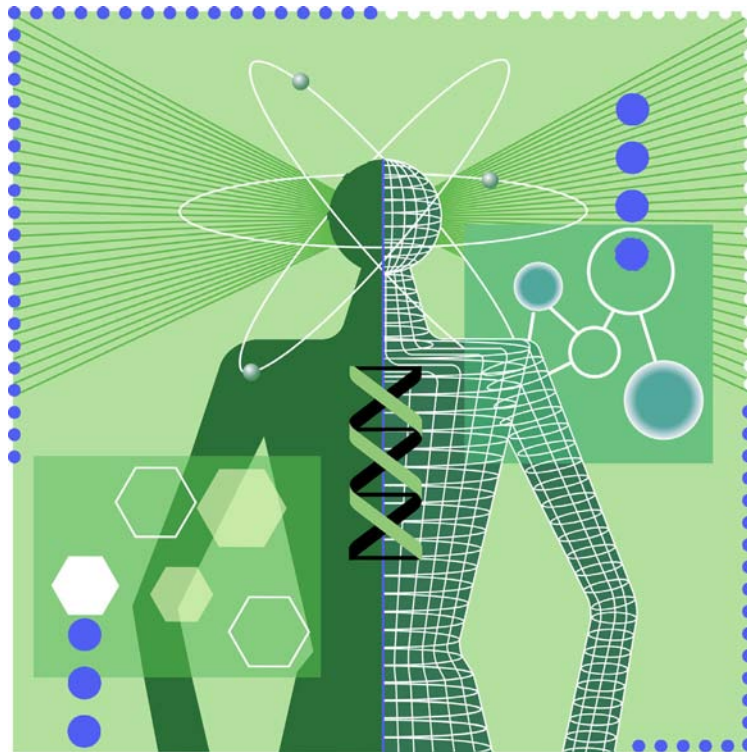


Ampliqon III

COMPETENT CELLS

BL21



Guide to Gene Expression in BL21

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de

Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)



TABLE OF CONTENTS

Section 1: Introduction to BL21	1
Section 2: Handling BL21 Cultures	1
Section 3: Expressing Your Protein	2
Section 4: Properties of BL21 Strains	4
Section 5: Factors That Affect Transformation with BL21	5
Section 6: Chemical Transformation	6
Section 7: Transformation Protocol	6
Section 8: Media and Formulations	8
Section 9: Troubleshooting Your Transformation	8
Section 10: Frequently Asked Questions	9
Section 11: Competent Cells - Technical Features	10
Section 12: General References	10
Section 13: Ampliqon Ordering Information	11

This guide describes how to use the T7 expression system in BL21 strains. High efficiency transformation for cloning purposes is covered in Ampliqon's Guide to High Efficiency Transformation.

SECTION 1: INTRODUCTION TO BL21

BL21 is by far the most widely used bacterial gene expression host. BL21 is a hardy strain that grows in minimal medium and is deficient in key proteases. BL21(DE3) cells have the T7 promoter expression system, which normally is capable of producing more protein than any other bacterial expression system. BL21(DE3) pLysS is a strain in which background expression of the T7 promoter is dampened by the LysS gene.

There are dozens of derivatives of BL21, each with special modifications. The resulting vast array of choices can be confusing. This Guide describes the use of BL21(DE3) and BL21(DE3) pLysS (referred to as BL21 and pLysS for short). The properties of BL21 strains are described in detail in Section 4, "Properties of BL21 Strains" on page 4. For gene expression, BL21 (DE3) should be used UNLESS the protein being expressed is toxic. If the protein is toxic, use pLysS.

If your protein is not made in your *E. coli* strain, then using BL21 should not be used. An amino terminal fusion protein, like thioredoxin, is needed to make the polypeptide. Then the protein may be made, but it may not be soluble.

If your protein is not soluble in *E. coli*, then try the procedure for insoluble proteins in Section 3, "Expressing Your Protein" on page 2.

An Overview of Gene Expression in BL21

BL21 will express proteins from *trc*, *tac*, λ PL, and *araD* promoters better than other commonly used strains of *E. coli*. Many people use BL21(DE3), a specially modified BL21 that will express genes from the T7 promoter. If a T7 promoter is not being used, BL21(DE3) can still be used, but not pLysS.

Most commonly used strains of *E. coli* are K12 strains that were originally developed as cloning hosts. They are usually *recA* mutants. BL21 comes from *E. coli* strain B and is not a *recA* mutant. The *recA* mutation keeps *E. coli* hosts from recombining homologous

sequences of DNA in genomic clones. For gene expression work, healthy bacteria are needed and *E. coli* needs the *recA* function to stay healthy. Expressing an ORF from a strong promoter presents all kinds of problems, but recombination is not one of them.

Strains that are good at protein expression:

BL21

Strains that are not as good at protein expression:

DH5 α TM, DH10BTM, XL1Blue, HB101, GeneHogs[®], MC1061, MM294, C600, JM83, K802, LE392, TOP10, X1776, Y1090

(DH5 α TM, DH10BTM, and GeneHogs[®] are trademarks of Invitrogen Corporation)

Another reason that BL21 is a good expression host is that BL21 is deficient in two key proteases, *lon* and *ompT*. The *lon* protease is an intracellular protease that *E. coli* makes to degrade abnormal proteins such as the one you want to make. It degrades the protein before the cells are even lysed. *OmpT* is a protease that *E. coli* makes to degrade extracellular proteins. It degrades the protein after your cells are lysed. People who prefer intact protein demand that all *E. coli* proteases be eliminated; but, this desire cannot be fully satisfied. *E. coli* is a living system and needs to be able to turn over protein and feed on peptides to stay healthy and productive. BL21's elimination of the two most offending proteases represents a good balance.

