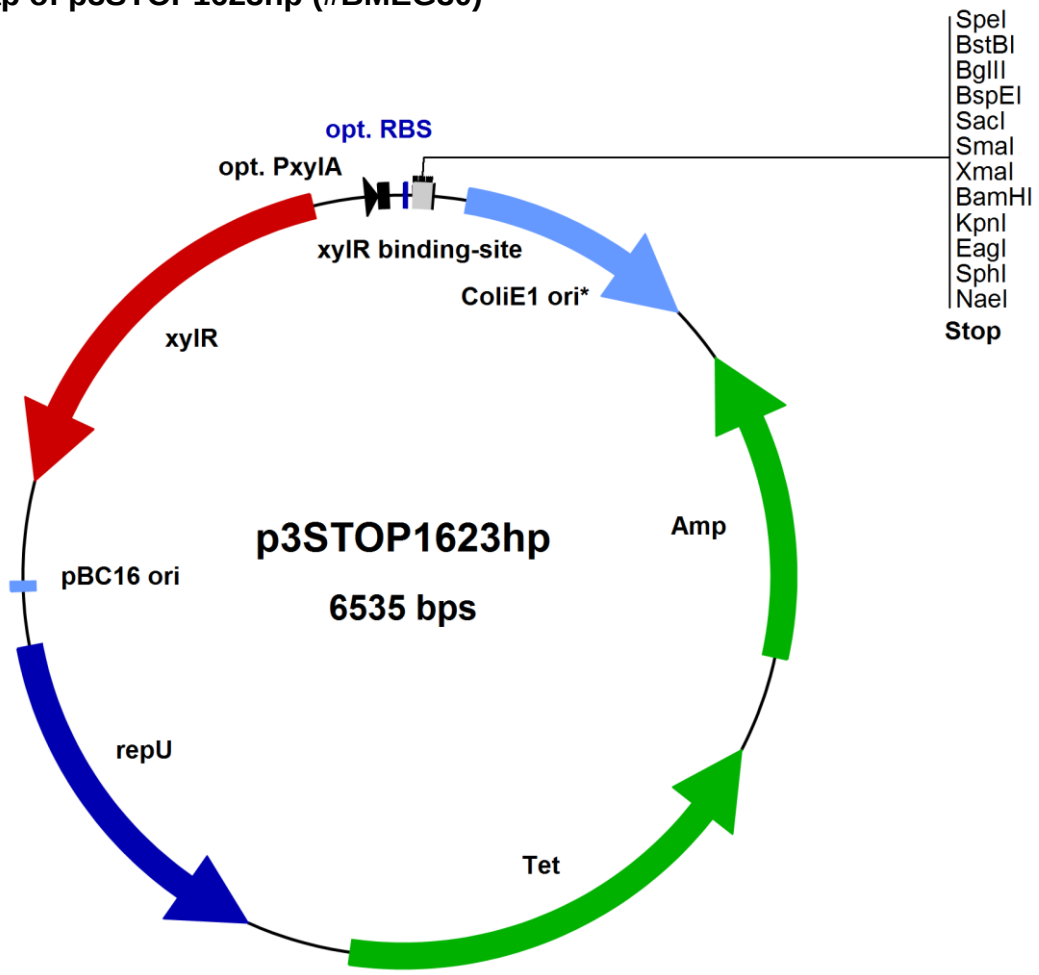
Vary, P. S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W.-D. & Jahn, D. (2007) *Bacillus megaterium* – from simple soil bacterium to industrial protein production host. Appl. Microbiol. Biotechnol. *76*, 957-967.

Wittchen, K. & Meinhardt, F. (1995). Inactivation of the major extracellular protease from *Bacillus megaterium* DSM319 by gene replacement. Appl. Microbiol. Biotechnol. *42*, 871-877.

7. Vector Maps

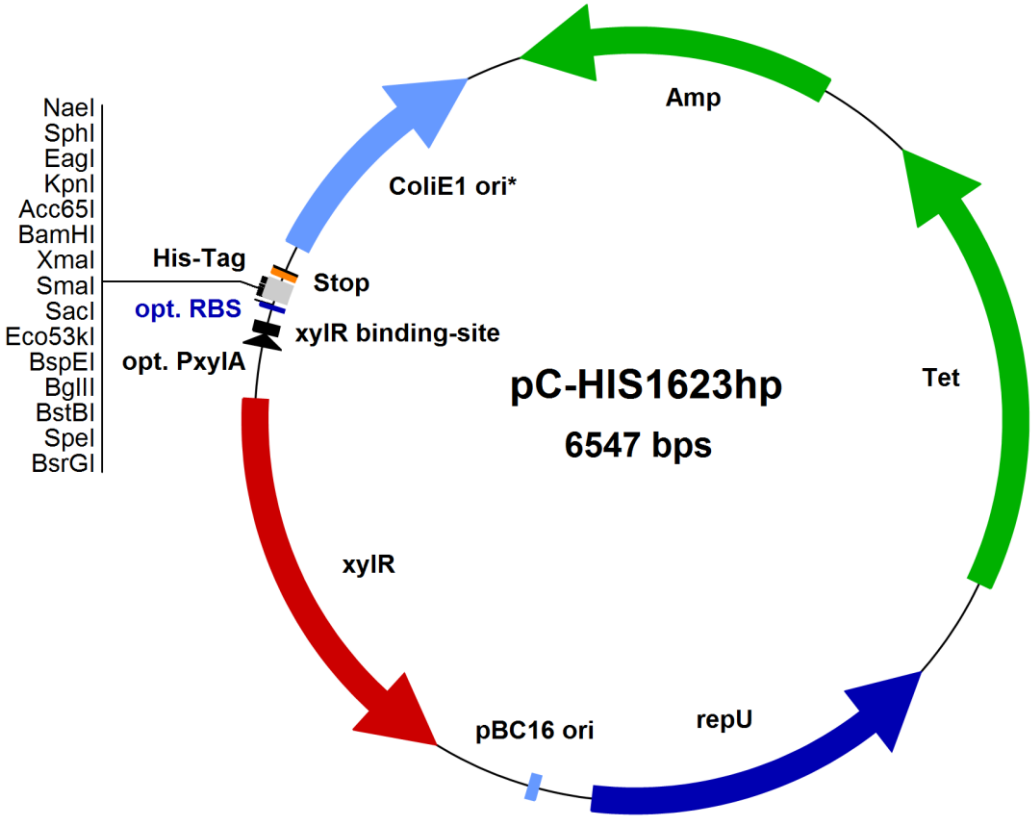
All vector maps and the DNA sequences are available for download on our web page at http://www.mobitec.com.

