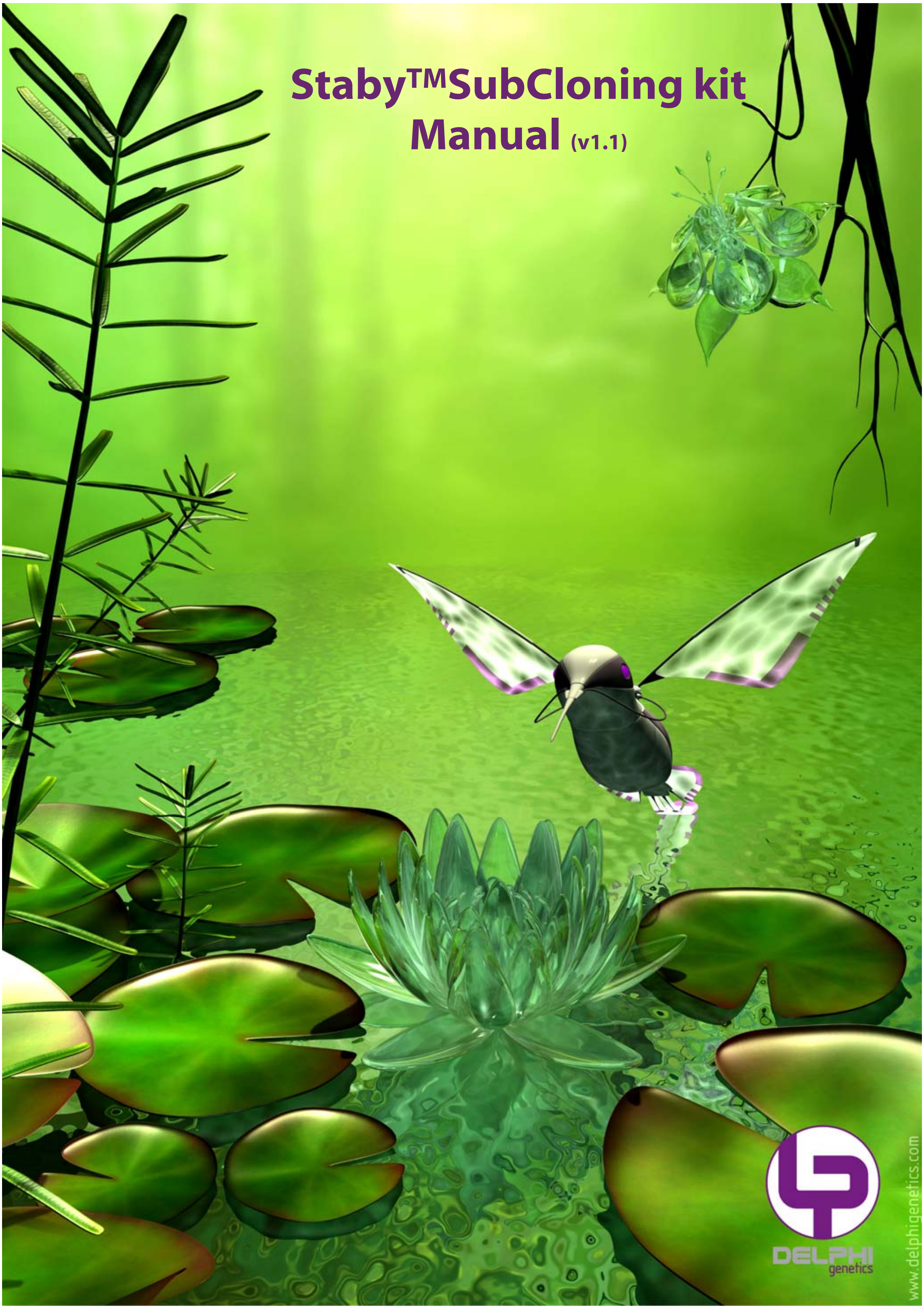
Staby™SubCloning kit Manual (v1.1)



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Content and storage :

The Staby™SubCloning expression kit is shipped on **dry ice**.

Storage: -80°C

Two different types of Staby™SubCloning kits are available: one containing electrocompetent cells (in non self-standing tubes), and the other type containing chemically-competent cells (in self standing tubes).

Each kit contains one box with the following items:

Name	Concentration / remarks	Amount (SSC1-10, SSC1-12)	Amount (SSC2-10, SSC2-12)
pSSC-Native1 DNA	0.1µg/µl	1 tube of 50µl	
pSSC-His1 DNA	0.1µg/µl	1 tube of 50µl	
pSSC-Cherry1 DNA	0.1µg/µl	1 tube of 50µl	
pSSC-Native2 DNA	0.1µg/µl		1 tube of 50µl
pSSC-His2 DNA	0.1µg/µl		1 tube of 50µl
pSSC-Cherry2 DNA	0.1µg/µl		1 tube of 50µl
CYS21 strain (for cloning) Pink cap	Competent cells	10 tubes	10 tubes
SE1 strain (for expression) Blue cap	Competent cells	10 tubes	10 tubes
Staby reverse primer Red cap	0.1µg/µl in water 5'-CCAACTCAGCTTCCTTTCG-3'	1 tube of 20µl	1 tube of 20µl
Staby forward primer Red cap	0.1µg/µl in water 5'-GCGTCCGGCGTAGAGGATC-3'	1 tube of 20µl	1 tube of 20µl
Cherry™ booster Orange cap	δ-amino levulinic acid, 1M in water	1 tube of 200µl	1 tube of 200µl
Expression control	SE1 bacteria carrying a plasmid with an insert encoding a His-tagged protein of 70kDa	1tube: glycerol stock	1 tube: glycerol stock
Regeneration medium White cap	2% Tryptone 0.5% Yeast extract 0.05% NaCl 2.5mM KCl 10mM $MgCl_2$	10 tubes of 1.5ml	10 tubes of 1.5 ml
Manual		1	1

The genotype of the CYS21 strain is: F^-, Cm^R, *mcr*A, *end*A1, Δ(*mrr-hsd*RMS-*mcr*BC) (restriction-, modification-), Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, Δ(*ara*, *leu*)7697, *ara*D139, *galU*, *galK*, *nup*G, *rpsl*, *ccdB*$^+$.

The genotype of the SE1 strain is: derivates from *E. coli* B strain, F^-, Cm^R, *ompT*, *lon*, *hsdS*$_B$ (restriction$^-$, modification$^-$), *gal, dcm,* DE3 (*lacI*, T7polymerase under the control of the PlacUV5 promoter), *ccdB*$^+$.

Material Safety Data Sheet:

Product and company identification:

Delphi Genetics SA
Rue C. Ader, 16
B-6041 Charleroi, Belgium
Tel: +32.71.37.85.25
Fax: +32.71.37.60.57
e-mail: delphigenetics@delphigenetics.com

Hazards identification

No specific hazard concerning the products of the StabySubCloning kit.

First aid measures

- Inhalation: If one of the products of the StabySubCloning kit is inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.
- Ingestion: Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of the products of the StabySubCloning kit are swallowed, call a physician immediately.
- Skin contact: In case of contact, immediately flush skin with plenty of water. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.
- Eye contact: In case of contact with one of the products of the StabySubCloning kit, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Fire-fighting measures

Use foam or all purpose dry chemicals to extinguish. Fire fighters should wear positive self-contained breathing apparatus and full turnout gear.

Accidental release measures

Immediately contact emergency personnel. Use suitable protective equipment (see below exposure controls and personal protection). For small spills add absorbent, scoop up material and place in a sealed, liquid-proof container for disposal. For large spills dike spilled material or otherwise contain material to ensure runoff does not reach a waterway. Place spilled material in an appropriate container for disposal. Minimize contact of spilled material with soils to prevent runoff to surface waterways.

Handling and storing

Keep the container tightly closed, in a cool and well-ventilated area.

Personal protection

The occupational exposure limits were not determined. Protect your skin and body using uniform or laboratory coat, chemical resistant, impervious gloves. Use safety glasses, face shield or other full-face protection if potential exists for direct exposure to aerosols or splashes.

Disposal consideration

Waste must be disposed of in accordance with federal, state and local environmental control regulations.