Can You Clone in BL21?

Yes. Success depends on how efficient the cloning is. If using a fancy cloning method with high yield and low background, then you can transform straight in BL21 to save time. If you are cutting, pasting, and screening, then life is full of surprises. You are better off using a cloning strain like GC5 until you get what you want. Then put the constructed plasmid into BL21.

SECTION 2: HANDLING BL21 CULTURES

The main concern with using BL21 is stability of the construct. The combination of your gene and a strong promoter may be deleterious. If it is, then there is a danger that the clones wanted will be lost and the mutants that don't make your protein will be robust and healthy. This danger can be avoided with a few simple steps.

Principles:

- In log growth, the T7 system is repressed and all cells are competitive (more or less).
- In stationary phase, cells are stressed and many die. Cells with the least amount of baggage will survive.
- Never make stock cultures from cells that have been induced.

Make a Stock Culture

The way to work with BL21 clones is to make stock cultures from fresh transformants and use these stock cultures for the expression work. This insures that the clone does not change and that each expression run gives optimal performance.

1. Transform the BL21 strain to be used with your plasmid.
2. Pick a single transformant colony from a fresh plate into 30 mL of LB + ampicillin (+ chloramphenicol as well for pLysS). A huge amount isn't needed - a little dab'll do.
3. Grow overnight. Room temperature is best. Turn the heater off on the shaker. Don't worry, the cells will grow. Many people cannot just shut the heat off on a shared lab shaker. In that case, grow the cells at 30°C. If a 30°C shaker isn't available and they have to grow at 37°C, make three cultures: one at full strength, the second a 10-fold dilution of the first flask, and the third a 10-fold dilution of that (100X dilution of the culture you inoculated with the colony). Use the flask that grew from the most diluted inoculum (that's the one that spent the least time in stationary phase).
4. In the morning, dilute 10 mL of the overnight with 10 mL of LB-20% glycerol.
5. Distribute 1 mL each into 1.2 mL cryotubes (5 to 20 tubes). Freeze and store at -70°C. These are stocks. As long as they stay at -70°C, they will be unchanged.
6. Each time you do an expression, thaw out a stock culture and use that to start the culture.

When down to the last tube, make a new stock culture. Using the original plasmid, make it the same way. Just subculture from the stock culture, and do a new test expression first to make sure that the strain has not lost viability. Don't use a culture that has been thawed more than once.

To subculture from a master tube without thawing it, remove the tube from the freezer and place it on dry ice. Open the tube and scrape some material from the top with an inoculation loop or a toothpick. Inoculate 1 mL of culture media with the scraping. Replace the tube in the freezer.

Please do not store the cultures as stabs, on plates, or in a tube in the refrigerator or on the bench. Even if

it's been done this way many times before, it's not good practice. When this is done, most of the cells die. Often, the ones that do not die are the ones that will not make your protein anymore. See Section 5 "Factors that Affect Transformation with BL21" on page 5 for proper storage.

SECTION 3: EXPRESSING YOUR PROTEIN

The key to successful gene expression is to do a test expression first.

Test Expression

To save time, do a test expression to determine:

- Does the clone really make the desired protein?
- How much protein is made?
- Is the protein soluble or insoluble?

Growth and Sampling

The following is a generic protocol that lacks some detail because people use different types of media and methods. Cells in LB grow to a maximum of about 3 OD with good aeration. In rich media such as PlasmidPro, Terrific Broth, or CIRCLEGROW[®], culture OD can reach 20 with excellent aeration. Minimal media can give various results as low as 0.5 OD or as high as 20 OD, depending on conditions.

(CIRCLEGROW[®] is a registered trademark of Qbiogene, Inc.)

1. Dilute 1 mL of stock culture into 100 mL of media + ampicillin (+ chloramphenicol as well for pLysS) in a 500 mL baffled flask. [Amp = 100 µL/mL, Cam = 25 µL/mL]
2. Grow the cells to 0.5 OD at 37°C. This should take about 2-3 hours. During this time, mark 5 conical 15 mL centrifuge tubes: 0, 1, 2, 3, O/N.
3. Harvest a 10 mL sample of the uninduced (0 hours) sample. Spin the tube at 4,000 rpm for 20 minutes. Pour off the supernatant. Freeze the pellets.
4. Add 1 mL of 100 mM IPTG to the culture [final conc. 10 mM].
5. Measure the OD of the cells for each of the next 3 hours. Harvest 10 mL samples of the culture each time: 1 hour, 2 hours, and 3 hours after induction. Store the pellets at -20°C.
6. Continue to express the cells overnight.
7. The next morning, harvest 10 mL of the cells. Note the time.

Protein Determination

The approximate amount of total protein (in mg) that can be expected in each sample depends on the OD of the culture when it was sampled (see the Protein Determination Table on page 3).

Protein Determination									
OD of culture when sampled:									
0.5	1	2	3	4	5	7.5	10	15	20
Approximate concentration of protein in the 1 mL resuspension of the sample pellet (mg/mL):									
0.25	0.5	1	1.5	2	2.5	3.8	5	7.5	10

Resuspend the pellets in 1 mL TE. Measure the total protein in each of the 1 mL samples using Bradford, BCA, or equivalent with a standard curve. It is important to do this, because the gel analysis is much easier if each lane has the same amount of protein.