7.1. Vector map of p3STOP1623hp (#BMEG30)



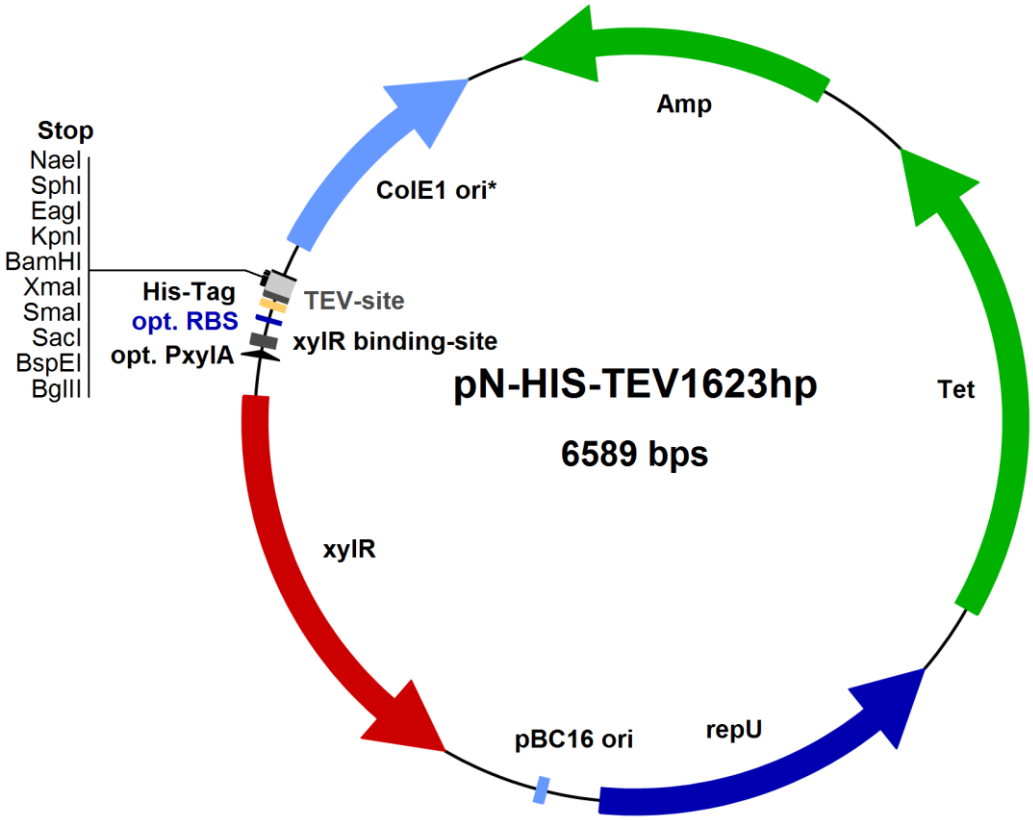
	Type	Start	End	Name	Description
	Region	1	10	Opt. RBS	Optimized ribosomal binding site
	MCS	27	80	MCS	Multiple Cloning Site
	Region	81	83	Stop	Stop Codon
	Origin of replication	175	837	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group
	Selectable genetic marker	1859	1001	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	3413	2130	Tet	Tetracycline resistance (*Bacillus*)
	Gene	4707	3706	repU	Gene encoding replication protein RepU
	Origin of replication	4859	4885	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	6285	5164	xylR	Gene encoding xylose repressor protein XylR
	Promoter	6431	6465	Opt. PxylA	Xylose-inducible promoter P_{xylA} including an optimized -35 region
	Region	6467	6495	xylR BS	xylR binding-site

