nextgensciences™

> guide to high efficiency transformation

competentcells

No matter where you get the DNA you wish to use in your transformation, you had to work to get it. You want to make the most of it and know that this step can be automated. Competent cells have to be reliable and as efficient as possible. This guide describes the efficiencies and strain properties that are available to make it easier to make the choice for automating transformations.

INTRODUCTION TO COMPETENT *E. COLI*

There are two ways to transform *E. coli:* chemical transformation and electroporation. Chemical transformation is more convenient and electroporation is more efficient. Each method, and factors that affect them, are described in detail below.

Types of *E. coli*

Many different strains of *E. coli* are available, and nearly all of them are derivatives of a single strain called K12. K12 was first isolated in 1922. BL21 comes from *E. coli* strain B, first described in 1946. Labs have passed around (mostly K12) strains and made mutants and shuffled around mutations until the average strain had a long list of genetic markers called a genotype.

Strains may 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 molecular biology experiments. **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. **Specialty strains** have particular properties that are occasionally used.

PROPERTIES OF CLONING STRAINS

This section features the properties of cloning strains that people find most useful. The genotypes that are responsible for these features appear in parentheses.

Blue/white Screening (Δ(*lac*ZYA-*arg*F)U169 Φ80d*lac* Δ(*lac*Z)M15) pUC19 and similar plasmids code for β-galactosidase (*lac*Z), which cleaves X-gal and turns colonies blue on an X-gal plate. Inserts cloned into the plasmid disrupt the β-galactosidase gene and the colonies are white. Actually, the plasmids only code for a small part of the β-galactosidase gene (called the α peptide), and the chromosome codes for the rest. Both parts are required for activity. Since the plasmid is complementing the chromosomal mutation, this effect is called "α complementation". More on blue/white screening is discussed in the "Special Technical Features" section below.

Recombination Deficient (*rec*A)

E. coli has a repair system which will recombine homologous sequences. Many people fear that recombination can cause plasmids to rearrange or delete insertions because of recombination. For this reason, cloning strains are traditionally *rec*A mutants. *Rec*A strains have the advantage of having simple plasmid profiles (mostly CC

monomer) whereas *Rec*+ strains have dimers, trimers, etc. and their nicked relatives that make uncut plasmid lanes complicated.

Genomic clones often have duplicated regions, but these duplications are usually rather short, tandem duplications. They are unstable, but this instability is not due to *rec*A function. If the length of the duplicated sequence is less than 200 bp, *rec*A has no effect whatsoever.

The *rec*A repair system is useful to *E. coli* and disabling it causes the cells to grow slower and be less healthy. For this reason, some expression strains (like BL21) are not *rec*A mutants.

Endonuclease Deficient (*end*A)

E. coli has a powerful endonuclease on the outside of the cell that degrades any type of DNA. *End*Amutants are devoid of this activity. The *end*A endonuclease has little or no effect on transformation efficiency, but can have a profound effect on the quality of plasmid DNA preparation. If plasmid DNA preps degrade when placed in magnesiumcontaining buffers, it is usually because the DNA was made from an *End*A+ bug.

Restriction Deficient (hsdRK-)

Most lab strains are *E. coli* K12 derivatives. K12 strains methylate their DNA at K12 sites (AAC(N6)GTGC and GCAC(N6)GTT). In K12 strains, DNA that is not methylated at these sites is degraded by a restriction enzyme. Many, but not all, cloning strains of *E. coli* are mutated in the gene that codes for this restriction enzyme. BL21 does not methylate, nor does it restrict unmethylated DNA.

Methyl Restriction Deficient (mcrA, mcrB, mrr)

E. coli has a system of enzymes that degrade DNA if it is methylated at the "wrong" sites. Genomic DNA from eukaryotic sources is methylated at all the wrong sites, as far as *E. coli* is concerned. When cloning genomic DNA from eukaryotic cells, it is essential to use a host that is deficient in all three of these methyl restriction systems. On the other hand, when cloning PCR fragments, cDNA, or fragments from previously made clones, there is no methyl restriction and it is not necessary to use a methyl restriction deficient host.

Single Strand Ability (F+ or F')

F is a huge plasmid (99 kb) that is naturally found in *E. coli* K12. There are derivatives of the F plasmid that also contain chromosomal DNA. These F derivatives with a bit of chromosomal DNA are called F' ("F prime") plasmid. *E. coli* with the F (or F') plasmid make special surface features that allow them to be infected with M13 and similar phage. This property is useful if one wants to make single-stranded DNA or generate phage display libraries.

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. This is described in

more detail in the section "Competent Cells - Special Technical Features."