Separation of Insoluble and Soluble Protein

Dilute the samples to 2 mg/mL protein. Sonicate the samples to disrupt the cells. Remove 10 µL and electrophorese. This is the total protein.

Spin 13,000 rpm for 5 minutes. Remove 10 µL and electrophorese. This is the soluble fraction.

Gel Electrophoresis

Load the same amount of protein in each lane of the gel. Analyze the samples by gel electrophoresis looking at all the samples: 0 (uninduced), 1, 2, 3, and O/N hours of induction. It is a good idea to compare soluble protein and total protein. Insoluble protein is the total minus the soluble. The amount of protein in each sample depends on the amount of cells. The amount of cells in each sample depends on the clone and the media being used. Measure the amount of protein and load the same amount of protein in each lane of the gel.

Gel Electrophoresis Sample Form						
Date: _____						
Host Strain: _____						
Plasmid: _____						
Medium: _____						
Antibiotics: Ampicillin _____ Chloramphenicol _____ Other _____						
Time	Time Post-inoc.	OD	Vol. Assayed	Reading	Protein/mL	Total Protein
_____	0	_____	_____	_____	_____	_____
_____	1	_____	_____	_____	_____	_____
_____	2	_____	_____	_____	_____	_____
_____	3	_____	_____	_____	_____	_____
_____	O/N	_____	_____	_____	_____	_____
Standard Protein			Vol. Assayed	Reading	Protein/mL	
			0	_____	0	
			_____	_____	_____	
			_____	_____	_____	
			_____	_____	_____	
<p>Time - Time of day time points were taken.</p> <p>Time post-inoc - Hours after inoculation. The target times are shown. The actual times should be recorded next to the target times.</p> <p>OD - OD of the cell culture when it was sampled. The wavelength is not crucial; 550, 590, 595, 600 nm all give similar results. Pick one.</p> <p>Vol. Assayed - The volume of resuspension that was assayed. The amount that should be sampled is suggested by the table above, which gives an estimate of the concentration of protein in the sample.</p> <p>Reading - The reading of the protein assay (e.g., OD₅₆₂ for BCA or OD₅₉₅ for Bradford).</p> <p>Protein/mL - With the standard protein, this is calculated by the concentration of the standard, the volume you assayed, and the volume of the assay. For the cell samples, the protein concentration/mL is determined by the standard curve and the sample reading, and the conversion factor determined by your results.</p> <p>Total Protein - "resusp vol" x "protein/mL".</p>						

The test expression experiment can be recorded in any way you like. A form such as this (see page 3) or a derivative can be used.

Analysis of Test Expression Results

There are many possible outcomes to the test expression experiment. Let's look at a few:

- **Cells not inhibited by induction and make a lot of soluble protein.** Isn't life grand? Go ahead and scale up.
- **Cells not inhibited by induction but do not make very much protein.** This is not good news; however, an amino terminal fusion can be made with a protein that *E. coli* does express well, like thioredoxin. If the fusion protein is made, then initiation of translation of your protein is inefficient. If using *E. coli*, stay with the amino fusion.
- **Cells inhibited by induction and make a lot of protein.** This is good. Induce at the highest OD possible. This depends on the media being used and aeration. Induce at 1/3 the final OD obtained when not inducing.
- **Cells inhibited by induction and do not make very much protein.** This is not good. Induce at the highest OD possible. This depends on the media being used and aeration. Induce at 1/3 the final OD obtained when not inducing. Whatever protein is being produced is killing off your cells. This is the best that you can achieve in this system.

SECTION 4: PROPERTIES OF BL21 STRAINS

Types of *E. coli*

The kind of *E. coli* that gives church picnics and hamburger stands a bad name is very different from the harmless strains of *E. coli* that everybody uses in the lab. Many different strains of *E. coli* are available, and they can be divided into three groups:

Cloning strains are efficiently transformed, offer blue/white screening, and do not restrict foreign DNA. These are the cells that are most often used in cloning and sequencing, but are not well suited to gene expression work. The use of these strains is described in Ampliqon's *Guide to High Efficiency Transformation*.

Expression strains are used to express a protein efficiently from a given construct. Expression strains do not need to be efficiently transformed to be useful. They are described here, with the focus heavily on BL21.

Specialty strains have particular properties that are only occasionally used. Call the Biomol tech line if you are interested in a custom strain.

BL21 comes from *E. coli* strain B, first identified in 1946. *E. coli* B is naturally deficient in the *lon* protease due to an insertion of a stray piece of DNA in the *lon* promoter. The *E. coli* world is replete with cloning strains that are derivatives of a single strain called K12. K12 was first isolated in 1922. Most of these strains, as noted above, are not as good at producing protein as BL21. For starters, they are not *lon* mutants; most of them are *recA* mutants, which makes them weak.

The Genotype of BL21 and BL21(DE3)

Labs have passed around *E. coli* strains and made mutants and shuffled around mutations until the average strain had a long list of genetic markers called a genotype. To most people, a genotype looks like it is written in Martian. Many markers are not relevant to most people. The following may help in what strain to use.

BL21	F ⁻ , <i>ompT</i> , <i>hsdS</i> (r _B , m _B), <i>gal</i> , <i>dcm</i>
λDE3	<i>lacI</i> , <i>lacUV5-T7 gene 1</i> , <i>ind1</i> , <i>sam7</i> , <i>nin5</i>
BL21 (DE3)	F ⁻ , <i>ompT</i> , <i>hsdS</i> (r _B , m _B), <i>gal</i> , <i>dcm</i> , λDE3 (<i>lacI</i> , <i>lacUV5-T7 gene 1</i> , <i>ind1</i> , <i>sam7</i> , <i>nin5</i>)

The genotype of BL21 is listed above. The genotype of the phage λDE3 is listed just below it. Yes, phage can have a genotype. The genotype of BL21(DE3) is listed below that. Note that BL21(DE3) is just BL21 with the phage DE3 added.