7.2. Vector map of pC-HIS1623hp (#BMEG31)



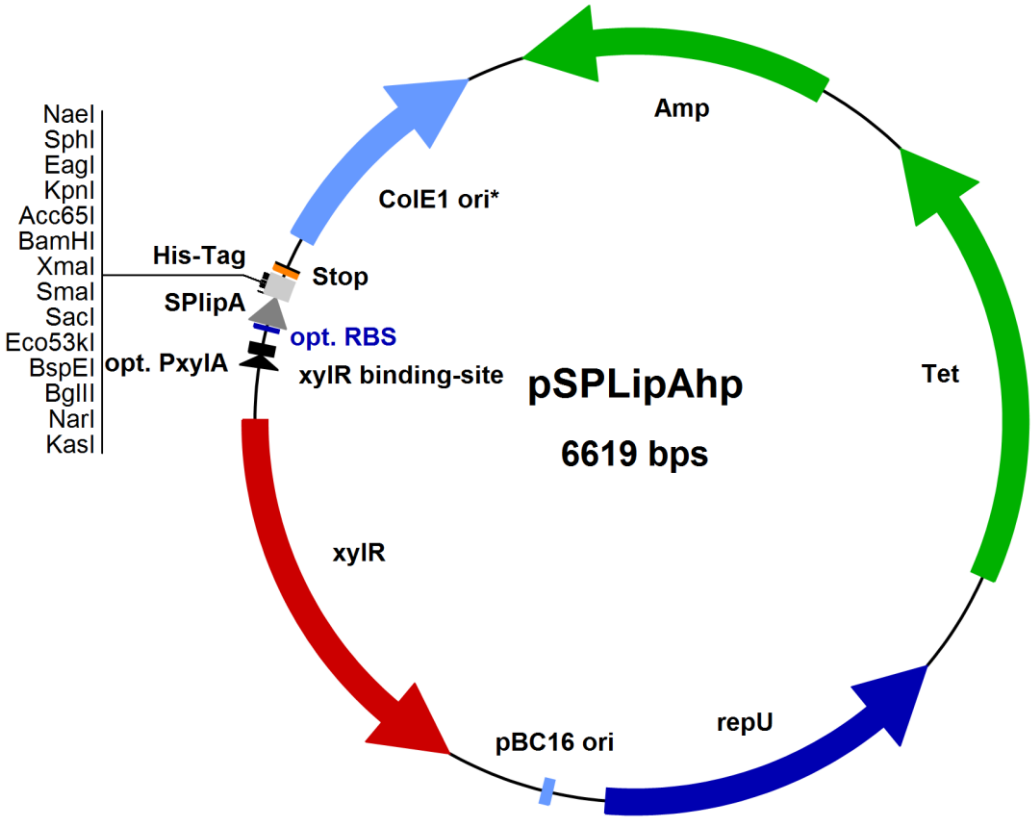
	Type	Start	End	Name	Description
	Selectable genetic marker	541	6232	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	2097	814	Tet	Tetracycline resistance (*Bacillus*)
	Gene	3391	2390	repU	Gene of replication protein RepU
	Origin of replication	3543	3569	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	4969	3848	xylR	Xylose repressor gene
	Promoter	5115	5149	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	5151	5179	xylR BS	xylR binding-site
	Region	5220	5229	Opt. RBS	Optimized ribosomal binding site
	MCS	5246	5299	MCS	Multiple Cloning Site
	Tag	5312	5329	His-Tag	6x histidine tag
	Region	5330	5332	Stop	Stop Codon
	Origin of replication	5406	6069	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

7.3. Vector map of pN-HIS-TEV1623hp (#BMEG32)



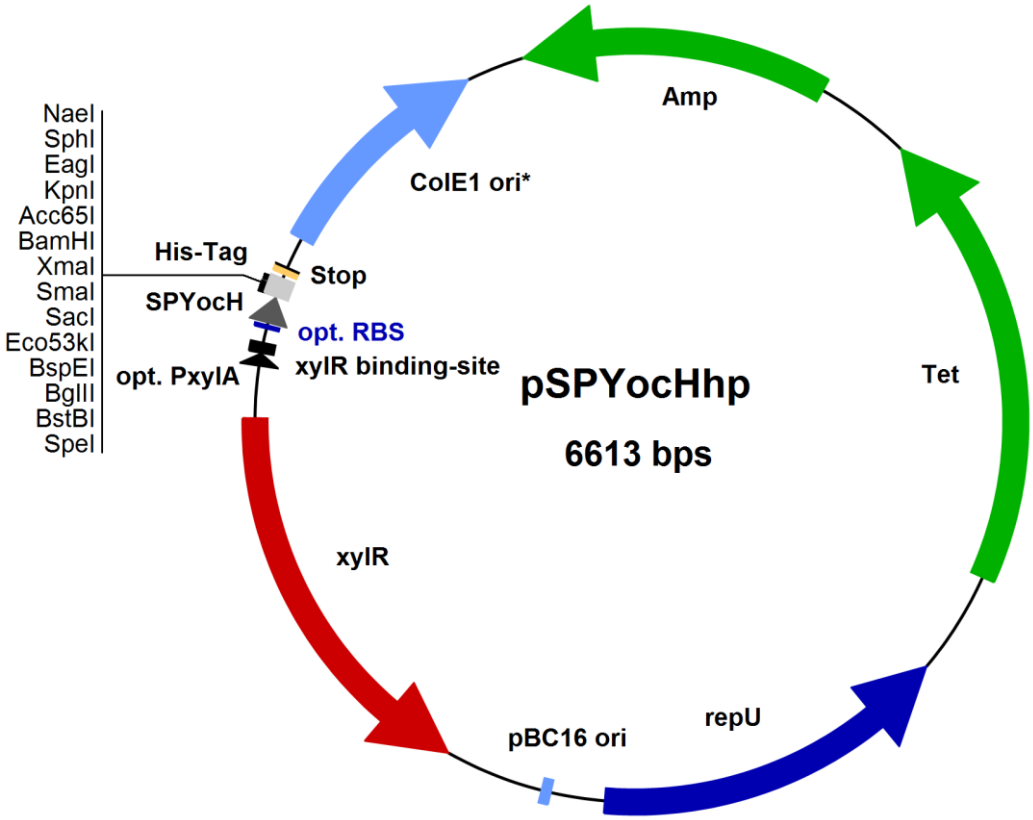
	Type	Start	End	Name	Description
	Selectable genetic marker	542	6274	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	2189	814	Tet	Tetracycline resistance (*Bacillus*)
	Gene	3391	2390	repU	Gene of replication protein RepU
	Origin of replication	3543	3569	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	5011	3845	xylR	Xylose repressor gene
	Promoter	5120	5138	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	5151	5179	xylR BS	xylR binding-site
	Region	5220	5229	Opt. RBS	Optimized ribosomal binding site
	Tag	5258	5275	His-Tag	6x histidine tag
	Cleavage site	5285	5305	TEV	TEV cleavage site
	MCS	5303	5354	MCS	Multiple Cloning Site
	Region	5354	5356	Stop	Stop Codon
	Origin of replication	5448	6111	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