N.B.: Final determination of suitability of any material is the sole responsibility of the user. All materials may present unknown hazards and should be used with caution. To the best of our knowledge, the information contained herein is accurate. However, neither Delphi Genetics SA nor any of its subsidiaries assumes any liability whatsoever for the accuracy or completeness of the information contained herein.

Licenses

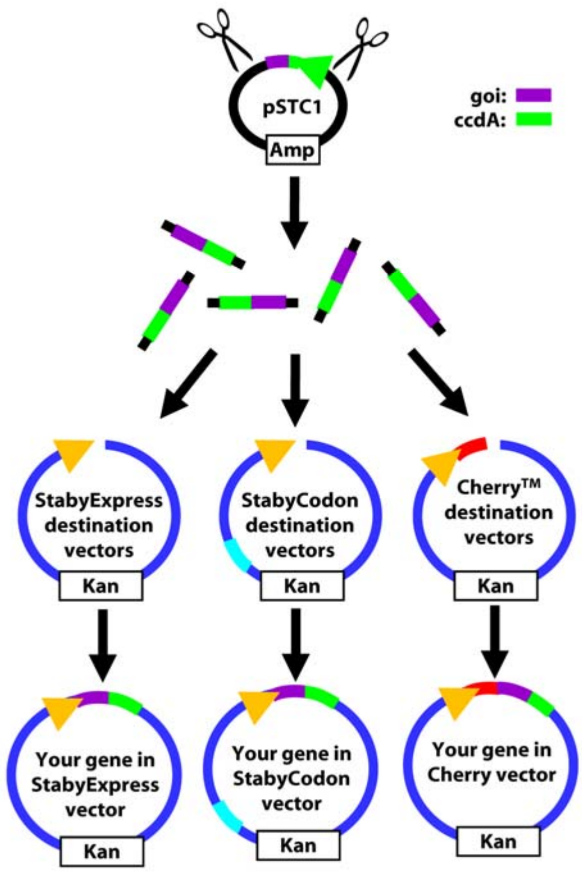
The Staby™SubCloning expression kit is covered by worldwide patents (US7183097, US7595186, US7595185 and other patents pending). The kit is sold under a license from the Université Libre de Bruxelles (Belgium). **The kit is sold for research purpose only**. A license from Delphi Genetics SA is required for any commercial use.
(Please, contact Delphi Genetics at delphigenetics@delphigenetics.com)

T7 expression kit is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is the subject of patent applications assigned to Brookhaven Science Associate (BSA) in the United States of America.

User Guide

Overview of the Staby™SubCloning system

The StabySubCloning kit is designed to transfer easily the genes cloned first with StabyCloning™ into expression vectors encoding all advantages of the Staby™ technology.
The Staby™SubCloning system is based on the use of CYS21 and SE1 bacterial strains. These bacteria contain a natural bacterial poison gene encoding the poison protein CcdB in their chromosomes. The CcdB protein is only toxic for bacteria and is counteracted by the antidote protein CcdA. The presence of CcdA is required to allow bacterial survival. During PCR cloning using the StabyCloning™ vector (pSTC1 vectors), the gene of interest is oriented and linked to the ccdA antidote gene (for more information, please, consult the StabyCloning™ section of our website www.delphigenetics.com). Using restriction sites in the pSTC1 vector, it is easy to transfer the gene of interest (linked to the ccdA antidote gene) into a sub-cloning vector. The ccdA presence linked to your gene allows selection of recombinants in CYS21 or SE1 bacteria. The restriction-sites choice allows selection of insert orientation. After sub-cloning, the vector is ready for protein expression in SE1 bacteria and the presence of the ccdA antidote gene on the plasmid will allow a perfect plasmid stabilization even without antibiotics (see

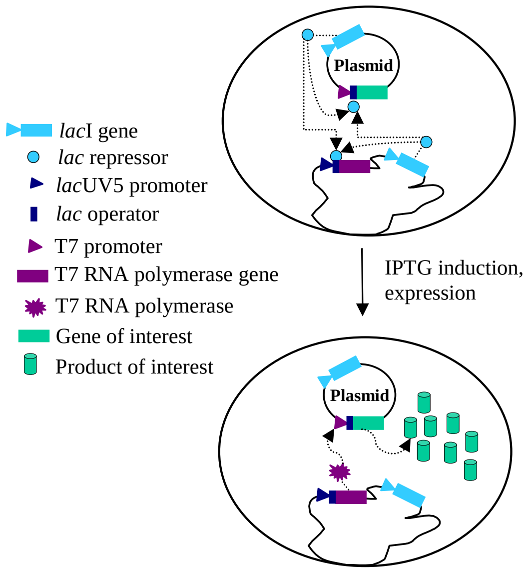


below, overview of the stabilization system).
The restriction sites of the StabySubCloning vectors were chosen to be compatible with sites of pSTC1 vector and to place your gene in the correct orientation and in frame with the protein tags. Several sub-cloning vectors were developed for each application (protein expression with his-tag, no tag, Cherry™ tag,…). All destination vectors contain the same compatible restriction sites. Consequently, one restriction of the pSTC1 vector is compatible with all StabySubCloning vectors.
The StabySubCloning vectors for protein expression in bacteria (GE-SSC1-10, GE-SSC1-12, GE-SSC2-10 and GE-SSC2-12) contain the T7 promoter to reach high yield of proteins.

Overview of the T7 expression system

The T7 expression system is based on the use of the T7 bacteriophage promoter and RNA polymerase. The T7 RNA polymerase is useful for synthesizing large amounts of RNA selectively: the T7 RNA polymerase only recognizes the T7 promoter and not the *E. coli* promoters. Conversely, the *E. coli* RNA polymerase does not recognize the T7 promoter (see below). The T7 RNA polymerase is able to transcribe genes five times faster than the *E. coli* RNA polymerase (Chamberlin and Ring, 1973; Golomb and Chamberlin, 1974). The gene encoding the T7 RNA polymerase is inserted into the chromosome of the expression bacteria (SE1, figure 1). The expression of this gene is under the control of the *lacUV5* promoter and therefore is basically controlled by the same mechanisms as the *lac* operon. Thus, the expression of the T7 RNA polymerase is repressed by the binding of the *lac* repressor (encoded by the *lacI* gene) to the *lacO* operator sequence. The gene encoding the repressor is present in the bacterial chromosome and also in the StabySubCloning vectors containing the T7 promoter to ensure high amount of repressor molecules. Consequently, in normal conditions, the T7 RNA polymerase is not or very weakly expressed. An additional repression of the *lac* promoter can be obtained using medium containing glucose (Grossman et al., 1998). The presence of glucose in the medium (especially in the stationary phase) induces the metabolic repression: the bacteria will first use glucose as a carbon source and will reduce the concentration of cyclic AMP, ensuring a better repression of the *lac* promoter (cyclic AMP stimulates the *lac* and *lacUV5* promoters). Moreover, Studier et al. (1990) have shown that a better regulation of the expression of the gene of interest is obtained by adding the *lacO* operator sequence between the T7 promoter and the beginning of the gene of interest. This sequence is present in the StabySubCloning vectors. Consequently, the *lac* repressor will also repress the expression of the gene of interest.

Figure 1: The T7 expression system used in the SE1 strain



Adding isopropyl-β-D-thiogalactoside (IPTG) to the medium will induce the expression of (i) the T7 RNA polymerase and of (ii) the gene of interest by removing the *lac* repressor bound to the *lacO* sequence (figure 1).
A powerful feature of the T7 expression system is the ability to clone the gene of interest under conditions of extremely low or no transcriptional activity, that is, in the absence of the T7 RNA polymerase (as the CYS21 genetic background). The expression of the gene of interest is minimal in the absence of the T7 RNA polymerase because this gene is under the control of the T7 promoter which is only recognized by the T7 RNA polymerase and not by the *E. coli* RNA polymerase. If the target gene is cloned directly into the expression strain, even a low basal expression of the T7 RNA polymerase can interfere with growth and selection of the right construct. After the cloning step into a cloning strain lacking the T7 RNA polymerase (CYS21), the plasmid construct is transferred into the expression strain encoding the T7 RNA polymerase (SE1) to produce the protein of interest.