The features that are found in these BL21 strains are listed below. The genotypes that are responsible for these features appear in parentheses.

Lon Protease Deficient (*lon*)

The serine protease encoded by the *lon* gene was not created by nature to frustrate biochemists. The *lon* protein serves two functions in *E. coli*. It degrades misfolded proteins, so it keeps trash from accumulating in *E. coli* cells. In this role, it's a housekeeping gene. The *lon* protease also degrades normal proteins. This function of *lon* serves to give certain proteins a half-life. This is also a useful function, because some proteins are made to do one specific job and are bad when they remain after their usefulness is over. For example, the *sulA* protein, induced by DNA damage, is made to keep cells from completing division until all the DNA snarls are straightened out. This is good. If *sulA* remains, it continues to prevent the cells from dividing even after all the DNA damage is repaired and all systems are "go". This is bad. Bacteria that do not divide are dead; thus, *sulA* responds to DNA damage by killing the cell unless *lon* degrades *sulA*.

over time. There are a lot of proteins like *sulA* that are great for their function, but must be discarded afterward. The *lon* protease performs this function.

Deficient in the Outer Membrane Protease *OmpT* (*ompT*)

Most bacteria would rather degrade extracellular protein than make their own amino acids, so they have proteases on their surfaces to chop up whatever is out there. *OmpT* is a protease that sits on the outer membrane of *E. coli*. There are other proteases on the outer membrane of *E. coli*, but *ompT* is the one that causes the most trouble.

Restriction Deficient (*hsdS_B*-)

When transforming your plasmid into a strain, you do not want that strain to degrade your plasmid. For that reason, BL21 is deficient in restriction endonuclease activity. *E. coli* B strains methylate their DNA at B sites (TGA(N)₈TGCT). In B strains, DNA that is not methylated at these sites is degraded by a restriction enzyme. BL21 does not methylate, nor does it restrict unmethylated DNA at B sites. This is because BL21 is mutant in the S gene in the restriction-modification system (which is at the *hsd* locus). The S gene is required both for methylation and for degradation.

T7 RNA Polymerase (λ DE3 with *lacUV5*-T7 gene 1)

When most say BL21, what they usually mean is BL21(DE3), a derivative of BL21 that has the T7 RNA polymerase gene (i.e., gene 1 of T7) under the control of the *lacUV5* promoter. The *lacUV5* promoter is a mutant *lac* promoter that is stronger than wild type *lac* promoter. This whole arrangement is on a λ phage genome, and this particular version of the λ phage is called λ DE3. λ DE3 is inserted into the chromosome of BL21 to make BL21(DE3). There are other markers on this λ DE3 genome (*lacI*, *ind1*, *sam7*, *nin5*), but none will affect expression.

pLysS

pLysS is a plasmid that has the T7 lysozyme gene (*LysS*) on it. The T7 lysozyme protein has two effects: it degrades the cell wall and it inhibits T7 RNA polymerase. Because T7 lysozyme inhibits T7 RNA polymerase, it helps to keep expression of the T7 promoter off until the IPTG is added. When IPTG is added, the amount of T7 RNA polymerase increases and overcomes the inhibition by *LysS*.

Phage Resistance (*fhuA*, *tonA* or T1R)

Commercial cDNA banks are sometimes infected with T1 phage, and there are many other ways to get into trouble with T1 or a T1 relative. Unlike other phage, T1 is resistant to drying and subsequently is

almost impossible to eliminate. Cells which are resistant to T1 are fast becoming standard in laboratories. More description is in Section 11, "Technical Features" on page 10.

Deficient in Cytosine Methylation (*dcm*)

Nobody knows why *E. coli* K12 methylates certain sites (CCAGG and CCTGG). *E. coli* B does not do this; therefore, neither does BL21.

Deficiency in Galactose Metabolism (*gal*)

It was once popular to make mutants in genes like *gal* and so this vestige of prehistoric *E. coli*-ology prevents BL21 from using galactose as a carbon source.

SECTION 5: FACTORS THAT AFFECT TRANSFORMATION WITH BL21

Storage & Handling of Competent Cells

Arrival. Competent cells arrive in dry ice and need to be kept at -70°C to keep them at their peak. You can keep your cells in peak form if you take a few precautions. First, clear out the space in the -70°C freezer where the cells are going to be stored. Unpack the box at the freezer, not on the other side of the lab. Place the cells in the -70°C freezer and shut the door. You don't have to rush, but please don't take the cells out for a stroll, or leave them on the bench while you rummage around trying to clear out a space for them. Even when they stay frozen, cells lose efficiency when they warm up.

Storage. We've all worked in labs, so we all know that -70°C space is shared, cold, cramped, and confusing. While the cells are stored in the freezer, people will need to get something that's behind them or under them; and, over time, the cells will spend some time in places warmer than -70°C. This is unavoidable but can be minimized by placing the cells where everybody can get to them but they aren't in the way of other things. When defrosting freezers, making major re-arrangements, etc., please take the time to grab some dry ice and place the cells in (not on) the dry ice while everything is shuffled around. You'll be glad you did. If your freezer dies but your cells don't thaw, you may lose efficiency. If your cells thaw, you probably need new cells.