7.4. Vector map of pSP$_{LipA}$-hp (#BMEG33)



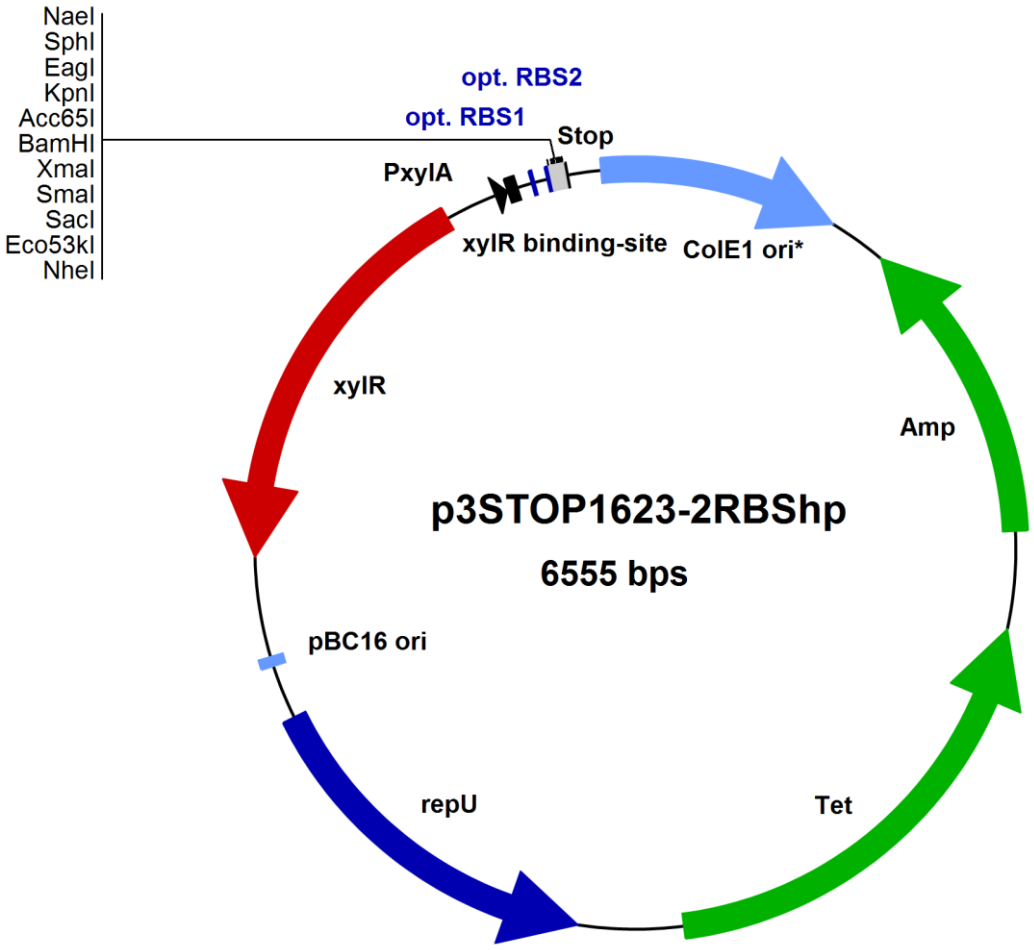
	Type	Start	End	Name	Description
	Selectable genetic marker	542	6304	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	2097	814	Tet	Tetracycline resistance (*Bacillus*)
	Gene	3391	2390	repU	Gene of replication protein RepU
	Origin of replication	3543	3569	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	4969	3848	xylR	Xylose repressor gene
	Promoter	5115	5149	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	5151	5179	xylR BS	xylR binding-site
	Region	5220	5229	Opt. RBS	Optimized ribosomal binding site
	Signal peptide	5237	5314	SPlipA	Signal sequence of *lipA* gene
	MCS	5315	5372	MCS	Multiple Cloning Site
	Tag	5384	5401	His-Tag	6x histidine tag
	Region	5402	5404	Stop	Stop Codon
	Origin of replication	5493	6139	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

7.5. Vector map of pSP$_{YocH}$-hp (#BMEG34)



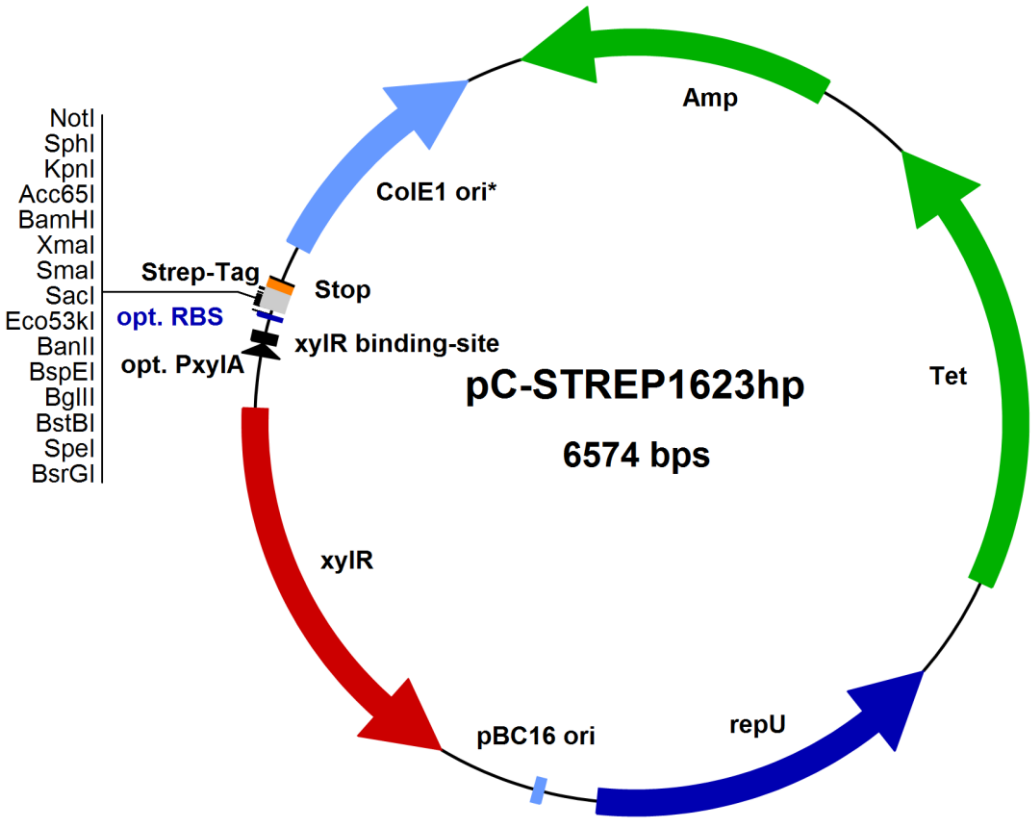
	Type	Start	End	Name	Description
	Selectable genetic marker	542	6304	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	2097	814	Tet	Tetracycline resistance (*Bacillus*)
	Gene	3391	2390	repU	Gene of replication protein RepU
	Origin of replication	3543	3569	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	4969	3848	xylR	Xylose repressor gene
	Promoter	5115	5149	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	5151	5179	xylR BS	xylR binding-site
	Region	5220	5229	Opt. RBS	Optimized ribosomal binding site
	Signal peptide	5237	5308	SPYocH	Signal sequence of *YocH* gene
	MCS	5312	5365	MCS	Multiple Cloning Site
	Tag	5378	5395	His-Tag	6x histidine tag
	Region	5396	5398	Stop	Stop Codon
	Origin of replication	5487	6133	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