Lac Promoter Control (LacIQ)

Even though gene expression is not being covered in this guide, it is important when making plasmid constructs to keep your expression promoter off until you are ready to turn it on. High level expression of many genes is deterimental for the host. When this happens, mutants that do not express the gene at a high level grow faster and take over the culture. Some expression systems use the lac, tac, or trc promoters to express cloned genes on high copy plasmids, but do not have the cognate repressors on the expression plasmid. Under these circumstances, there is not enough lac repressor to keep the promoters off. A mutant that produces more lac repressor (LacIQ) can repress lac, tac, and trc promoters until IPTG is added to induce them.

	GC5™	GC10™	JM109
Blue/white screening	✓	✓	✓
Recombination deficient	✓	✓	✓
Endonuclease deficient	✓	✓	✓
Restriction deficient	✓	✓	✓
Methyl restriction deficient	—	✓	—
Single strand ability	—	—	✓
Phage resistance	✓	✓	—
Lac promoter control	—	—	✓

FACTORS THAT AFFECT TRANSFORMATION

Forms of DNA

Relaxed plasmids, which are formed in ligation reactions, transform *E. coli* with the same effciency as supercoiled plasmids. Linear plasmids and single-stranded plasmids transform very poorly (< 1% as efficiently as double strand circles). A special host is needed to achieve chromosomal transformation, which is very inefficient. Usually, a transformation involves a mixture of linear (nontransforming) and circular (transforming) DNA.

Amount of DNA

It seems obvious that if you add more DNA to a transformation, you get more transformants. For chemically competent cells; however, adding more than 10 ng of pUC19 DNA does not result in significantly more transformants. The point of diminishing returns is about 100 ng of pUC19 for electrocompetent cells.

But what happens with ligations? A ligation will have insert DNA, linear vector, re-circularized vector, and vector with insert (both circular and linear). The concentration of all these components together is usually about 50 ng/µL. Even on a good day, the nontransforming DNA will be in the majority—will it compete out the transforming DNA? Not usually. With 20 ng of total DNA per reaction, the non transforming DNA will decrease the efficiency of the transforming DNA only about 2-fold for chemically competent cells, and not at all for electrocompetent cells.

If the ligation reaction is concentrated by precipitation and 500 ng are added to a single reaction, the competition effects can drop the transformation efficiency 10-fold for chemically competent cells, but will still not affect electroporation.

Source of DNA

DNA from eukaryotes is heavily methylated. *E. coli* have restriction systems that restrict these types of methylation. When cloning any genomic DNA, it is wise to use a *mcr* mutant like GC10. DNA generated by PCR is unmethylated, so cloning a PCR fragment from genomic DNA does not require a *mcr* mutant.

Other problems with Donor DNA

Donor DNA should not have detergent, phenol, alcohol, PEG, or DNA-binding protein in it. For electroporation, donor DNA cannot have salt in it either. DNA in TE buffer works well. Ligase and PEG strongly inhibit transformation. A central problem in molecular biology is that both of these are components of most ligation reactions. The best thing to do is to precipitate the ligation mixture (see the Section on "Protocols for Transformation" for details). Most people just dilute the ligation mixture 3-fold and transform with one µL.

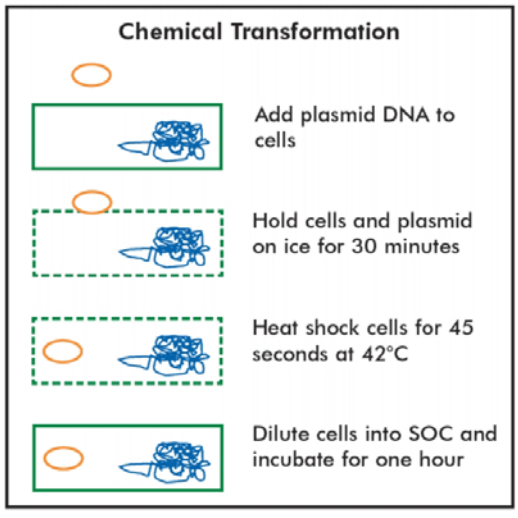
Storage & Handling

On arrival: Competent cells arrive in dry ice and need to be kept at -80°C to keep them at their efficiency. You can keep your cells for a number of months if you take a few precautions. First, clear out the space in the -80°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 -80°C freezer and shut the door. You don't have to rush, but even when they stay frozen, cells lose efficiency as they warm up.

Storage. We've all worked in labs, so we all know that -80°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 -80°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 take some dry ice and place the cells in (not on) the dry ice while everything is shuffled around. If your freezer fails but your cells don't thaw, you may lose efficiency. If your cells thaw, you probably need to replace them.

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 -80°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.

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 to10^7 for most home-made cells).

WHAT'S IMPORTANT IN CHEMICAL TRANSFORMATION?