Refreezing cells. If you thaw out a tube of competent cells on purpose and want to refreeze the remainder, you can. Place the tube in crushed dry ice or in a dry ice-ethanol bath (best), buried in a bed of dry ice (second best), or by itself on a metal shelf at -70°C for an hour before putting it in the box (third best). You'll lose about 2-fold in efficiency. If you simply put the

tube back in the box and place it in the freezer, you could lose 5- to 10-fold in efficiency.

SECTION 6: CHEMICAL TRANSFORMATION

Chemical transformation is achieved by suspending the cells in an ice-cold buffer that contains calcium chloride and other salts. Typically, these cells are stored frozen. When desired, the cells are thawed and DNA is added. Transformation occurs when the cells are warmed briefly. Transformed cells are diluted into media; and after a time, they are plated onto media that selects for transformants. Transformation efficiencies vary from 10^8 to 10^9 transformants per microgram of pUC19 DNA with commercially prepared cells (10^6 to 10^7 for most home-made cells).

What's Important in Chemical Transformation

Transformation protocols are described in detail under Section 7, "Transformation Protocol". Let's face it, nobody does transformations EXACTLY like they are supposed to. What's important?

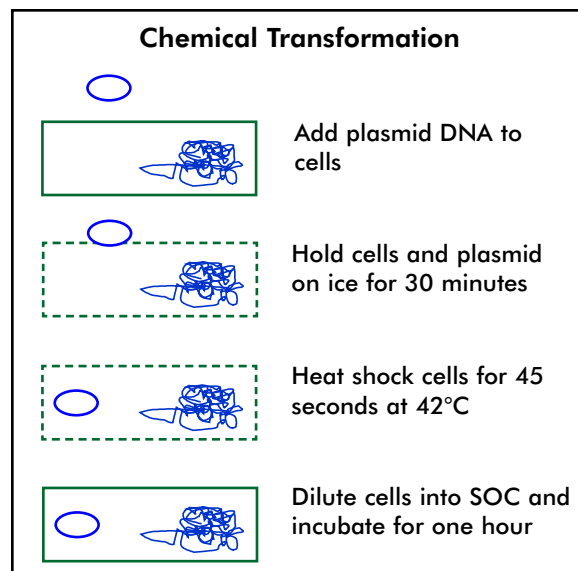
Thawing the cells. Thaw cells for 5 to 10 minutes directly on ice. If you are in a hurry, you can thaw the cells by rolling the tube in your fingers until the ice melts. DO NOT run water over the tube or put the tube in a water bath. If you do this, you can't stop when the cells reach 0° . If you are busy and the cells stay in the ice bucket for an hour, that's fine. Beyond that, you'll start dropping in efficiency about 2-fold every hour or so. If you leave the cells in the ice bucket overnight, don't bother using them.

Incubating the DNA with the cells on ice.

Incubating on ice is necessary for chemically competent cells. If you heat shock right away, your efficiencies will be down 10-fold. If you incubate for only 15 minutes, you'll be down 3-fold. Occasionally, it's a corner to cut if you are pressed for time and efficiency is not a critical issue.

The heat shock. The heat shock works best in a Falcon 2059 tube with a 42°C bath. Lots of people have 37°C but not 42°C baths. A 45 second heat shock at 42°C gives the best results; but, one minute at 37°C works almost as well (down 2-fold).

Expression. The effect of the expression time depends on the plasmid and strain. With pUC19 and GC5, the efficiency is down 10-fold if you plate without any expression time at all, down 7-fold if you plate after 15 minutes, and down 3-fold if you plate after 30 minutes. SOC medium gives 2-fold better results than LB medium for chemically competent cells.



Plates. Some plates give better results than other plates, but there are no magic plates. If you use plates that are less than six months old and are not too dry, you should have good results. The only plates to be careful about are tetracycline plates. Tetracycline breaks down, particularly in the light, to toxic products that kill everything except contaminants. Put the tetracycline in when the agar's cooled down and ready to pour. Throw the plates out after 3 months.

SECTION 7: TRANSFORMATION PROTOCOL

(from Ampliqon product inserts)

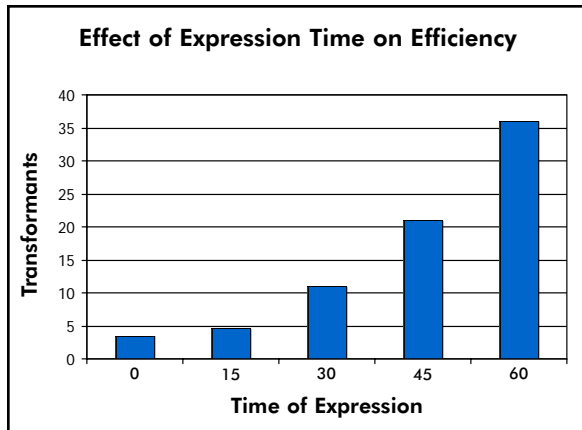
Notes on Ligation Reactions:

Ligation reactions inhibit transformation. Less transformants are observed from ligation reactions than from transformations with plasmid DNA.

Use of 0.5 μL of a ligation reaction per 50 μL of competent cells. For best results, either purify the ligation mixture by ethanol precipitation prior to transformation or dilute the ligation reaction 3-fold in TE buffer and use 1 μL per 50 μL competent cells.

General Handling of Competent Cells

- Competent cells are very sensitive to any change in temperature. Cells must be thawed on ice. The transformation should be started immediately after the cells are thawed.
- Competent cells must be treated gently. Mix cells by swirling or gently tapping the reaction tube. Do not mix by pipetting or vortexing.