7.6. Vector map of p3STOP1623-2RBShp (#BMEG35)



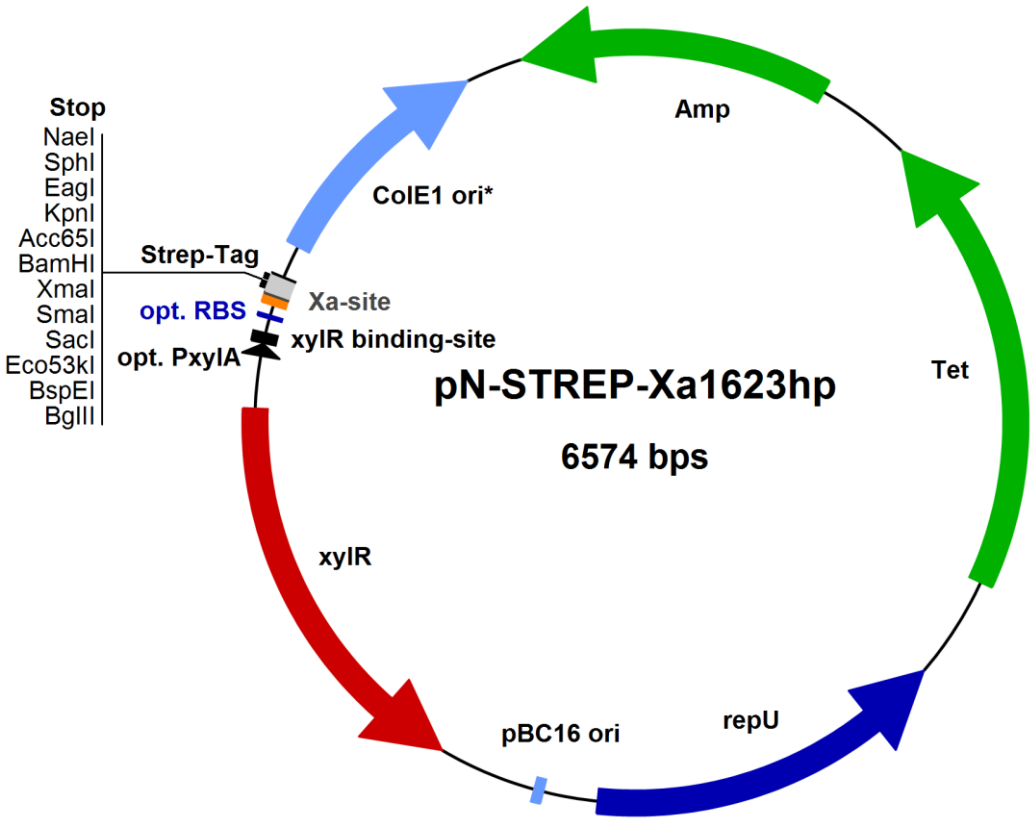
	Type	Start	End	Name	Description
	Origin of replication	692	1338	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group
	Selectable genetic marker	1589	732	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	3144	1861	Tet	Tetracycline resistance (*Bacillus*)
	Gene	4438	3437	repU	Gene of replication protein RepU
	Origin of replication	4590	4616	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	6016	4895	xylR	Xylose repressor gene
	Promoter	6162	6196	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	6198	6226	xylR BS	xylR binding-site
	Region	6267	6275	Opt. RBS1	Optimized ribosomal binding site 1
	Region	6311	6321	Opt. RBS2	Optimized ribosomal binding site 2
	MCS	6323	6366	MCS	Multiple Cloning Site
	Region	6367	6369	Stop	Stop Codon