Expression of heterologous genes in *E. coli*:

In all organisms, most amino acids are encoded by more than one codon: 61 codons are available for 20 amino acids. But each organism is characterized by a specific "codon bias" (see table below), *i.e.* it preferentially uses some codons over others. In practice, when a heterologous gene is expressed in *E. coli*, this gene might exhibit some codons that are common in the original host but are rarely used in *E. coli*. Whereas, the presence of only a small number of rare codons might not severely depress target protein synthesis, the presence of clusters of and/or numerous rare codons generates a demand for one or more rare tRNAs. In turn, the rarity of some tRNAs leads to very low expression of the target protein due to premature translation termination, translation frameshifting, amino acid misincorporation, growth inhibition and plasmid instability. Six rare codons can cause problems in *E. coli* B (*e.g.;* BL21(DE3) or SE1): AGG and AGA (both encoding arginine using the *argU* tRNA), AUA (isoleucine, *ileX* tRNA), CUA (leucine, *leuW* tRNA), GGA (glycine, *glyT* tRNA), and CCC (proline, *proL* tRNA). An analysis of your gene-of-interest can be performed using **Staby™Soft**. In the **Staby™SubCloning kit**, we solve the problem by the use of vectors encoding the tRNA genes of the six rare codons (SSC2-10 and SSC2-12 kits). Hence, the vectors contain the T7 promoter for a strong expression, the *ccdA* gene for plasmid stabilization and supply the rare tRNAs.

Overview of the Cherry™ tag system:

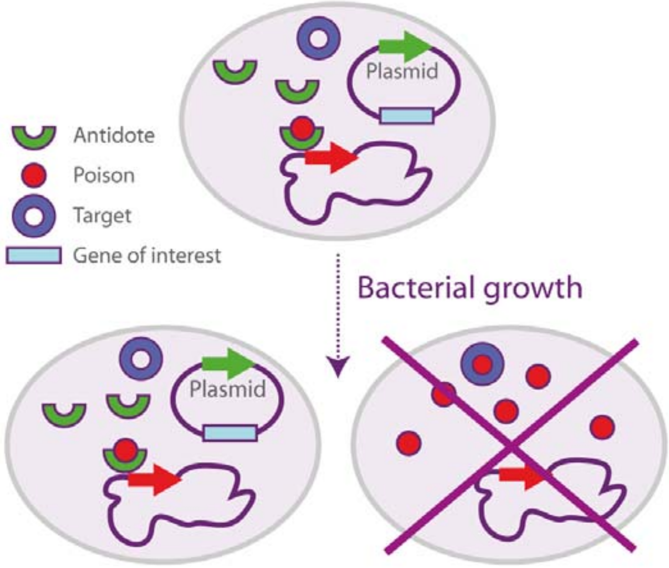
When using the Cherry™ vectors (pSSC-Cherry1 or pSSC-Cherry2), your gene of interest is fused to a small sequence encoding a red polypeptide (heme binding part of cytochrome, 11 kDa) providing a visual aid for estimating expression level and solubility: bacteria expressing the fusion protein are red when the fused protein is soluble. As lack of solubility is a major problem when expressing recombinant protein in *E. coli*, the Cherry™ vectors are convenient for rapid screening and optimization of protein solubility. The tag itself being highly soluble, it can increase the solubility of the target protein. The red color also constitutes a **visual marker** throughout protein purification: it is easy to visualize binding of the protein to the column (affinity or ion exchange) and to verify the absence of remaining protein of interest in the effluent. During elution, it is not necessary to collect multiple fractions and to analyse it to localize the target protein. Indeed, the Cherry™ tag allows you to collect only the fraction containing the protein of interest. When using the Cherry™ tag, it is possible to quantify the protein concentration at any step (from protein production to the end of purification): a simple absorbance measurement at **413nm** allows specific and accurate calculation of the target protein concentration. When using the pSSC-Cherry1 or pSSC-Cherry2 vectors, the tag can be cleaved after purification using enterokinase (a recognition site is inserted at the C-terminal end of the tag sequence).

Overview of the stabilization system:

Higher plasmid stability= More proteins

Principle: In the StabySubCloning vectors, the antidote gene (*ccdA*) is under the control of a constitutive promoter. On the other hand, the toxic gene (*ccdB*) is introduced in the chromosome of the bacteria (cf. fig. below). Expression of the poison gene is under the control of a promoter strongly repressed in the presence of the plasmid. When the plasmid is lost, the antidote is degraded and the production of the toxin is induced, causing cell death.

Figure 2: Principle of the stabilization system



Practically this means that when during the pre-induction phase bacteria are grown, 100% of the bacteria will carry the vector. If they lose the vector, they will not obtain a growth advantage, but will die. **Upon induction 100% of the bacteria will start producing the recombinant protein leading to higher yields of the target protein and less background caused by unwanted proteins**.

For manufacturers of recombinant proteins this system offers a great benefit because it is an antibiotic free expression system. Therefore the manufactured protein will also be free of traces of antibiotics.

Benefits of the Staby™ system for plasmid stabilization:

- The production of the protein of interest is 3 to 5 times higher;
- Plasmid are perfectly stabilised without the use of antibiotics;
- Reduced background of "parasite proteins";
- The promoters typically used in protein production remain available;
- System is usable in any culture medium

Experimental outline: Easy 4 steps procedure.

1. Cloning of your gene of interest in the StabySubCloning vectors

↓

2. Transformation into the CYS21 *E. coli* cells and selection of the desired construction

↓

3. Transformation of your plasmid DNA into the SE1 *E. coli* competent cells

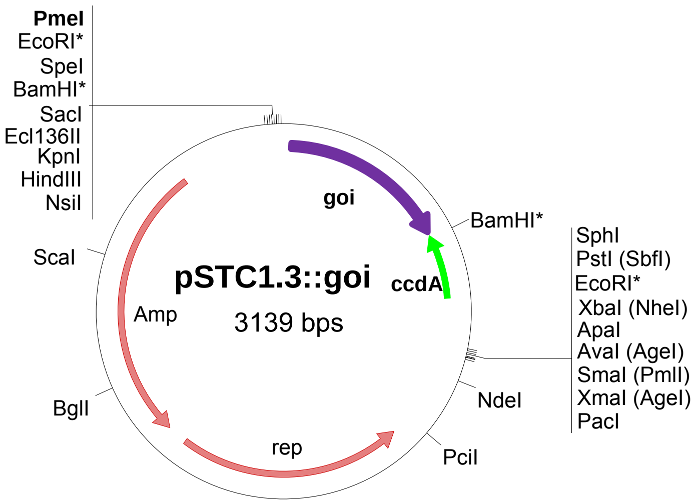
↓

4. Expression of your gene of interest with or without antibiotic

Step 1: Sub-cloning of your gene of interest in the StabySubCloning vectors:

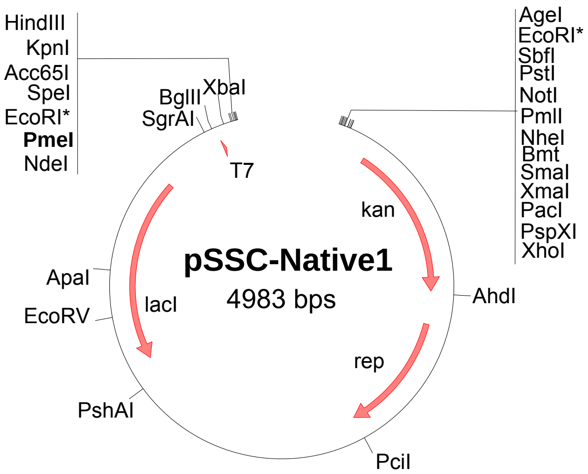
Many strategies can be used for cloning your gene of interest (goi) into the StabySubCloning vectors. The most convenient strategy is to use restriction enzymes: the single-cutter enzymes from the multiple cloning sites are indicated on the maps below. It is important to always check that your gene of interest does not contain the restriction sites chosen for sub-cloning.
If your gene of interest (goi) was cloned in pSTC1.3 starting from ATG start codon to stop codon, **PmeI** (which is a very rare restriction site) allows in frame sub-cloning into the StabySubCloning vectors. If your gene was not cloned in pSTC1.3 vector starting with the ATG start codon, you must take into account the reading frame when choosing the restriction sites for subcloning. Use of PmeI will add only four amino acids to the N-terminal end of your protein. However, **if you need to avoid any fusion** of amino acid at the N-terminal end of your protein, you can use the NdeI restriction site. To do it, first, check that your gene does not contain any NdeI restriction site. Next, amplify it by PCR and add the "CAT" bases to the forward primer 5' end (just before the ATG start codon) to create the NdeI recognition sequence ("CATATG"). Clone your gene into the pSTC1.3 vector and next, you can use NdeI restriction enzyme to subclone your gene into StabySubCloning vectors without any fusion.
Use the buffer and incubation conditions provided by the restriction enzyme manufacturer. After restriction, the DNA fragment encoding the gene of interest is inserted by ligation using compatible ends. You will find below plasmid maps and enzyme couples usable to transfer your gene of interest from StabyCloning vector (pSTC1) to destination vectors of the StabySubCloning system (pSSC vectors).

