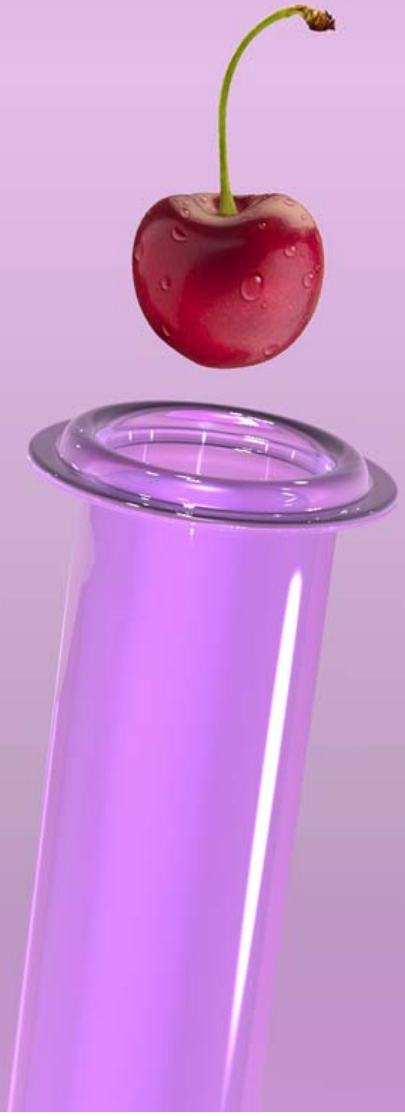
Cherry™Codon kit

Manual (v1.5)



DELPHI genetics

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Content and storage :

The Cherry™Codon kit is shipped on **dry ice**.

Storage: -80°C

Two different types of Cherry™Codon kits are available: one containing electrocompetent cells (in non self-standing tubes), and the other type containing chemically-competent cells (in self-standing tubes). Electroporation is more efficient that chemical transformation.

Each kit contains one box with the following items:

Name	**Concentration/remarks**	**Amount (CCT7-05 and CCT7-07)**	**Amount (CCT7-10 and CCT7-12)**
pSCherry2 DNA Grey cap	0.1µg/µl	1 tube of 50µl	1 tube of 50µl
pSCodon1.2 DNA Grey cap	0.1µg/µl	1 tube of 50µl	1 tube of 50µl
CYS21 strain (for cloning) Pink cap	Competent cells	5 tubes	10 tubes
SE1 strain (for expression) Blue cap	Competent cells	5 tubes	10 tubes
Staby reverse primer Red cap	0.1µg/µl in water 5'-CCA ACT CAG CTT CCT TTC G-3'	1 tube of 20µl	1 tube of 20µl
Staby forward primer Red cap	0.1µg/µl in water 5'-GCG TCC GGC GTA GAG GAT C-3'	1 tube of 20µl	1 tube of 20µl
Regeneration medium White cap	2% Tryptone 0.5% Yeast extract 0.05% NaCl 2.5mM KCl 10mM $MgCl_2$	5 tubes of 1.5 ml	10 tubes of 1.5 ml
Cherry™ booster Orange cap	δ-amino levulinic acid, 1M in water	1 tube of 200µl	1 tube of 200µl
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, *rps*l, *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:

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Hazards identification

No specific hazard concerning the products of the CherryCodon kit.

First aid measures

- Inhalation: If one of the products of the CherryCodon 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 CherryCodon 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 CherryCodon 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 CherryCodon kit is covered by worldwide patents. 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 (see assurance letter below).

User Guide

The Cherry™Codon kit combines multiple advantages: T7 expression, plasmid stabilization and protein visualization to obtain a high yield of colored heterologous-protein expression.