7.7. Vector map of pC-STREP1623hp (#BMEG36)



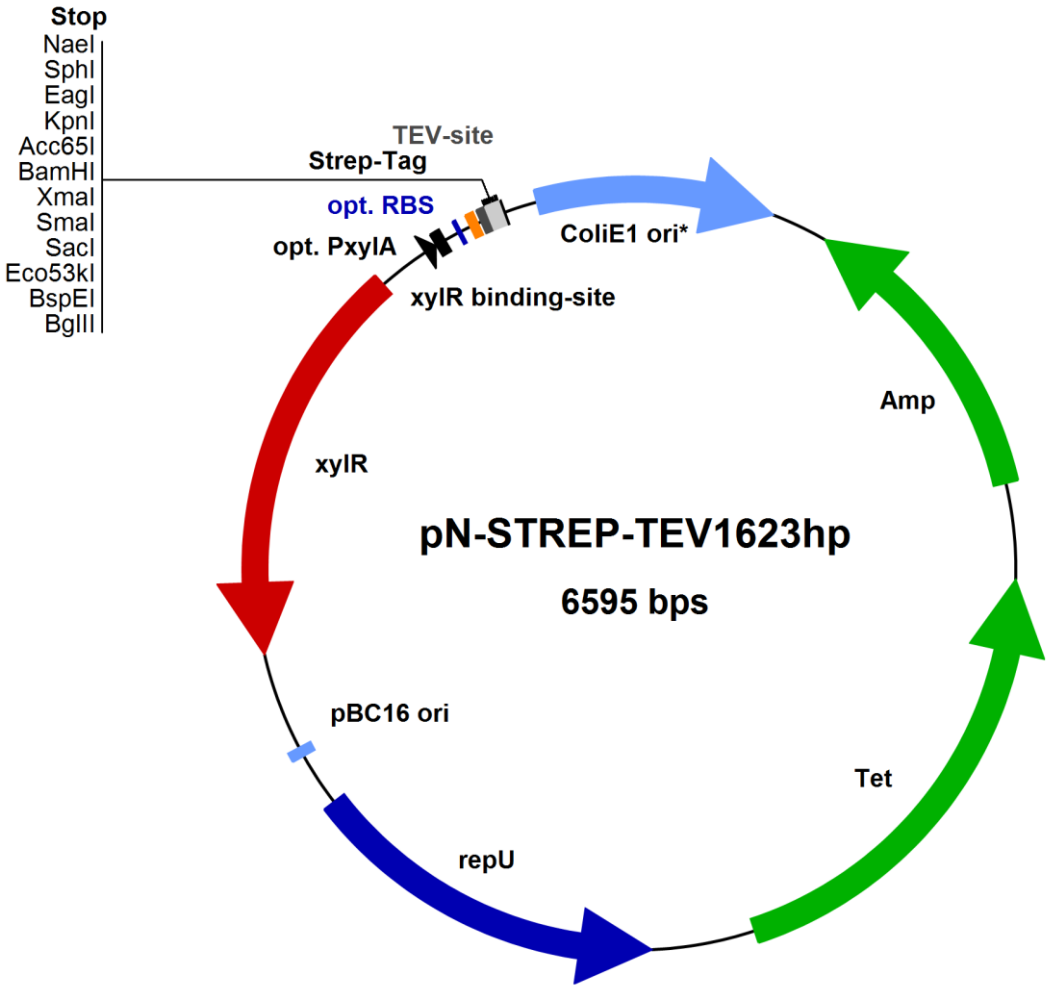
	Type	Start	End	Name	Description
	Selectable genetic marker	542	6256	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	2097	814	Tet	Tetracycline resistance (*Bacillus*)
	Gene	3391	2390	repU	Gene of replication protein RepU
	Origin of replication	3543	3569	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	4969	3848	xylR	Xylose repressor gene
	Promoter	5115	5149	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	5151	5179	xylR BS	xylR binding-site
	Region	5220	5229	Opt. RBS	Optimized ribosomal binding site
	MCS	5246	5302	MCS	Multiple Cloning Site
	Tag	5303	5326	Strep-Tag	Streptavidin tag II
	Region	5327	5329	Stop	Stop Codon
	Origin of replication	5433	6096	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

7.8. Vector map of pN-STREP-Xa1623hp (#BMEG37)



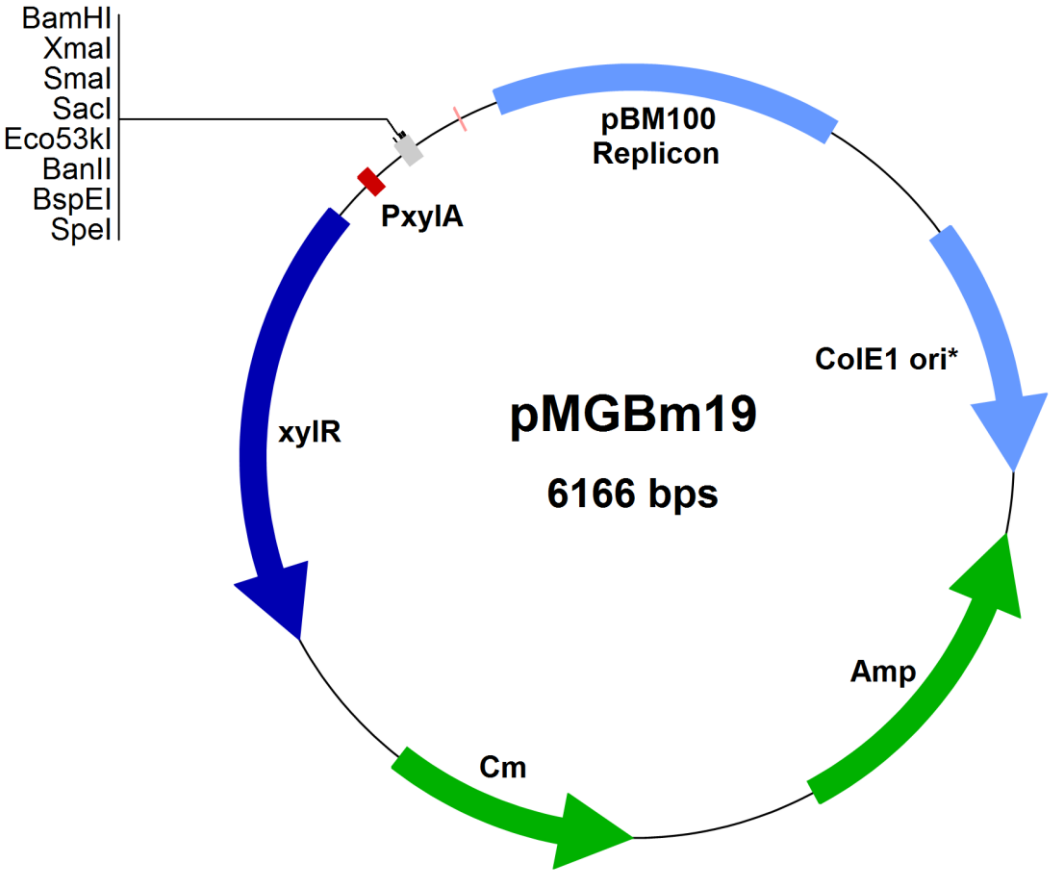
	Type	Start	End	Name	Description
	Tag	5258	5281	Strep tag	Streptavidin tag II
	Cleavage site	5258	5293	Xa	Factor Xa cleavage site
	MCS	5289	5338	MCS	Multiple Cloning Site
	Region	5339	5341	Stop	Stop codon
	Origin of replication	5433	6095	ColE1 ori*	origin of replication (*E. coli*); ColE1 incompatibility group
	Selectable genetic marker	542	6256	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	2097	811	Tet	Tetracycline resistance (*Bacillus*)
	Gene	3391	2387	repU	Gene of replication protein RepU
	Origin of replication	3543	3569	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	4969	3845	xylR	Xylose repressor gene
	Promoter	5115	5149	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	5151	5179	xylR BS	xylR binding-site
	Region	5220	5229	Opt. RBS	Optimized ribosomal binding site