Map of pSTC1.3 containing a gene of interest (goi) to be transferred into StabySubCloning vectors.



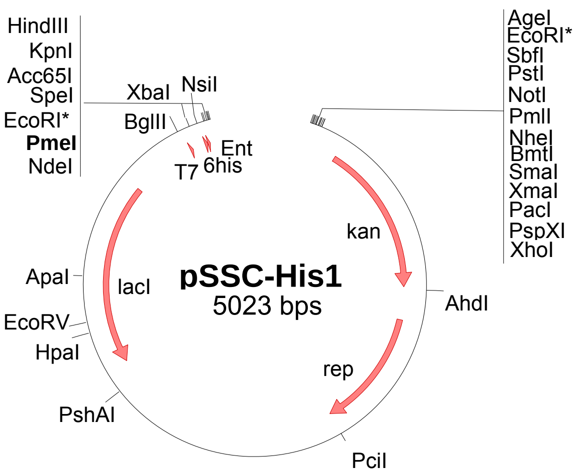
Usable restriction sites are indicated. Brackets contain compatible restriction sites present in StabySubCloning vectors.
*: double-cutter enzyme

Maps of Staby™ Sub-Cloning vectors provided in SSC1-10 and SCC1-12 kits :



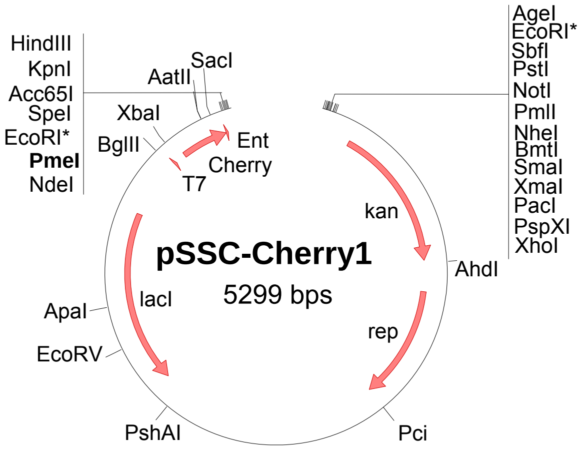
Features: T7 promoter, no tag, Kan resistance, lacI repressor to reduce basal expression, plasmid stabilization for high yields in CYS21 and SE1 bacteria after sub-cloning.

The complete vector sequence is available on our website (www.delphigenetics.com)



Features: T7 promoter, His tag, Enterokinase cleavage site, Kan resistance, lacI repressor to reduce basal expression, plasmid stabilization in CYS21 and SE1 bacteria after sub-cloning.

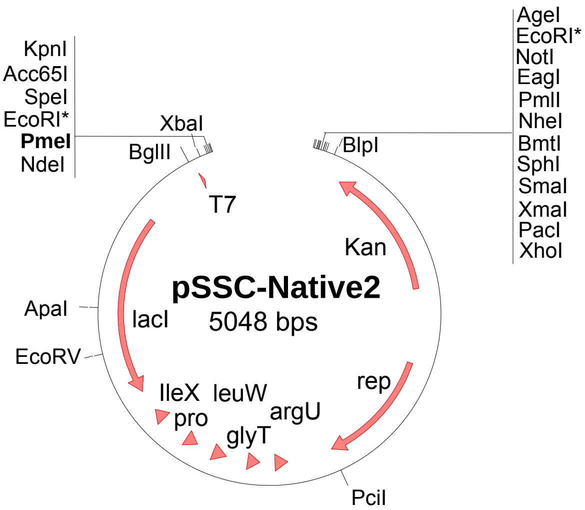
The complete vector sequence is available on our website (www.delphigenetics.com)



Features: T7 promoter, Cherry tag, Enterokinase cleavage site, Kan resistance, lacI repressor to reduce basal expression, plasmid stabilization in CYS21 and SE1 bacteria after sub-cloning.

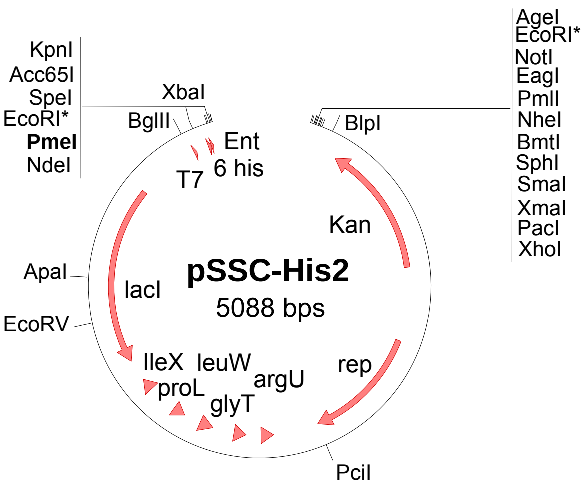
The complete vector sequence is available on our website (www.delphigenetics.com)

Maps of Staby™ Sub-Cloning vectors provided in SSC2-10 and SCC2-12 kits :



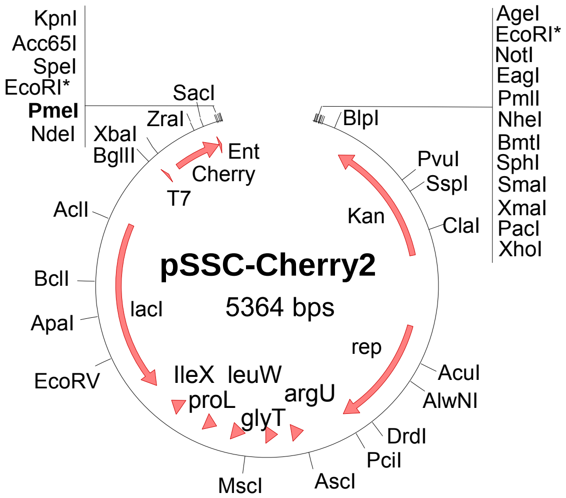
Features: T7 promoter, no tag, additional t-RNAs, Kan resistance, lacI repressor to reduce basal expression, plasmid stabilization in CYS21 and SE1 bacteria after sub-cloning.

The complete vector sequence is available on our website (www.delphigenetics.com)



Features: T7 promoter, His tag, Enterokinase cleavage site, additional t-RNAs, Kan resistance, lacI repressor to reduce basal expression, plasmid stabilization in CYS21 and SE1 bacteria after sub-cloning.

The complete vector sequence is available on our website (www.delphigenetics.com)



Features: T7 promoter, Cherry tag, Enterokinase cleavage site, additional t-RNAs, Kan resistance, lacI repressor to reduce basal expression, plasmid stabilization in CYS21 and SE1 bacteria after sub-cloning.