Overview of the Cherry™ tag system:

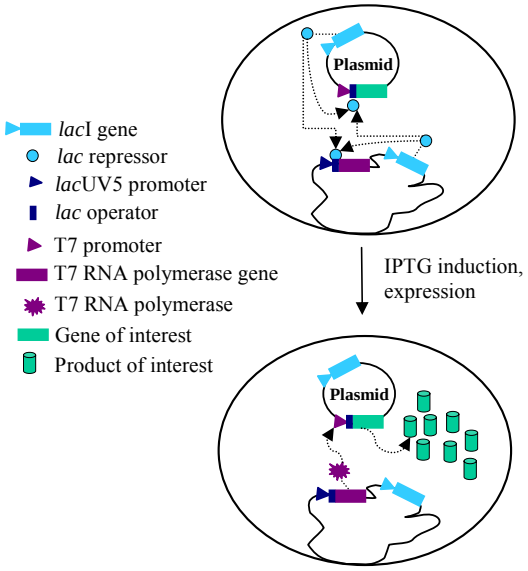
When using the Cherry™Codon vector (pSCherry2), your gene of interest is fused to a small sequence encoding a red polypeptide (heme binding part of cytochrome, 11 kDa) providing a visual aid for estimating expression level and solubility: bacteria expressing the fusion protein are red when the fused protein is soluble. As lack of solubility is a major problem when expressing recombinant protein in *E. coli*, the Cherry™Codon kit is 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. After purification, the tag can be cleaved using enterokinase (a recognition site is inserted at the C-terminal end of the tag sequence).

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 *lac*I gene) to the *lacO* operator sequence. The gene encoding the repressor is present in the bacterial chromosome and also in the pSCherry2 plasmid 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. 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 pSCherry2 and pSCodon1.2 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

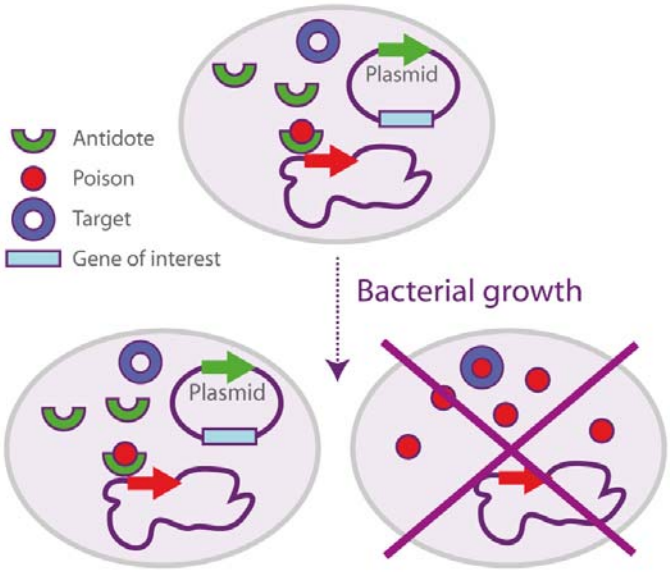
(CYS21), the plasmid construct is transferred into the expression strain encoding the T7 RNA polymerase (SE1) to produce the protein of interest.
As the the Cherry™Codon system is based on commonly used T7 promoter, you can easily change between your existing expression system and Cherry™Codon.

Overview of the stabilization system:

Higher plasmid stability= More proteins

Principle: The stabilization system is based on the use of bacterial antidote/poison *ccdA*/*ccdB* genes naturally found in the F plasmid of *Escherichia coli* (for more information about this system, see Bernard *et al*., 1992 and 1994 or Gabant *et al*. 1998 and 2002). In the Cherry™Codon system, the antidote gene (*ccdA,* 218bp) is introduced in the plasmid DNA under the control of a constitutive promoter. On the other hand, the bacterial toxic gene (*ccdB*, 305bp) 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 protein 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.

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** (contact us at stabysoft@delphigenetics.com).