- Once thawed, the cells should be used. Re-freezing thawed competent cells will result in a significant drop in transformation efficiency.

Amount of DNA

Usually, the construct has been made and the BL21 cells are being transformed with a single plasmid species. When transforming BL21, use less not more DNA. The cells are saturated with anything over 10 ng, meaning that you are transforming all of the cells that can be transformed. 1 μL of any plasmid prep is plenty. If using more, the result is fewer colonies because the impurities in the DNA will inhibit some of the cells from being transformed.

Advance Preparations

- Equilibrate a non-shaking water bath to 42°C.
- Place SOC Medium at room temperature.
- Prepare LB agar plates with the appropriate antibiotic.
- Agar plates should be placed in a 37°C incubator for about 30 minutes prior to plating.

Procedure

- Remove competent cells from -70°C and place directly in ice. Thaw cells for 5 to 10 minutes.
- Gently mix cells by tapping tube. Aliquot 50 μL into transformation tubes. Falcon 2059 tubes are best for this.
- Add 1-50 ng of DNA (or 1 μL control DNA) into 50 μL competent cells. Gently tap tube to mix.
- Place the tubes on ice for 30 minutes.
- Freeze any remaining cells.
- Heat-shock the cells for 45 seconds in a 42°C water bath or 60 seconds in a 37°C water bath. Do not shake.
- Add 450 μL of room temperature SOC medium to each transformation reaction.
- Incubate at 37°C for one hour with shaking (225 to 250 rpm).
- Spread on LB agar plates containing appropriate

antibiotic (e.g., for control pUC19: use 100 $\mu\text{g}/\text{mL}$ ampicillin for BL21(DE3), and 100 $\mu\text{g}/\text{mL}$ ampicillin plus 25 $\mu\text{g}/\text{mL}$ chloramphenicol for BL21(DE3)pLysS).

- Incubate the plates at 37°C overnight (12 to 16 hours).

Transformation Efficiency: What It Means and How It Is Calculated

Transformation efficiency is a measure of the ability of cells to be transformed. Transformation efficiency is expressed as the number of transformants per microgram of pUC19. It can be written as transformants/ μg , T/ μg , as colony forming units per microgram, or cfu/ μg . The higher the efficiency, the higher the fraction of cells in the reaction that are actually transformable, and the more transformants for the same DNA.

If you transform BL21 (DE3) competent cells (10^6 transformants/ μg) with 1 μg of pUC19, you will not get 10^6 transformants because the cells are saturated at 0.01 μg of pUC19 in a 100 μL reaction. The efficiency is often measured with 50 picograms of pUC19 in a reaction; an efficiency of 10^6 transformants/ μg is the same as 50 transformants/pg. At Ampliqon, we plate 0.1 mL, and look for 50 colonies.

Sample Calculation:
colonies on control plate / ng of control DNA plated
 $\times 1000 \text{ ng} / \mu\text{g} = \text{T}/\mu\text{g}$

Assume you added 0.1 ng of control DNA (1 μL of 0.1 ng/ μL , freshly diluted) to 100 μL of competent cells. 900 μL of SOC medium is added prior to expression.

100 μL (equivalent to 0.01 ng DNA) is then plated.

If 100 colonies are counted on the plate, calculate the transformation efficiency as follows:

100 cfu on control plate / 0.01 ng of control DNA plated
 $\times 1000 \text{ ng} / \mu\text{g} = \text{T}/\mu\text{g}$
 100 colonies / 0.01 ng $\times 1000 \text{ ng}/\mu\text{g} = 1 \times 10^7 \text{ T}/\mu\text{g}$.

Although transformation efficiency is an important consideration in initial cloning experiments (with cloning strains such as GC5 and GC10), it is not a critical factor in protein expression. With BL21 strains, usually only one colony is needed. If more colonies are needed, consider making your construct in a high efficiency cloning strain. Then, once you have your clone, transform that clone into BL21 for gene expression.

SECTION 8: MEDIA AND FORMULATIONS

If you use USBio Media (Biomol), you can skip this section; just add water to the bottle and autoclave (see Section 13, "Ordering Information" on page 11).

LB Medium

10 g	Tryptone
5 g	Yeast Extract
10 g	NaCl
to 1 Liter	water

Autoclave. Cool to room temperature before using. Store at room temperature.

LB Agar

10 g	Tryptone
5 g	Yeast Extract
10 g	NaCl
15 g	agar
1	stir bar
to 1 Liter	water

Autoclave. Cool to 45-50°C in a water bath. Place on stir plate. Add antibiotics while stirring. Pour 25-35 mL per 87 mm plate. Let plates sit at room temperature overnight. Bag plates and store at 4°C.

SOC Medium

2 g	Tryptone
0.5 g	Yeast Extract
1 mL	1 M NaCl
0.25 mL	1 M KCl
1 mL	1 M MgCl ₂
1 mL	1 M MgSO ₄
1 mL	2 M glucose
to 100 mL	water

Add tryptone, yeast extract, NaCl, KCl to 97 mL water. Stir, autoclave, and cool to room temperature. Prepare MgCl₂, MgSO₄, and glucose stocks and filter sterilize. Add magnesium and glucose solutions to cooled medium. Filter sterilize. pH should be 7.0. Store at room temperature.

LB + 20% glycerol

80 mL	LB
20 mL	glycerol

Put 80 mL LB in a small flask. Add 20 mL glycerol. Run the LB up and down in the pipette a couple of times to dissolve the residual glycerol. Filter sterilize. Store at room temperature.