The complete vector sequence is available on our website (www.delphigenetics.com)

Examples of compatible restriction sites:

Enzymes used to cut pSTC1.3 containing the gene of interest		**Enzymes used to cut StabySubCloning vector**		**Comments**
PmeI	PacI	PmeI	PacI	Gene cloned from ATG to stop codon in pSTC1.3; automatically in frame in StabySubCloning vectors
PmeI	XbaI	PmeI	NheI	XbaI and NheI have compatible ends
SpeI	SmaI	SpeI	PmlI	SmaI and PmlI have compatible ends. Using SpeI, take into account the reading frame in the StabySubCloning vectors

Ligation conditions:

1. Inactivate the restriction enzymes (80°C, 20min) and dialyze restricted DNAs to remove salts (or purify your insert on agarose gel).
   Note: To dialyze your DNA fragments against sterile water, use a 0.025µm filter. Add the sterile water in a Petri dish and carefully place the filter on the water surface. Delicately, put the restriction mix on the filter. Wait 10min, pipet back the DNA mix and use it for ligation.

2. Mix digested DNAs with buffer and ligase in a sterile microcentrifuge tube. It is better to use about 3 times more insert molecules than SSC vector molecules. Use the buffer and incubation conditions provided by the ligase manufacturer. We recommend to fully thaw (and mix) the ligase buffer before use.
   After ligation proceed to transformation (see step 2 below).

Important:
Delphi Genetics can help you for cloning and sub-cloning or for optimization of the nucleotide sequence of your gene-of-interest for a best protein production (please contact us at delphigenetics@delphigenetics.com).

Step 2. Transformation into the CYS21 strain and selection of the desired construction:

Selection of the desired construction is performed in CYS21 *E. coli* cells lacking the T7 RNA polymerase gene (*). These cells contain the *ccdB* gene in their chromosome.

This enables:

- *(i)* High efficiency of transformation (the transformation efficiency of SE1 is lower than that of CYS21),
- *(ii)* Stabilization of plasmids for high DNA production,
- *(iii)* Selection of the desired construction without expression of the gene of interest (goi).

(*)**Remark:**
It is not recommended to clone directly the goi into the expression host containing the T7 RNA polymerase gene: the T7 gene basal expression, and the resulting goi basal expression, would reduce the efficiency of recovery of the desired construction.

Protocol:
Two different types of StabySubCloning kits are available: one containing electrocompetent cells, and the other type containing chemically-competent cells. Generally, electroporation is more efficient than chemical transformation.

a) Transformation by electroporation:

1) Prepare LB plates containing 50 to 100 µg/ml Kanamycin. Let the plates dry and then warm them at 37°C.
2) Set up your electroporator for bacterial transformation. Use the manufacturer's instructions. Classically, electroporation conditions are: 2,5 kV, 25 µF, and 200 Ohms.
3) For each cloning reaction, place one vial of the **CYS21** electrocompetent cells (pink cap) and one electroporation cuvette on ice. Allow the cells to thaw on ice for 5-10 minutes.
4) Add 1 or 2 µl of the ligation to the vial of the **CYS21** electrocompetent cells (pink cap). Stir gently to mix. Do not mix by pipetting up and down.

   If you wish to use more than 2µl of the ligation mix, it is recommended to dialyze it against sterile water using a 0.025µm filter. Add the sterile water in a Petri dish and carefully place the filter on the water surface. Delicately, put the ligation mix on the filter. Wait 10min, pipet back the ligation mix and add the dialyzed solution to the electrocompetent cells.

5) Transfer all the content of the tube (cells+DNA) to the pre-chilled electroporation cuvette.
6) Electroporate the cells according to the manufacturer's instructions.
7) Quickly add 500µl of the regeneration medium (white cap) at room temperature and mix well. Incubate the mixture at **37°C** during **1 hour**.

8) Spread 20 to 150µl on pre-warmed plates containing kanamycin.
9) Incubate the plates overnight at 37°C.
10) Pick about 10 colonies and culture them overnight in 10ml of LB medium with or without kanamycin (50 to 100 µg/ml).

    Note: The stabilization is now effective; the ccdB gene is activated. Consequently, the plasmid is stabilized in the CYS21 strain and no antibiotic is needed to select bacteria containing the plasmid. However, the kanamycin resistance is still available. The stabilization system will insure high yield of plasmid DNA.

11) Extract plasmid DNA and analyze the constructions using your method of choice (restriction, sequencing, …). Sequencing primers (Staby reverse and Staby forward primers) are included in the kit (0.1 µg/µl). The complete sequences of the vectors are available on our website: http://www.delphigenetics.com.
12) Optional: choose one of the clones containing the desired construct. Mix well 800µl of the culture with 800µl of sterile glycerol and transfer to a cryo-vial. Store at -80°C.

b) Transformation using chemically competent cells:

1. Prepare LB plates containing 50 to100 µg/ml Kanamycin. Let the plates dry and then warm them up at 37°C.
2. Set a water bath or a heating-bloc to 42°C
3. Thaw (bring to room temperature) one vial of regeneration medium (white cap) per cloning reaction.
4. For each cloning reaction, place one vial of the **CYS21** chemically-competent cells (self-standing tube with pink cap) on ice. Allow the cells to thaw on ice for 5-10 minutes.
5. Add 5 µl of the ligation product to one vial of the **CYS21** chemically competent cells (self-standing tube with pink cap). Stir gently to mix. Do not mix by pipetting up and down.
6. Incubate on ice for 30 minutes.
7. Heat-shock the bacteria by placing the vial at 42°C for 30 seconds without shaking.
8. Immediately transfer the tubes to ice.
9. Add 250µl of room-temperature regeneration medium (white cap) and mix well. Incubate at **37°C** during **1 hour**.
10. Spread 20 to 150µl of the product (from step 9) on pre-warmed plates containing kanamycin.
11. Incubate the plates overnight at 37°C.
12. Pick about 10 colonies and culture them overnight in 10ml of LB medium with or without Kanamycin (50 to 100 µg/ml).

Note: The stabilization is now effective; the ccdB gene is activated. Consequently, the plasmid is stabilized in the CYS21 strain and no antibiotic is needed to select bacteria containing the plasmid. However, the kanamycin resistance is still available. The stabilization system will insure high yield of plasmid DNA.

13. Extract plasmid DNA and analyze the constructions using your method of choice (restriction, sequencing, …). Sequencing primers (Staby reverse and Staby forward primers) are included in the kit (0.1 µg/µl). The complete sequences of the StabySubCloning vectors are available on our website: http://www.delphigenetics.com
14. Optional: choose one of the clones containing the desired construct. Mix well 800µl of the culture with 800µl of sterile glycerol and transfer to a cryo-vial. Store at -80°C.

Step 3. Transformation in the expression host (SE1):

a) Transformation by electroporation:

1) Prepare LB plates containing 50 to 100 µg/ml Kanamycin. Let the plates dry and then warm them at 37°C.

   Note: Addition of 1% glucose (from a sterile filtered 20% stock solution) in the plates can be useful to better repress the promoter and to avoid basal expression.

2) Set up your electroporator for bacterial transformation. Use the manufacturer's instructions. Classically, electroporation conditions are: 2,5 kV, 25 µF, and 200 Ohms.
3) For each transformation, place one vial of the **SE1** electrocompetent cells (blue cap) and one electroporation cuvette on ice. Allow the cells to thaw on ice for 5-10 minutes.
4) Add 1µl of the selected plasmid DNA (steps 11 and 12 above) to the **SE1** cells and mix gently.
5) Transfer all the content of the tube (cells+DNA) to the pre-chilled electroporation cuvette.
6) Electroporate the cells according to the manufacturer's instructions.
7) Quickly add 500µl of the regeneration medium (white cap) at room temperature and mix well. Incubate at 37°C during 1 hour.
8) Spread 20 to 150µl on the pre-warmed LB plates.
9) Incubate the plates overnight at 37°C.
10) Optional: Pick about 5 colonies and culture them overnight in 10ml of LB medium.

    Note: The plasmid is now stabilized in the SE1 strain using the StabyExpress™ system, no antibiotic is needed to select bacteria containing the plasmid. However, the kanamycin resistance gene is still available. Addition of 1% glucose (from a sterile filtered 20% stock solution) in the medium can be useful to better repress the promoter and to avoid basal expression.

11) Optional: Extract plasmid DNA and analyze the constructions using your method of choice (restriction, sequencing, …). Sequencing primers are included in the kit (0.1 µg/µl). The complete sequences of the StabySubCloning vectors are available on our website: http://www.delphigenetics.com

12) Optional: Select one of the clones containing the desired construction. Mix well 800µl of the culture with 800µl of sterile glycerol and transfer to a cryovial. Store at -80°C.