Amino acid	Codon	Frequency in E. coli B (SE1) (%)	Frequency in Homo sapiens (%)	Frequency in Arabidopsis thaliana (%)	Frequency in Saccharomyces cerevisiae (%)
Arginine	CGT	35	**8**	17	14
	CGC	40	19	**7**	**6**
	CGA*	**5***	11	12	**7**
	CGG	11	20	**9**	**4**
	AGA	**5**	21	35	48
	AGG	**4**	21	20	21
Glycine	GGT	30	16	34	47
	GGC	41	34	14	19
	GGA	**10**	25	37	22
	GGG	18	25	15	12
Isoleucine	ATT	48	36	41	46
	ATC	44	47	35	27
	ATA	**7**	17	24	27
Leucine	TTA	14	**8**	13	28
	TTG	15	13	22	28
	CTT	11	13	26	13
	CTC	12	20	17	**6**
	CTA	**3**	**7**	11	14
	CTG	45	40	11	11
Proline	CCT	14	28	38	31
	CCC	**6**	33	11	16
	CCA	18	27	33	41
	CCG	61	11	18	12

*: CGA codon does not cause problem because large amounts of the corresponding tRNA are present

In the **Cherry™Codon kit**, we solve the problem by the use of the pSCherry2 expression plasmid encoding the tRNA genes of the six rare codons. Hence, this plasmid contains the T7 promoter for a strong expression, the *ccdA* gene for plasmid stabilization and supplies the rare tRNAs.

Benefits of the Cherry™Codon system:

- Direct visualization of the protein of interest
- No need of any special apparatus or reagents
- Rapid screening and optimization of protein expression and solubility
- Easy quantification of the protein of interest
- High yield of heterologous-protein expression even when the protein contains rare codons;
- High yield of protein production using Staby™ systems with or without antibiotics

Experimental outline: Easy 4 steps procedure.

1. Cloning of your gene of interest in the pSCherry2 vector

↓

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 antibiotics and visualize the target protein

Control your experiment:

Using the Cherry™Codon kit, it is possible to perform two controls of the experiment: 1°) Cloning of the gene of interest into the pSCodon1.2 vector to test expression without the Cherry™ tag

2°) Transformation of the pSCherry2 vector in the SE1 bacteria and test of the Cherry™ tag expression.

Step 1: Cloning of your gene of interest in the pSCherry2 vector:

Many strategies can be used for cloning your gene of interest (*goi*) into the pSCherry2 vector. The most convenient strategy is to use restriction enzymes: the single-cutter enzymes from the multiple cloning site are indicated on the map (see

below: from NdeI to XhoI). 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. The complete vector sequence is available on our website: http://www.delphigenetics.com

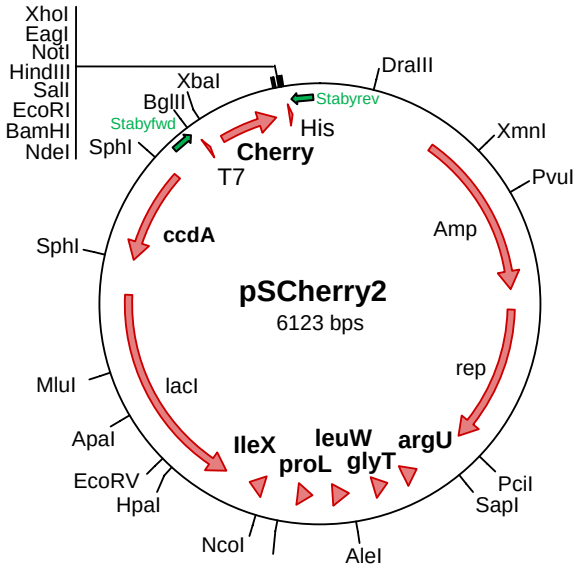


Figure 3: Restriction map of the pSCherry2 vector

Features:
-Staby forward primer: 5474-5492
-T7 promoter: 5514-5530
-Cherry tag: 5603-5899
-His: 5967-5984
-Staby reverse primer: 6027-6009 (C)

Experiment control: Using the same strategy, clone your gene of interest in the pSCoon1.2 vector. This vector is used as a control; the protein expressed using pSCodon1.2 will not be colored. The complete vector sequence is available on our website: http://www.delphigenetics.com