Transformation protocols are described in detail in the Section "Protocols for Transformation."

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°C. If you are busy and the cells stay in the ice bucket for an hour, that's fine. Beyond that, efficiency drops by 2-fold every hour.

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, if you are performing the transformations manually, it's a corner to cut if you are pressed for time and efficiency is not a critical issue.

The heat shock

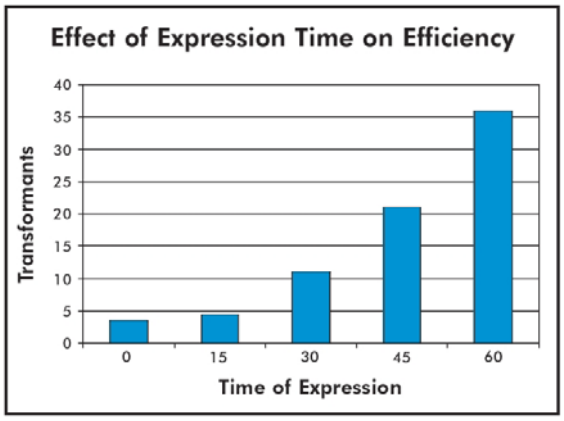
The heat shock works best in a Falcon 2059 tube with a 42°C bath. Lots of people have 37°C but not 42°C baths. A 45 second heat shock at 42°C gives the best results; but, one minute at 37°C works almost as well (down 2-fold).

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.

Agar 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; and, throw the plates out after 3 months.



PROTOCOLS FOR TRANSFORMATION

Notes on Ligation Reactions:

Ligation reactions inhibit transformation.

Less transformants are observed from ligation reactions than from transformations with plasmid DNA. Use of 0.2 µL of a ligation reaction per 20 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 0.5 µL per 20 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. Refreezing thawed competent cells will result in a significant drop in transformation efficiency.

Transformation of Chemically Competent Cells Advance Preparations

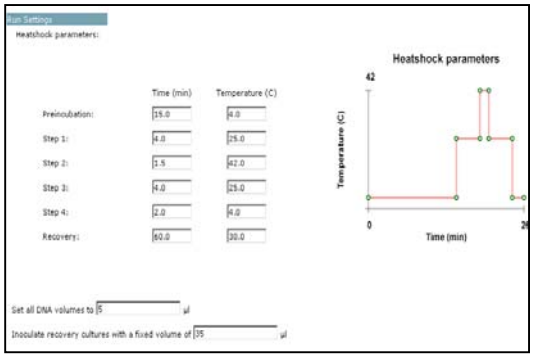
- Equilibrate a non-shaking water bath to 42°C.
- Place SOC medium at room temperature.
- Prepare LB agar plates with the appropriate antibiotic. If blue/white screening for recombinants is desired, the plates should include 40 µg/mL X-gal and 1 mM IPTG.
- Agar plates should be placed in a 37°C incubator for about 30 minutes prior to plating.

Manual procedure

1. Remove competent cells from -70 °C and place directly in ice. Thaw cells for 5 to 10 minutes.
2. Gently mix cells by tapping the plate.
3. Add 1-20 ng of DNA (or 1 µL control DNA) into the 20 µL competent cells. Swirl the pipettor tip through the cells while dispensing DNA. Gently tap tube to mix.
4. Place the tubes on ice for 30 minutes.
5. Heat-shock the cells for 45 seconds in a 42 °C water bath. Do not shake.
6. Add 450 µL of room temperature SOC medium to each transformation reaction.

7. Incubate at 37 °C for one hour with shaking (225 to 250 rpm).
8. Spread on LB agar plates containing appropriate antibiotic (e.g., 100 µg/mL ampicillin for controlpUC19).
9. Incubate the plates at 37 °C overnight (12 to 16hours).

Automated procedures for transformation are performed by the expressionfactory™ and the expressionworkstation™. Contact NextGen Sciences for details.



Set-up screen from the NextGen Sciences' orchestratorIMS software that controls the robotics systems

TROUBLESHOOTING YOUR TRANSFORM-ATION

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

Impurities in the DNA

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

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

Inhibition of Chemical Transformation by Ligation Mixture

The section "Factors That Affect Transformation" covers this subject in detail. The best way is to ethanol precipitate the ligation and resuspend the pellet in an equal volume of TE. In this case, use 1 µL of resuspended DNA per 100 µL competent cells. Alternatively, dilute the ligation 3X and add 1 µL per 50 µL competent cells.

Adding Too Much Ligation 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 µL 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. For electrotransformation, the added salt lowers efficiency or causes arcing. 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 the section, "Protocols for Transformation."
2. Dilute the ligation 3-fold in TE and use 1 µL per transformation.