<u>b) Transformation using chemically competent cells:</u>

1. Prepare LB plates containing 50 to 100 µg/ml Kanamycin. Let the plates dry and then warm them up at 37°C.

   Note: Addition of 1% glucose (from a sterile filtered 20% stock solution) in the plates can be useful to better repress the promoter and to avoid undesirable expression.

2. Set a water bath or a heating-bloc to 42°C
3. Thaw (bring to room temperature) one vial of regeneration medium (white cap) per cloning reaction.
4. For each transformation, place one vial of the **SE1** chemically-competent cells (self-standing tube with bue cap) on ice. Allow the cells to thaw on ice for 5-10 minutes.
5. Add 1µl or 2µl of the selected plasmid DNA (steps 11 and 12 above) to one vial of the **SE1** chemically competent cells (self-standing tube with blue cap). Stir gently to mix. Do not mix by pipetting up and down.
6. Incubate on ice for 30 minutes.
7. Heat-shock the bacteria by placing the vial at 42°C for 30 seconds without shaking.
8. Immediately transfer the tubes to ice.
9. Add 250µl of room-temperature regeneration medium (white cap) and mix well. Incubate at 37°C during 1 hour.
10. Spread 20 to 150µl of the product (from step 9) on pre-warmed plates.
11. Incubate the plates overnight at 37°C.
12. Optional: Pick about 5 colonies and culture them overnight in 10ml of LB medium.

    Note: The plasmid is now stabilized in the SE1 strain using the StabyExpress™ system, no antibiotic is needed to select bacteria containing the plasmid. However, the kanamycin resistance gene is still available. Addition of 1% glucose (from a sterile filtered 20% stock solution) in the medium can be useful to better repress the promoter and to avoid basal expression.

13. Optional: Extract plasmid DNA and analyze the constructions using your method of choice (restriction, sequencing, …). Sequencing primers are included in the kit (0.1 µg/µl). The complete sequences of the StabySubCloning vectors are available on our website: http://www.eurogentec.com or http://www.delphigenetics.com
14. Optional: Select one of the clones containing the desired construction. Mix well 800µl of the culture with 800µl of sterile glycerol and transfer to a cryovial. Store at -80°C.

Step 4. Gene expression and protein extraction:

The T7 RNA polymerase is under the control of the PlacUV5 promoter (Studier and Moffat, 1986; Studier *et al*., 1990). Both the SE1 strain and the StabySubCloning vector carry the *lacI* gene. LacI represses both the expression of the T7 RNA polymerase and the transcription of the gene of interest. Consequently, the expression of the T7 RNA polymerase is inducible by isopropyl-β-D-thiogalactoside (IPTG): addition of IPTG to the culture of the SE1 strain containing the StabySubCloning plasmid will induce the expression of the T7 RNA polymerase which, in turn, will transcribe the gene of interest.
Expression can also be performed using **Staby™Switch medium** (auto-inducible medium without IPTG). Perform the small-scale expression following the manual instructions.
The small-scale protocols below will allow you to verify that the target protein is produced upon induction and to verify for the presence of detection tags in the target protein.
The glycerol stock of the expression positive control (provided in the kit) will allow you to produce his-tagged test protein of 69kDa. Use the same protocol for your gene and for the positive control.

Protocol for a small-scale expression using Staby™Switch auto-inducible medium:

1. Inoculate two containers containing the desired volume of pre-warmed *Staby™Switch* medium with a few microliters (1 or 2 µl / 10ml culture) from a glycerol stock of the SE1 strain containing your construction in the StabySubCloning vector. Alternatively, inoculate containers with a single colony from a plate streaked with this strain.
   For a 10ml culture volume, the use of 50ml tubes with conical bottom (28mm x 114mm) is ideal. The tubes can be maintained closed during all the whole expression experiment. For bigger culture volumes, use Erlenmeyer flasks with a capacity of 5 times the culture volume. For 96 well plates, use 1 single colony or 0.001 volume of a glycerol stock per well.
   Antibiotics are not required but can be used.

Note: When using pSSC-Cherry1 or pSSC-Cherry2 vectors, to enhance bacterial color, add 1mM of Cherry™ booster to the medium. A tube containing 1M Cherry™ booster is provided in the kit (orange cap).

2. Add 1% sterile glucose (from a sterile-filtered 20% stock solution) to one of the two containers. This culture will be used as a non-induced control and/or to prepare a glycerol stock.
3. Incubate the containers at 37°C for approximately 24 hours with shaking (200rpm max, rotary shaker, 2.54cm orbit).
   Note: (1) If your protein is unstable, add 1% lactose (from a sterile-filtered 20% stock solution) 2 hours before the end of the culture.
   (2) It is essential to grow the bacteria to stationary phase for full

induction. If you want to incubate your cultures at lower temperature (<37°C), it is necessary to adapt the incubation time. Continue incubation for several hours (8 to 10 hours) after saturation. The first time, it is recommended to take a sample every hour and to check the protein expression (red colour or SDS-PAGE analysis).

4. After incubation, measure the Optical Density at 600nm for each culture. Transfer 1ml sample of each flask in a microcentrifuge tube. Centrigufe at maximum speed (13000 g) for 10 min (if possible at 4°C).

Protocol for a small-scale expression using IPTG:

1) Inoculate two Erlenmeyer flasks containing 10ml of LB medium with a few microliters from a glycerol stock. Alternatively, pick two single colonies from a plate streaked with the SE1 bacteria containing your construction; inoculate two flasks containing 10ml of LB medium.

Note: When using pSSC-Cherry1 or pSSC-Cherry2 vectors, to enhance bacterial color, add 1mM of Cherry™ booster to the medium. A tube containing 1M Cherry™ booster is provided in the kit (orange cap).

2) Incubate with shaking at 37°C until OD_{600} reaches 0.4-1 (the best range is between 0.6 and 0.8).
3) In one of the two flasks, add IPTG (100µl of a fresh 100mM stock solution) to reach a final concentration of 1mM. The other flask is used as a non-induced control. Continue incubation of both flasks for 2-3 hours.
4) Measure the Optical Density at 600nm for each culture. Transfer 1ml sample of each flask in a microcentrifuge tube.

Protein extraction under denaturing conditions

Note: the red color of the Cherry™ tag (when using pSSC-Cherry1 or pSSC-Cherry2 vectors) will disappear when using denaturing conditions (during protein extraction or SDS-PAGE). If you want to keep color to track your protein during the next steps (purification, quantification, interaction,…), it is necessary to avoid any denaturant (TCA, SDS or other detergents,…) during protein extraction (see below: protein extraction under non-denaturing conditions).

1) Transfer 1ml sample of each flask in a microcentrifuge tube. Centrigufe at maximum speed (13000 g) for 10 min (if possible at 4°C). Discard the supernatant, add 1ml H_2O and resuspend the bacteria. Add 50µl of cold 100% Trichloroacetic acid (TCA) (w/v) to each sample and vortex for a few seconds.

   Note: The TCA precipitation allows the analysis of the total protein content of the cells. Other methods can be used to specifically analyze different fractions (soluble, insoluble, periplasm, …) in order to identify the cellular localization of the target protein. For more information, please, check specialized literature or protocols (e.g., Sambrook et al., Ausubel et al.).

2) Place on ice for 10 min.
3) Centrifuge at maximum speed (13000 g) for 10 min (if possible at 4°C).
4) Remove carefully and discard the supernatant.
5) Wash the pellet with cold acetone (+4°C): add 500µl of acetone, vortex, and centrifuge for 5 min at maximum speed (if possible at 4°C).
6) Repeat steps 7 and 8
7) Remove carefully the supernatant. Air dry the final pellet: leave the tube opened on the bench or use vacuum drying.
8) Add (OD_{600} x 200)µl of 1X sample buffer (2X sample buffer= 100mM DTT, 2% SDS, 80mM Tris-HCl, pH 6.8, 0.006% bromophenol blue, 15% glycerol). Vortex vigorously to resuspend the pellet.