Ampicillin 10 mg/mL

1 g	ampicillin
100 mL	water

Dissolve 1 g ampicillin in 100 mL water. Filter sterilize and store at 4°C.

LB + ampicillin (BL21 DE3 clones)

1 mL	10 mg/mL filtered ampicillin
1 Liter	autoclaved, cooled LB

Add components and swirl to mix. Store at room temperature.

Chloramphenicol 1 mg/mL

100 mg	chloramphenicol
100 mL	water

Dissolve 100 mg chloramphenicol in 100 mL water. Filter sterilize and store at 4°C.

LB + ampicillin + chloramphenicol (BL21 DE3 pLysS clones)

1 mL	10 mg/mL ampicillin
1 mL	1 mg/mL chloramphenicol
1 Liter	autoclaved, cooled LB

Add components and swirl to mix. Store at room temperature.

IPTG 100 mM

23.8 mg	IPTG
1 mL	water

Dissolve 23.8 mg IPTG in 1 mL water. Filter sterilize. Aliquot in working volumes and store at 20°C.

SECTION 9: TROUBLESHOOTING YOUR TRANSFORMATION

Transformation frequency is affected by the purity of the DNA, how the cells are handled, and how the transformation was actually performed.

Impurities in the DNA

Easy-to-use spin columns can be used to purify DNA from PCR reactions, ligations, endonuclease digestions, or other treatments. Experienced personnel can use phenol-chloroform extraction, followed by ethanol precipitation. The presence of salts is indicated if the donor DNA causes electrocompetent cells to explode.

Proteins	column purify or phenol extraction/ethanol precipitation
Detergents	ethanol precipitation

PEG	column purify or phenol extraction/ethanol precipitation
Ethanol	dry pellet before resuspending in TE
Salts	ethanol precipitation

Adding Too Much Ligation Mix to the Transformation

The most common mistake when transforming *E. coli* is to put too much ligation mix in the transformation. As suggested in the protocols, less than 1 μ L of a ligation is sufficient for any type of transformation. Adding more LOWERS the number of transformants. For chemically competent cells, the ligase and PEG in the mix inhibits transformation. Sometimes you have to squeeze every possible transformant out of a ligation. To do this, there are two options:

1. Precipitate the ligation and resuspend it in TE as described in Section 7, "Transformation Protocol" on page 6.
2. Dilute the ligation 3-fold in TE and use 1 μ L per transformation.

SECTION 10: FREQUENTLY ASKED QUESTIONS

Working with a heterologous gene under strong promoter control is not easy. If you have a question that is not listed below, try the Biomol Technical Service at +49-40-853260-23/-27/-37 or e-mail ts@biomol.de.

I store my cultures on plates that I keep in my refrigerator. When I inoculate media with these cultures, the cells are all stringy and gross, and they take forever to grow.

The stringy gunk are dead cells. The survivors won't make as much of your protein. Retransform BL21 with the original plasmid and make stock cultures. Never store BL21 on a plate because this discriminates against cells that are making your protein. That is, if you store BL21 (colonies) on a plate, most of the cells will die and those that survive are most likely not the ones that make your protein. Storage on a plate "favors" the wrong cells. Please refer to Section 2, "Handling BL21 Cultures" on page 1.

I'm working with BL21(DE3) pLysS and I'm getting a lot of background expression.

Are you selecting for pLysS as well as your plasmid? You should, by putting chloramphenicol (Cam) in selective plates and growth media. If you are selecting with Cam and still getting a lot of background, then your construct is super hot. Sometimes, this isn't a problem. If this is a problem, then you might switch to a promoter system that has better control,

like PL. You may not need as much power as T7 has to offer and you may be going backwards if the background is killing you. Some researchers use the BL21 (DE3) pLysE strain because it produces even more inhibitor of background T7 expression. The strain is tricky to use and sometimes grows poorly, probably because of the increased background expression of T7 lysozyme from the *tet* promoter.

I'm working with BL21(DE3) pLysS and I'm getting different-sized colonies.

Are you putting chloramphenicol in the plates? If not, the big colonies may be ones in which the pLysS plasmid has been lost, because it inhibits growth. On the other hand, the big colonies might be the ones WITH pLysS, because background expression of your gene is killing the cells in the absence of pLysS. Streak the big and little on chloramphenicol to see what you have.

When I induce my cells, they stop growing. After 3 hours, there is stringy stuff in the flask. After incubating overnight, there are a lot of cells; but, I can hardly see my protein. What gives?

If the OD level is off when you induce the cells, that's normal. If the OD drops, that means that the induced protein is lethal. Overnight, SOMETHING grows, and it's probably something that has killed your protein gene. *So what do I do?* Grow to 1 OD before induction, and harvest in 1 to 3 hours, whatever your test expression told you to do.

I left my cells in the ice bucket overnight. Can I still use them?

Don't waste your time unless you are desperate.

How come I didn't get any transformants?

- Try the control DNA to make sure the cells are competent.
- Does this donor work on any cells?
- Use less DNA.
- Is the selection appropriate to the plasmid?
- If you concentrate the cells by centrifugation, be gentle. Try plating 0.1 mL before concentrating the rest of them.

My plates look like one big colony.

Is there antibiotic in the plate? If not, remember it next time. Was the plate wet? These cells are swimmers (much more so than cloning strains are).

When I do the control and calculate transformation efficiency, I get a number that is 2-4 times as high as the specification.

The spec is a minimum. We are getting BL21's at 10^8 quite often.

My freezer died, but the temperature only went to -50°C before I transferred them. Can I still use them? Expect a 2- to 5-fold loss in efficiency.