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 GC5 High Efficiency competent cells (10^9 transformants/µg) with 1 µg of pUC19, you will not get 10^9 transformants because the cells are saturated at 0.01 µg of pUC19 in a 100 µL reaction. The efficiency is instead measured with 50 picograms of pUC19 in a reaction. An efficiency of 10^9 transformants/ µg is the same as 10^3 transformants/pg; so 50 pg should generate 50 x 10^3 transformants. On the expressionfactory and expressionworkstation, we dilute such a transformation 100-fold, plate 0.1 mL, and look for 50 colonies.

Likewise, an efficiency of 10^{10} transformants per µg (10^4 transformants/pg) can be measured by putting 20 pg in a reaction and looking for 20 x 10^4 transformants, i.e., 200 colonies when 0.1 mL of a 100-fold dilution is plated.

Sample Calculation:

colonies on control plate / ng of control DNA plated X 1000 ng / µg = T/µg

For example, adding 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 diluted in 900 µL SOC and 100 µL is plated (equivalent to 0.001 ng DNA). If 100 colonies are counted on the plate, the transformation efficiency is calculated as follows:

100 cfu on control plate / 0.001 ng of control DNA plated X 1000 ng / µg = T/µg

100 colonies / 0.001 ng X 1000 ng/µg = 1 x 108 T/µg.

FREQUENTLY ASKED QUESTIONS

How come I didn't get any transformants (chemical transformation)?

- Try the control DNA to make sure the cells are competent.
- Does this donor work on any cells? Maybe the ligation did not work.
- Is the selection appropriate to the plasmid? (don't laugh - it happens)
- If you concentrate the cells by centrifugation, be gentle. Try plating 0.1 mL before concentrating the rest of them.

My plate looks like the stars in the sky - big colonies down to tiny colonies, all sizes.

The selection is off. Streak cells that should grow on the selection, and cells that should not. The cells that should grow are struggling; you have too much antibiotic. If the cells that should not grow are growing where the streak is heaviest, you don't have enough antibiotic. If there is too much antibiotic, you are losing transformants. Try half as much. If there is not enough antibiotic, then you are getting "breakthrough" of nontransformed cells that are mutants to a low level of resistance. Try using twice as much antibiotic.

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

The spec is a minimum you can expect to achieved from our cells and you may find that the efficiencies are higher than specified.

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

Not recommended.

My freezer died but the temperature only went to-50°C before I transferred the cells. Can I still use them?

Expect a 2- to 5-fold loss inefficiency.

People in my lab keep putting my cells on the bench while they root around for their samples in the -80°C. Can I still use them?

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.

By the time I get through with my ligation, there is only an hour left before I have to go home. Is there any way to shorten the protocol?

You can, as described in the section "What's Important in Chemical Transformation" on page 4. You will sacrifice some efficiency. If you need the most possible transformants, don't cut corners. If you usually get more colonies than you test anyway, start by cutting the expression time to 30 minutes, and thaw the tube with your fingers to save time. Alternatively, you can always automate this process …..

NextGen Sciences' COMPETENT CELLS – SPECIAL TECHNICAL FEATURES

T1 Phage and Its Relatives

NextGen Sciences' GC5 and GC10 Competent Cells protect your work and laboratory from infection by bacteriophage T1 or one of its relatives.

The T1 resistance marker is important to have:

- 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.

Blue / white Screening

Blue/white screening is a popular tool for quick and easy identification of recombinants. NextGen Sciences' GC5, GC10, and JM109 strains all carry the *lac*ZΔM15 marker that enables blue/white screening.

*Genotypic markers*Δ*lac* - deletes β−gal gene

Φ80d*lac* - makes β peptide

*lac*ZΔM15 - makes β peptide

X-gal is a chromogenic substrate for β -gal (*lac*Z).

When cleaved, a blue color is produced.

How Does It Work?

Cells - Need to make the β peptide, but not the whole β -galactosidase (β -gal) molecule.

Plasmids - Carry the β peptide sequence for β -gal in the multiple cloning site (MCS).

Host strains - Genotypes with the *lac*ZM15 marker lack the β peptide sequence for β gal. The β peptide must be supplied by the plasmid. When DNA is inserted into the MCS of the plasmid, the β gene is interrupted. There is no β peptide to complement the β -gal protein and thus the β -gal is not functional. When transformed, colonies containing the plasmid + insert (recombinants) are white. If the β gene is still intact (non recombinants), β -gal is activated and the colonies are blue.

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nextgensciences™

North America and Canada
fax to: 1-888-566-4383 (toll free)

Europe and rest of World
Fax to: +44 (0) 1480 410 858

email to: sales@nextgensciences.com