7.9. Vector map of pN-STREP-TEV1623hp (#BMEG38)



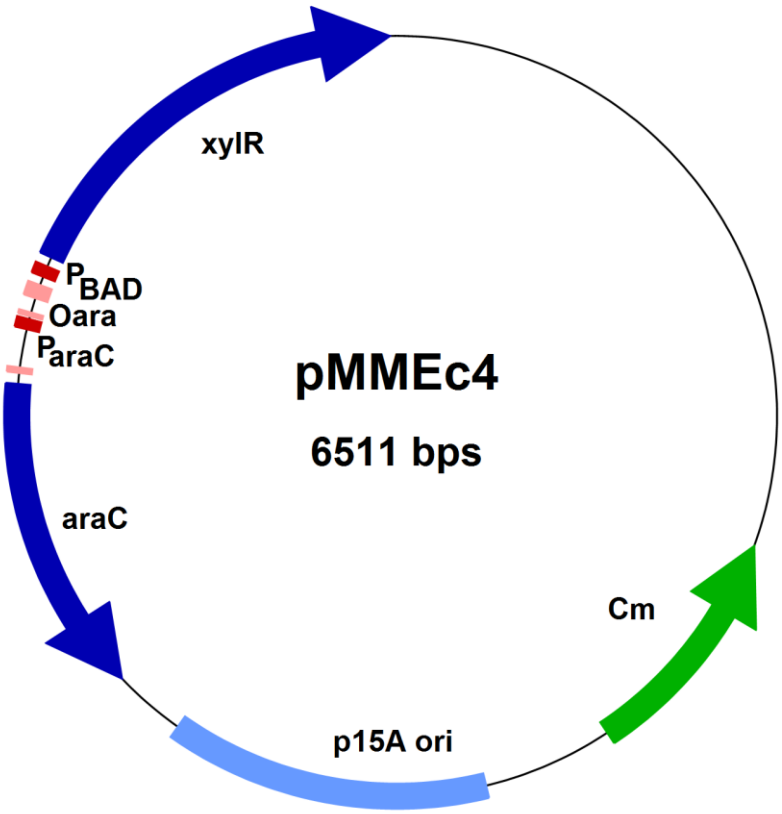
	Type	Start	End	Name	Description
	Selectable genetic marker	1408	551	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	2963	1677	Tet	Tetracycline resistance (*Bacillus*)
	Gene	4257	3253	repU	Gene of replication protein RepU
	Origin of replication	4409	4435	pBC16 ori	Origin of replication (*Bacillus*)
	Gene	5835	4711	xylR	Xylose repressor gene
	Promoter	5981	6015	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	6017	6045	xylR BS	xylR binding-site
	Region	6086	6095	Opt. RBS	Optimized ribosomal binding site
	Tag	6124	6147	Strep-Tag	Streptavidin tag II
	Cleavage site	6157	6177	TEV	TEV cleavage site
	MCS	6182	6225	MCS	Multiple Cloning Site
	Region	6226	6228	Stop	Stop Codon
	Origin of replication	6320	388	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

8.1. Vector map of pMGBM19 (#BMEG39)



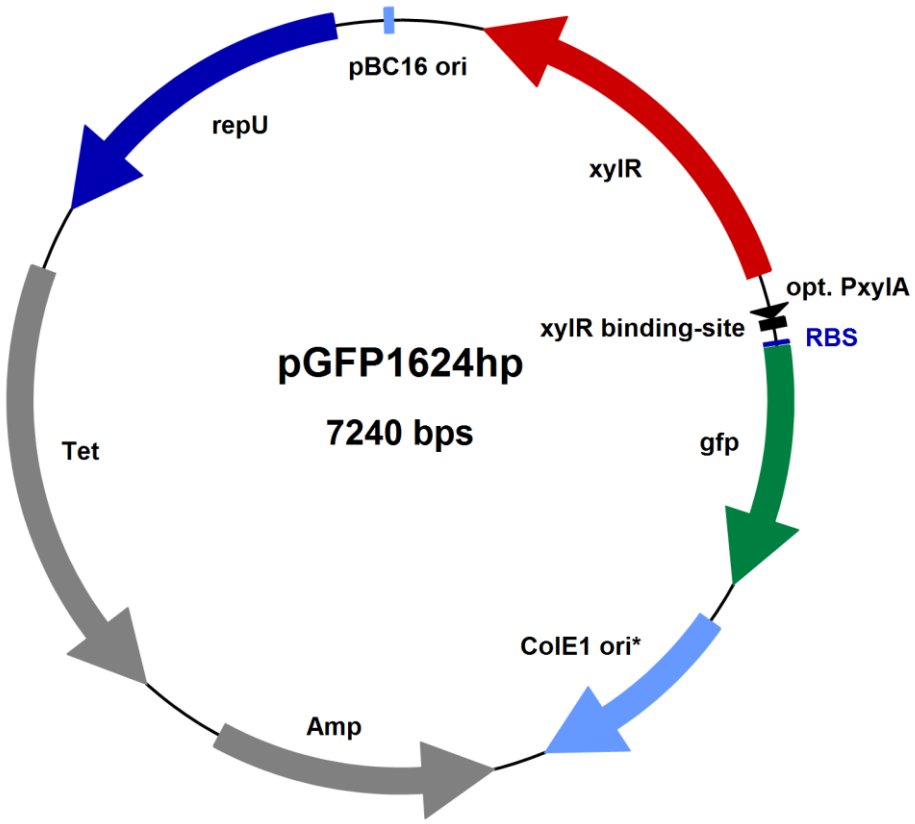
	Type	Start	End	Name	Description
	Origin of replication	922	1576	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group
	Selectable genetic marker	2598	1741	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	3733	3086	Cm	Chloramphenicol resistance (*Bacillus*)
	Gene	5299	4136	xylR	Xylose repressor gene
	Promoter	5403	5436	PxylA	Xylose-inducible promoter
	MCS	5525	5567	MCS	Multiple Cloning Site
	Region	5700	5702	Stop	Stop Codon
	Origin of replication	5808	535	pMB100 replicon	Origin of replication (*Bacillus*)