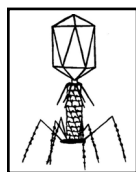
People in my lab keep putting my cells on the bench while they root around for their samples in the -70°C. Can I still use them?

People don't mean any harm. They probably figure that if the cells are only out for a minute and don't thaw, they're OK. That's natural. Wrong, but natural. Your cells are losing potency over time because of this, but only slowly. Try putting the cells in a different place so they are not in people's way.

I checked the plasmid in my BL21 transformant and the digest looks like it is all degraded.

BL21 derivatives have an endonuclease that degrades all DNA. Extract the plasmid prep with an equal volume of phenol, and then ethanol precipitate. The yield should be more than 50% and the resulting plasmid prep should be fine.

SECTION 11: AMPLIQON COMPETENT CELLS - TECHNICAL FEATURES



T1 Phage and its relatives

Ampliqon BL21 (DE3) Competent Cells have been genetically modified so that the cells are resistant to bacteriophage T1. Thus, they protect your work and laboratory from infection by phage T1 or one of its ugly relatives. It's a bonus! BL21 strains are typically sensitive to T1 and other phage.

The T1 resistance marker is nice to have. Here's why:

- T1 and its relatives are nasty phage that kill *E. coli*.
- T1 is present in some libraries of cDNA clones that get passed around between labs and genome centers.
- If you have experienced T1 phage contamination, you will never forget it. It's total devastation for your clones.
- The T1 resistance marker protects your clones. It does not impair them.

SECTION 12: GENERAL REFERENCES

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Rare codons. Zhan, K. (1996) Overexpression of an mRNA dependent on rare codons inhibits protein synthesis and cell growth. *J Bacteriol.* 178: 2926-2933.

Fusion proteins:

To a modified DsbA

Zhang, Y. DR Olsen, K.B. Nguyen, P.S. Olson, E.T. Rhodes and D. Mascarenhas (1998) Expression of eukaryotic proteins in soluble form in *Escherichia coli*. *Protein Expr. Purif.* 12:159-165.

To a phage protein

Ferrer P. and R. Jaussi (1998) High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage lambda head protein D. *Gene* 224: 45-52.

To maltose binding protein

Kapust, R.B. and D.S. Waugh (1999) *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci.* 8: 1668-1674.

To anything, but the order of fusion matters

Sachdev, D. and J.M. Chirgwin (1998) Order of fusions between bacterial and mammalian proteins can determine solubility in *Escherichia coli*. *Biochem Biophys. Res. Commun.* 244: 933-937.

Proteases. Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annu. Rev. Genet.* 30: 465-506.

SECTION 13: ORDERING INFORMATION

COMPETENT CELLS for PROTEIN EXPRESSION			
Size	Cat. No.	Price	
BL21 (DE3)			
5 x 200 µL aliquots	AMP852005	€112	Protein Expression
BL21 (DE3) pLysS			
5 x 200 µL aliquots	AMP862005	€112	Protein Expression
COMPETENT CELLS for CLONING			
GC5 HIGH EFFICIENCY			
5 x 200 µL aliquots	AMP802005	€116	> 1 x 10⁹ transformants/µg pUC19
20 x 50 µL aliquots	AMP805020	€126	
10 x 50 µL aliquots	AMP805010	€80	
GC5 IN PLATES inquire			> 1 x 10⁸ transformants/µg pUC19
GC5 VALUE EFFICIENCY			
10 x 200 µL aliquots	AMP812010	€70	> 1 x 10⁸ transformants/µg pUC19
GC10 HIGH EFFICIENCY			
5 x 200 µL aliquots	AMP822005	€116	> 1 x 10⁹ transformants/µg pUC19
20 x 50 µL aliquots	AMP825020	€136	
10 x 50 µL aliquots	AMP825010	€80	
GC10 SUPERPATH ELECTROCOMPETENT			
5 x 80 µL aliquots	AMP830805	€140	> 1 x 10¹⁰ transformants/µg pUC19
5 x 100 µL aliquots	AMP831005	€176	
JM109 HIGH EFFICIENCY			
5 x 200 µL aliquots	AMP842005	€112	> 1 x 10⁸ transformants/µg pUC19

USBio MEDIA



Pre-weighed media in the bottle

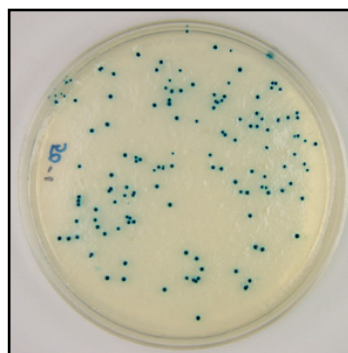
See www.biomol.de

Add water • Autoclave • Ready to grow

CONSISTENT RESULTS -
TOTAL CONFIDENCE!

REAGENTS

Size	Cat. No.	Price
X-GAL		
1 gram	02249-1	€62
IPTG		
5 grams	05684-2	€95
SOC MEDIUM		
10 x 10 mL	AMP800000	€26



TO ORDER:

Tel.: 0800-2466651 (D) • Fax: 0800-2466652 (D)
+49-40-8532600 • +49-40-85326022

FOR MORE INFORMATION:

Biomol Technical Service: ts@biomol.de • www.biomol.de
Tel.: +49-40-853260-23/-27/-37

Ampliqon III

Biomol GmbH • Waidmannstr. 35 • 22769 Hamburg • Tel.: 0800-2466651 • FAX 0800-2466652
www.biomol.de • Biomol Technical Service: ts@biomol.de • info@biomol.de