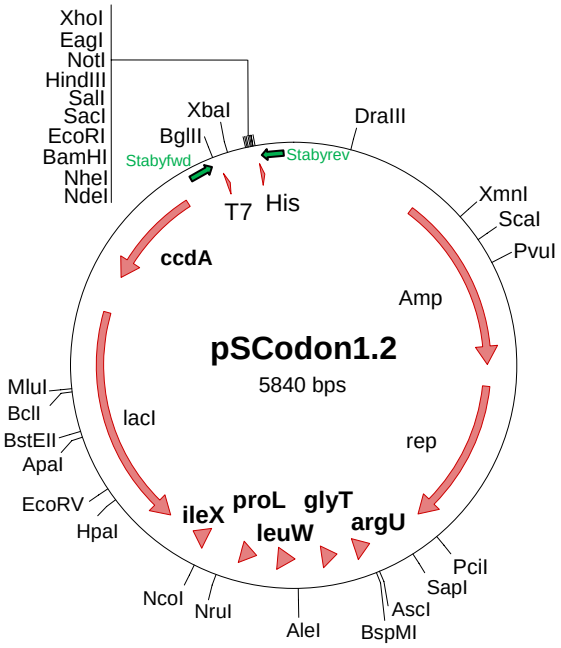


Figure 4: Restriction map of the pSCodon1.2 vector

Features:
-Staby forward primer: 5474-5492
-T7 promoter: 5514-5530
-His: 5684-5701
-Staby reverse primer: 5744-5726 (C)

Remarks:

Take into account the reading frame of the Cherry™ gene to fuse your sequence to the Cherry™ tag (cf. figure 5 below).

If you need to avoid any additional amino acid between the end of the Cherry™ tag and the N-terminal end of your protein, use the NdeI restriction site to clone your gene at the ATG codon (see figure 5).

Note that an **Enterokinase site** is present at the C-terminal end of the Cherry™ tag to cleave the fused protein (the cleavage position is indicated in figure 5, after the recognition site DDDDK).

The pSCherry2and pSCodon1.2 vectors allow the **fusion** of 6 histidine residues at the C-terminal end of the protein. This tag facilitates the purification of the target protein (and the detection when the protein is insoluble). If needed, the C-terminal fusion can be skipped by including a stop codon at the end of the gene of interest. Please, note that the 6 histidine residues are not in the same frame than the ATG start codon to avoid any fortuitous fusion (the 6 histidine codons are indicated in blue).

Fig. 5. Sequence of the cloning region in pSCherry2 vector: (unique restriction sites are indicated in bold or italized)

```
                                                                NdeI   BamHI
aag ata gcc aag cct tcg gaa acc ctt gat gat gac gat aag cat atg gga tcc
ttc tat cgg ttc gga agc ctt tgg gaa cta cta ctg cta ttc gat tac cct agg
 K   I   A   K   P   S   E   T   L   D   D   D   D   K   H   M   G   S
  Cherry tag C-terminal end        Enterokinase site

                                        EagI       AvaI
 EcoRI             SalI   HindIII      NotI       XhoI
gaa ttc gag ctc cgt cga caa gct tgc ggc cgc act cga g cac cac cac cac
ctt aag ctc gag gca gct gtt cga acg ccg gcg tga gct c gtg gtg gtg gtg
 E   F   E   L   R   R   Q   A   C   G   R   T   R
                                                  H   H   H   H
                                                    6 his

        Stop
cac cac tgagatccggctgctaa
gtg gtg actctaggccgacgatt
 H   H   -
```

Important:

Delphi Genetics can help you with a software-based 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 bacteria 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 - derivative of BL21 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 Cherry™Codon kits are available: one containing electrocompetent cells, and the other type containing chemically-competent cells. Electroporation is more efficient that chemical transformation.

Experiment Control: Do not forget to perform the same cloning experiment with the pSCodon1.2 vector as a control.

a) Transformation by electroporation:

1) Prepare LB plates containing 100µg/ml Ampicillin. 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.
8) Spread immediately 20 to 150µl on the pre-warmed plates containing ampicillin.
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 ampicillin (100µg/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 ampicilline 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 sequence of the vector is available on our website: http://www.delphigenetics.com

12) 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 100 µg/ml Ampicillin. 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.
10. Spread immediately 10, 20 and 100µl of the product (from step 9) on different pre-warmed plates.