7.10. Vector map of helper plasmid pMMEc4 (#PEC04)



	Type	Start	End	Name	Description
	Selectable genetic marker	2648	1989	Cm	Chloramphenicol resistance (*Bacillus*)
	Origin of replication	3010	3892	p15A ori	Origin of replication (*E. coli*)
	Gene	4971	4093	araC	Gene of AraC regulatory protein
	Operator	5001	5016	Oara	araO2, operator sequence
	Promoter	5122	5150	ParaC	Arabinose inducible promoter
	Operator	5158	5169	Oara	araO1, operator sequence
	Operator	5210	5248	Oara	araI1/2, operator sequence
	Promoter	5272	5302	pBAD	Arabinose inducible promoter
	Gene	5329	6492	xylR	Xylose repressor gene

7.11. Map of the control vector pGFP1624hp (#BMEG40C)



	Type	Start	End	Name	Description
	Gene	1421	255	xylR	Xylose repressor gene
	Promoter	1525	1559	Opt. PxylA	Xylose-inducible promoter including an optimized -35 region
	Region	1561	1589	xylR BS	xylR binding-site
	Region	1630	1639	Opt. RBS	Optimized ribosomal binding site
	Gene	1647	2390	gfp	Green fluorescent protein (Gfp)
	Origin of replication	2523	3169	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group
	Selectable genetic marker	4191	3334	Amp	Ampicillin resistance (*E. coli*)
	Selectable genetic marker	5836	4463	Tet	Tetracycline resistance (*Bacillus*)
	Gene	7040	6039	repU	Gene of replication protein RepU
	Origin of replication	7192	7218	pBC16 ori	Origin of replication (*Bacillus*)

8. Order Information, Shipping, and Storage

Order #	Description	Amount
BMEG30	*Bacillus megaterium* vector p3STOP1623hp, lyophilized	10 µg
BMEG31	*Bacillus megaterium* vector pC-HIS1623hp, lyophilized	10 µg
BMEG32	*Bacillus megaterium* vector pN-HIS-TEV1623hp, lyophilized	10 µg
BMEG33	*Bacillus megaterium* vector pSP_{LipA}-hp, lyophilized	10 µg
BMEG34	*Bacillus megaterium* vector pSP_{YocH}-hp, lyophilized	10 µg
BMEG35	*Bacillus megaterium* vector p3STOP1623-2RBShp, lyophilized	10 µg
BMEG36	*Bacillus megaterium* vector pC-STREP1623hp, lyophilized	10 µg
BMEG37	*Bacillus megaterium* vector pN-STREP-Xa1623hp, lyophilized	10 µg
BMEG38	*Bacillus megaterium* vector pN-STREP-TEV1623hp, lyophilized	10 µg
BMEG39	*Bacillus megaterium* vector, pMGBm19, lyophilized DNA	10 µg
BMEG40C	pGFP1624hp, high performance GFP expression vector, positive control	10 µg
PEC04	*Escherichia coli* P_{xylA} repressing vector, pMMEc4, lyophilized DNA	10 µg
Shipped at RT, store lyophilized vectors at 4 °C and reconstituted vectors at -20 °C Vectors are *E. coli*/ *B. megaterium* shuttle vectors.		

Related Products

Order #	Description	Amount
BMEG02	*Bacillus megaterium* protoplast, strain WH320	5 x 500 µl
BMEG04	*Bacillus megaterium* protoplast, strain YYBm1	5 x 500 µl
BMEG50	*Bacillus megaterium* protoplast, strain MS941	5 x 500 µl
PR-ETA10010-01	MobiTEV Protease, recombinant, His-Tag	1000 U
PR-ETA10010-05	MobiTEV Protease, recombinant, His-Tag	10 x 1000 U
Shipped on dry ice, store at -80 °C		
PR-ETA10050-01	TEV Protease(TurboTEV), recombinant, GST- & His-Tag	10 µg (100 U)
PR-ETA10050-02	TEV Protease(TurboTEV), recombinant, GST- & His-Tag	50 µg (500 U)
PR-ETA10050-03	TEV Protease(TurboTEV), recombinant, GST- & His-Tag	100 µg (1000 U)
PR-ETA10050-04	TEV Protease(TurboTEV), recombinant, GST- & His-Tag	1 mg (10,000 U)
GE-NUC10700-01	HS-Nuclease, recombinant Endonuclease (encoded by the same gene as Benzonase®)	50000 U
Shipped on blue ice, store at -20 °C		
For His-Tag Purification		
PR-HTK004	MoBiTec Ni-IDA Columns	4 columns
PR-HTK010	MoBiTec Ni-IDA Columns	10 columns
PR-HTK105	MobiSpin Ni-IDA Columns	5 columns
PR-HTK110	MobiSpin Ni-IDA Columns	10 columns
shipped at RT, store columns at RT		

9. Contact and Support

MoBiTec GmbH ◆ Lotzestrasse 22a ◆ D-37083 Goettingen ◆ Germany

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fax: +49 (0)551 707 22 22
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Version: 10/2017