   Note: Taking into account the OD_{600} allows comparison of Coomassie-stained band intensities between samples.
9) Heat the samples at 70°C-100°C (10min.) to resuspend and denature the proteins. The samples can be used directly for SDS-PAGE analysis or stored at -20°C.
10) Load 4 to 10 µl of each sample in a SDS-PAGE gel containing the appropriate concentration of polyacrylamide (according to the size of the overproduced protein). Add a molecular size marker.

    Note: The sample volume that needs to be loaded will depend on the gel size, the expression level, and the extraction efficiency.
11) After migration, visualize the proteins with Coomassie-blue staining or continue the analysis with western blot.

    Note: Western blot analysis is a more specific and sensitive method but needs protein-specific antibodies or fusion tag-specific antibodies. For more information, please, check specialized literature or protocols (e.g., Sambrook et al, Ausubel et al.).

Protein extraction under non-denaturing conditions

Note: the red color of the Cherry™ tag (when using pSSC-Cherry1 or pSSC-Cherry2 vectors) will disappear when using denaturing conditions (during protein extraction or SDS-PAGE). If you want to keep color to track your protein during the next steps (purification, quantification, interaction,…), it is necessary to avoid any denaturant (TCA, SDS or other detergents,…) during protein extraction (see below: protein extraction under non-denaturing conditions).

Efficient bacterial lysis without denaturation can be obtained using French press or sonication (see the manufacturer's instructions). Otherwise, satisfactory results are obtained with freeze-thaw cycles in the presence of lysozyme. This protocol isolates soluble protein.

1) Centrifuge the samples at maximum speed (13000 g) for 10 min (if possible at 4°C).
2) Remove and discard supernatant.
3) Freeze completely the pellet at -20°C or -70°C.

4) Completely thaw the pellet on ice.
5) Resuspend the pellet by pipetting up and down or gentle vortexing using buffer A (50mM Tris-HCl, pH 7-8). Use 20 to 50µl of buffer A per ml of culture. Optional: add lysozyme (10mg/ml) and protease inhibitors, incubate on ice 20 min.
6) Freeze the sample (at -20°C or -70°C).
7) Thaw on ice and vortex.
8) Centrifuge the samples at maximum speed (13000 g) for 20 min at 4°C to remove insoluble cell debris. Check the pellet for color to know if the lysis was complete. Repeat steps 6 to 8 if additional lysis is necessary.
9) Transfer the supernatant to a clean tube for analysis and/or purification. Store the samples on ice for short term storage (few hours) or at -20°C until needed.

Note: The supernatant should be red for samples expressing the Cherry™tag. It is possible to quantify the protein expression using the Cherry™ tag (see below, protein quantification).

10) Take 10µl of supernatant and add 10µl of 2X sample buffer (100mM DTT, 2% SDS, 80mM Tris-HCl, pH 6.8, 0.006% bromophenol blue, 15% glycerol). Vortex.

11) Heat the samples at 70°C-100°C (10min.) to denature the proteins. The samples can be used directly for SDS-PAGE analysis or stored at -20°C.

12) Load 4 to 10 µl of each sample in a SDS-PAGE gel containing the appropriate concentration of polyacrylamide (according to the size of the overproduced protein). Add a molecular size marker.

Note: The sample volume that needs to be loaded will depend on the gel size, the expression level, and the extraction efficiency.

13) After migration, visualize the proteins with Coomassie-blue staining or continue the analysis with western blot.

Note: Western blot analysis is a more specific and sensitive method but needs protein-specific antibodies or fusion tag-specific antibodies. For more information, please, check specialized literature or protocols (e.g., Sambrook et al, Ausubel et al.).

Protein quantification using the Cherry™ tag (pSSC-Cherry1 or pSSC-Cherry vectors)

When using the Cherry™ vectors (pSSC-Cherry1 or pSSC-Cherry2), it is possible to quantify the protein concentration at any step (from protein production to the end of purification): a simple absorbance measurement at 413nm allows specific and accurate calculation of the target protein concentration based on the Beer-Lambert law:

$C = A / \varepsilon\ l$ where C is the concentration (mole/liter), A is the absorbance of the solution, ε is the specific extinction coefficient (ε = 117000 cmxliter/mole for the Cherry™ tag), l is the light path (1cm using standard cuvette).

1) Measure the absorbance (A) at 413 nm (absorption wavelength of the Cherry™ tag) for each sample expressing a protein fused to the Cherry™ tag (do not forget to perform the calibration using buffer without proteins).

2) When Aborbance$_{413nm}$ is higher than 0.8, dilute 5 times the sample and repeat step 1 to verify measurement accuracy.
3) Divide the A_{413} value by 117000 to calculate the concentration (mole/liter) of the protein of interest fused to the Cherry™ tag. If the sample was diluted, multiply the result by the dilution factor.

Note: the limit of detection by eye is about 0.12 Aborbance$_{413}$ corresponding to 1 µmole/liter and the Cherry™ tag is clearly visible at 0.24 A_{413} (2 µmole/liter). On native PAGE gels, a band corresponding to 0.5 nanomole is visible without staining.

Troubleshooting:

Please note that problems with cloning or expression efficiencies can result from the following parameters. Most of these problems can be fixed as explained below. However, due to intrinsic and specific properties of your gene or protein, the cloning or expression efficiencies may vary.

Problem	Solution
Only a few or no colonies are observed after transformation (ligation mix into the CYS21 bacteria or plasmid construct into the SE1 bacteria).	- Check the DNA concentration of your insert and the ligation conditions. -Check your restriction on agarose gel -Use the conditions recommended by the manufacturer of the enzymes -If you are doing double digest (two different enzymes) check buffer compatibility and test single digestion - Check the quality of your insert (single bands must be visible after agarose gel electrophoresis, if not, purify your DNA insert from the gel before cloning). - Be sure that the DNA transformation was optimal. When using electrocompetent bacteria, check the electroporation conditions (see above). When using chemically competent bacteria: check the temperature of the water bath, incubate the transformation product during one hour at 37°C to allow regeneration of the bacteria before spreading. - Check your plates with another strain which is resistant to the kanamycin antibiotic. If no growth is observed, check your antibiotic solution. - Your cloned fragment could be toxic for the bacteria. Check the literature (if data are available). Add 1% glucose (from a sterile 20% stock solution) to the plates to better repress the promoter.
No expression	- Check the gene sequence for mutations. - Check the gene sequence for the presence of rare codons. If rare codons are present, use StabySubCloning vectors with the missing tRNAs (SSC2-10 or SCC2-12 kits) - Check your expression conditions. - Check expression using the expression control. - Check expression starting with a single colony or a glycerol stock (do not use pre-culture) - When using Staby™Swicth medium, pre-warm the medium at room temperature or 37°C before inoculation. - Your protein might be unstable, try different induction conditions (lower temperature, Staby™Switch medium, longer induction time,…). - Use Cherry™ vectors to follow your protein with your eyes (for more information, please consult our website).
No color when using pSSC-Cherry1 or pSSC-Cherry2 vectors	- Check the expression of your protein using SDS-PAGE. - Try to localize your protein (insoluble or soluble part). If the protein is visible on SDS-PAGE but not soluble, try different induction conditions (lower temperature, Staby™Switch medium, longer induction time,…). - Check the color when expressing the Cherry™ tag alone with SE1 bacteria containing pSSC-Cherry vector without gene of interest. - Check expression in the presence of Cherry™ booster (see section about induction with Cherry tag).

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Related Staby™ products and services:

The **StabyCloning™ kit** is designed for the rapid, precise and efficient DNA cloning of PCR products. The complete cloning procedure is performed in one hour (including plating), the background is basically nil (the bacteria containing vectors without insert are killed), the PCR product is oriented, the plasmid is stabilized, and the export of the insert to another vector is easily selected.

The **StabyExpress™ T7 kit** contains all the key elements for cloning of a gene-of-interest and its expression in *Escherichia coli*. The kit combines two technologies (T7 expression and plasmid stabilization) that allow high-yield protein expression and standardization of the production-protocol.

The **GetStaby™ kit** allows easy addition of Delphi-Genetics' stabilization technology into your favourite vector. The technology is compatible with any expression system. Using this technology, your vectors are perfectly stabilized even without antibiotics.