    If you wish to have more clones, incubate the product (from step 9) at 37°C for one hour for regeneration of the bacteria before spreading of 10, 20 and 100µl on different pre-warmed plates.

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 ampicillin (100µg/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 ampicilline 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 sequence of the vector is available on our website: http://www.delphigenetics.com
14. 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 bacteria):

Experiment control: 1°) Do not forget to perform the same experiment with the pSCodon1.2 vector containing the gene-of-interest as a control.
2°) Transform the pSCherry1 vector alone (without gene-of-interest) to test the Cherry™ tag expression.

a) Transformation by electroporation:

1) Prepare LB plates containing 100µg/ml Ampicillin. 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.
8) Spread immediately 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 + 1% glucose (from a sterile filtered 20% stock solution).

Note: The plasmid is now stabilized in the SE1 strain using the Staby™ system, no antibiotic is needed to select bacteria containing the plasmid. However, the ampicilline resistance gene is still available.

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 sequence of the vector is 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.

b) Transformation using chemically competent cells:

1. Prepare LB plates containing 100 µg/ml Ampicillin. 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 blue 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.
10. Spread immediately 10, 20 and 100µl of the product (from step 9) on different 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 + 1% glucose (from a sterile filtered 20% stock solution).

Note: The plasmid is now stabilized in the SE1 strain using the Staby™ system, no antibiotic is needed to select bacteria containing the plasmid. However, the ampicilline resistance gene is still available.

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 sequence of the vector is available on our website: 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. Expression, protein extraction and quantification:

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 pSCherry2 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 pSCherry2 or the pSCodon1.2 plasmid will induce the expression of the T7 RNA polymerase which, in turn, will transcribe the Cherry™ tag and the gene of interest.
To enhance the red color of bacteria, it is possible to add 1mM of Cherry™ booster (δ-amino levulinic acid) in the culture medium. This booster is a heme precursor that

affects efficient heme incorporation. A tube containing 1M of Cherry™ booster is included in the kit.
Expression can also be performed using **Staby™Switch medium** (auto-inducible medium without IPTG). Perform the small-scale expression following the manual instructions. Do not forget to perform the expression controls (SE1 bacteria containing the pSCodon1.2 with the gene-of interest and SE1 bacteria with the pSCherry2 alone).

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 (Cherry™ tag and his tag).

Experiment controls: SE1 bacteria containing the pSCodon1.2 with the gene-of interest and SE1 bacteria with the pSCherry2 alone. The SE1 bacteria containing the pSCherry2 vector alone will allow you to test the expression of the Cherry protein alone (without his tag, size: 11KDa).

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

1. Inoculate two containers containing the desired volume of *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 pSCherry2 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.
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). Check the pellet for color: when using the pSCherry2 vector and when the expressed protein is soluble, the bacterial pellet of the induced sample is red. Compare to uninduced bacterial pellet and to expression controls.

Protocol for a small-scale expression using IPTG:

The small-scale protocol 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 (Cherry™ tag and his tag).

Experiment control: The SE1 bacteria containing the pSCherry2 vector will allow you to test the expression of the Cherry™ protein alone (without his tag, size: 11KDa).

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 in the pSCherry2 vector; inoculate two flasks containing 10ml of LB medium.

   Repeat step 1 for the construction using the pSCodon1.2 vector (expression of the protein of interest without colored tag) and for the positive control (SE1 bacteria containing the pSCherry2 vector without gene-of-interest).

Note: 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) For each construction, in one of the two flasks, add IPTG (100µl of a fresh 100mM stock solution) to reach a final concentration of 1mM. In the other flask, add 1% glucose (from a sterile filtered 20% stock solution). The second flask is used as a non-induced control (the glucose will repress the T7 promoter). 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. Centrigufe at maximum speed (13000 g) for 10 min (if possible at 4°C). Check the pellet for color: when using the pSCherry2 vector and when the expressed protein is soluble, the bacterial pellet of the induced sample is red. Compare to uninduced bacterial pellet.

Protein extraction under denaturing conditions

The red color of the Cherry™ tag 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.)

The TCA precipitation will denature the proteins and the red color of the Cherry™ tag will disappeared. If you want to keep the color, use non-denaturing conditions to extract proteins (see below).

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 4 and 5
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

The red color of the Cherry™ tag 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. 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

When using the Cherry™Codon kit, 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 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 the quality of your insert (one single band must be visible after agarose gel electrophoresis of the purified DNA fragment). - 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 ampicillin 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 color	- 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 pSCherry2 without gene of interest. - Check expression in the presence of Cherry™ booster (see step 4).
No expression	- Check the gene sequence for mutations. - Check your expression conditions. Check expression of the Cherry™ tag alone with SE1 bacteria containing pSCherry2 without gene of interest. - Check expression using the pSCodon1.2 plasmid carrying your gene-of-interest. - Check expression using SE1 bacteria containing pSCherry2 without gene of interest. - Your protein might be unstable, try different induction conditions (lower temperature, Staby™Switch medium, longer induction time,…).

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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 reactions
GE-SET7-0707	StabyExpress T7 expression kit, chemically-competent cells cells	5 reactions
GE-SET7-1010	StabyExpress T7 expression kit, electro-competent cells	10 reactions
GE-SET7-1212	StabyExpress T7 expression kit, chemically-competent cells	10 reactions
GE-SET7-1111	Set of 10 cloning bacteria (CYS21) and 10 expression bacteria (SE1), electro-competent cells	10 reactions
GE-SET7-1313	Set of 10 cloning bacteria (CYS21) and 10 expression bacteria (SE1), chemically-competent cells	10 reactions
GE-SET7-2020	StabyExpress T7 expression kit, electro-competent cells	20 reactions
GE-SET7-2222	StabyExpress T7 expression kit, chemically-competent cells	20 reactions
GE-SET7-0020	Set of 20 expression bacteria (SE1), electro-competent cells, 50µl/tube	20 reactions
GE-SET7-0022	Set of 20 expression bacteria (SE1), chemically-competent cells, 100µl/tube	20 reactions
GetStaby™		
GE-GSA1-10	GetStaby kit, electro-competent cells	10 reactions
GE-GSA1-12	GetStaby kit, chemically-competent cells	10 reactions
StabyCloning™		
GE-STC1-10	StabyCloning kit, electro-competent cells	10 reactions
GE-STC1-12	StabyCloning kit, chemically-competent cells	10 reactions
GE-STC1-20	StabyCloning kit, electro-competent cells	20 reactions
GE-STC1-22	StabyCloning kit, chemically-competent cells	20 reactions
GE-STCB-20	Set of 20 cloning bacteria (CYS21) electro-competent cells (50µl/tube)	20 reactions
GE-STCB-22	Set of 20 cloning bacteria (CYS21) chemically-competent cells (100µl/tube)	20 reactions
Staby™Codon		
GE-SCT7-0505	StabyCodon T7 expression kit, electro-competent cells	5 reactions
GE-SCT7-0707	StabyCodon T7 expression kit, chimio-competent cells	5 reactions
GE-SCT7-1010	StabyCodon T7 expression kit, electro-competent cells	10 reactions
GE-SCT7-1212	StabyCodon T7 expression kit, chimio-competent cells	10 reactions
Staby™Switch		
GE-AIME-04	Auto-induction medium	2L
Cherry™Express		
GE-CET7-05	CherryExpress T7 expression kit, electrocompetent	5 reactions
GE-CET7-07	CherryExpress T7 expression kit, chimio-competent cells	5 reactions
GE-CET7-10	CherryExpress T7 expression kit, electrocompetent	10 reactions
GE-CET7-12	CherryExpress T7 expression kit, chimio-competent cells	10 reactions
Cherry™Codon		
GE-CCT7-05	CherryCodon T7 expression kit, electrocompetent	5 reactions
GE-CCT7-07	CherryCodon T7 expression kit, chimio-competent	5 reactions
GE-CCT7-10	CherryCodon T7 expression kit, electrocompetent	10 reactions
GE-CCT7-12	CherryCodon T7 expression kit, chimio-competent	10 reactions

Notes:

Notes:

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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