The **Staby™Codon T7 kit** combines three technologies to ensure high-yield and standardized expression of eukaryote proteins in *Escherichia coli*. These technologies are *(i)* T7-controled expression, *(ii)* plasmid stabilization, and *(iii)* codon-usage adaptation of *E. coli* for the efficient expression of proteins that contain rare codons.

The **Cherry™Express kit** allows direct visualization (by eye!) of your protein of interest during protein production in *E. coli* and protein purification. Special requirements or reagents are not needed. It is also possible to quantify the protein concentration at any step by spectral measurement. The Cherry™Express kit combines multiple advantages: protein visualization, T7 expression, plasmid stabilization and codon-usage adaptation.

The **Staby™Switch** medium is an auto-inducible medium (ready-to-use) designed for high-level protein expression using Staby™ products or any other IPTG-inducible bacterial expression system. Using Staby™Switch medium, protein expression is automatically induced when high cell density is reached. Thus, it is neither necessary to add IPTG nor to monitor optical density during bacterial growth.

Staby™ Soft was specifically designed by Delphi Genetics to support the users of the Staby™ Operating System. This software package can perform customized gene-of-interest analysis to choose the most adapted kit and to optimize protein production.

For more information, please, consult www.delphigenetics.com

Staby™ products ordering information:

StabyExpress™		
GE-SET7-0505	StabyExpress T7 expression kit, electro-competent cells	5 RXN
GE-SET7-0707	StabyExpress T7 expression kit, chemically-competent cells cells	5 RXN
GE-SET7-1010	StabyExpress T7 expression kit, electro-competent cells	10 RXN
GE-SET7-1212	StabyExpress T7 expression kit, chemically-competent cells	10 RXN
GE-SET7-2020	StabyExpress T7 expression kit, electro-competent cells	20 RXN
GE-SET7-2222	StabyExpress T7 expression kit, chemically-competent cells	20 RXN
GE-SEGST-0505	pStabyGST1.2 expression kit, electro-competent	5 RXN
GE-SEGST-0707	pStabyGST1.2 expression kit, chemically-competent	5 RXN
GE-SEGST-1010	pStabyGST1.2 expression kit, electro-competent	10 RXN
GE-SEGST-1212	pStabyGST1.2 expression kit, chemically-competent	10 RXN
GetStaby™		
GE-GSA1-10	GetStaby kit, electro-competent cells	10 RXN
GE-GSA1-12	GetStaby kit, chemically-competent cells	10 RXN
StabyCloning™		
GE-STC1-10	StabyCloning kit, electro-competent cells	10 RXN
GE-STC1-12	StabyCloning kit, chemically-competent cells	10 RXN
GE-STC1-20	StabyCloning kit, electro-competent cells	20 RXN
GE-STC1-22	StabyCloning kit, chemically-competent cells	20 RXN
Staby™Codon		
GE-SCT7-0505	StabyCodon T7 expression kit, electro-competent cells	5 RXN
GE-SCT7-0707	StabyCodon T7 expression kit, chimio-competent cells	5 RXN
GE-SCT7-1010	StabyCodon T7 expression kit, electro-competent cells	10 RXN
GE-SCT7-1212	StabyCodon T7 expression kit, chimio-competent cells	10 RXN
GE-SCGST-0505	pSCodonGST1.2 expression kit, electro-competent	5 RXN
GE-SCGST-0707	pSCodonGST1.2 expression kit, chimio-competent	5 RXN
GE-SCGST-1010	pSCodonGST1.2 expression kit, electro-competent	10 RXN
GE-SCGST-1212	pSCodonGST1.2 expression kit, chimio-competent	10 RXN
Staby™Switch		
GE-AIME-04	Auto-induction medium	2L
Cherry™Express		
GE-CET7-05	CherryExpress, pSCherry1 kit, 5 reactions, electrocompetent	5 RXN
GE-CET7-06	CherryExpress, pSCherry3 kit, 5 reactions, electrocompetent	5 RXN
GE-CET7-07	CherryExpress, pSCherry1 kit, 5 reactions, chimiocompetent	5 RXN
GE-CET7-08	CherryExpress, pSCherry3 kit, 5 reactions, chimiocompetent	5 RXN
GE-CET7-10	CherryExpress, pSCherry1 kit, 10 reactions, electrocompetent	10 RXN
GE-CET7-11	CherryExpress, pSCherry3 kit, 10 reactions, electrocompetent	10 RXN
GE-CET7-12	CherryExpress, pSCherry1 kit, 10 reactions, chimiocompetent	10 RXN
GE-CET7-13	CherryExpress, pSCherry3 kit, 10 reactions, chimiocompetent	10 RXN
Cherry™Codon		
GE-CCT7-05	CherryCodon, pSCherry2 kit, 5 reactions, electrocompetent	5 RXN
GE-CCT7-06	CherryCodon, pSCherry4 kit, 5 reactions, electrocompetent	5 RXN
GE-CCT7-07	CherryCodon, pSCherry2 kit, 5 reactions, chimiocompetent	5 RXN
GE-CCT7-08	CherryCodon, pSCherry4 kit, 5 reactions, chimiocompetent	5 RXN
GE-CCT7-10	CherryCodon, pSCherry2 kit, 10 reactions, electrocompetent	10 RXN
GE-CCT7-11	CherryCodon, pSCherry4 kit, 10 reactions, electrocompetent	10 RXN
GE-CCT7-12	CherryCodon, pSCherry2 kit, 10 reactions, chimiocompetent	10 RXN
GE-CCT7-13	CherryCodon, pSCherry4 kit, 10 reactions, chimiocompetent	10 RXN
Staby™SubCloning		
GE-SSC1-10	pSSC-Native1, pSSC-His1 and pSSC-Cherry1 vectors, electro-competent cells	10 RXN
GE-SSC1-12	pSSC-Native1, pSSC-His1 and pSSC-Cherry1 vectors, chemically-competent cells	10 RXN

GE-SSC2-10	pSSC-Native2, pSSC-His2 and pSSC-Cherry2 vectors, electro-competent cells	10 RXN
GE-SSC2-12	pSSC-Native2, pSSC-His2 and pSSC-Cherry2 vectors, chemically-competent cells	10 RXN
Staby™ competent cells (CYS21 and SE1)		
GE-SET7-1111	10 cloning bacteria (CYS21) and 10 expression bacteria (SE1), electro-competent cells	10 RXN
GE-SET7-1313	10 cloning bacteria (CYS21) and 10 expression bacteria (SE1), chemically-competent cells	10 RXN
GE-STCB-20	20 cloning bacteria (CYS21) electro-competent cells, 50µl/tube	20 RXN
GE-STCB-22	20 cloning bacteria (CYS21) chemically-competent cells, 100µl/tube	20 RXN
GE-SET7-0020	20 expression bacteria (SE1), electro-competent cells, 50µl/tube	20 RXN
GE-SET7-0022	20 expression bacteria (SE1), chemically-competent cells, 100µl/tube	20 RXN

Worldwide ordering:

You can order our products directly from Delphi Genetics worldwide (with the exception of Asia) using our online ordering platform (www.delphigenetics.com). Should you prefer to work with a local dealer, you can find a list of distributors on our website: http://www.delphigenetics.com/international-distributors.html

ACADEMIC AND NON-PROFIT LABORATORY ASSURANCE LETTER REGARDING THE USE OF THE T7 EXPRESSION SYSTEM

The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U. S. Department of Energy and is the subject of patent applications assigned to Brookhaven Science Associates, LLC. (BSA). BSA will grant a non-exclusive license for use of this technology, including the enclosed materials, based upon the following assurances:

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2. No materials that contain the cloned copy of T7 gene 1, the gene for T7 RNA polymerase, may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this license and agrees to be bound by its terms. This limitation applies to strains BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE and SE1 and any derivatives you may make of them.

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Contact us:

Delphi Genetics SA
Rue C. Ader, 16
B-6041 Charleroi
Belgium
Tel: +32.71.25.10.00
Fax: +32.71.37.60.57
www.delphigenetics.com

delphigenetics@delphigenetics.com