

TECHNIQUES IN MOLECULAR BIOLOGY – BASIC *E. COLI* INFORMATION FOR MOLECULAR BIOLOGY

Common *E. coli* strains used in molecular biology. Most *Escherichia coli* (*E. coli*) strains used by most molecular biology applications are rod-shaped bacteria named after Dr. Theodor Escherich, who first described these intestinal gram-negative bacteria. There are two parent strains for the hundreds of *E. coli* commonly used. These are K-12 and B strain bacteria. K-12, isolated from feces of a diphtheria patient lead to DH5a while the B strain eventually became BL21 strain after passing through the laboratories of several scientists. The *E. coli* strains are commonly used because they easily grow on solid and liquid medium at a fairly high doubling rate of 20-30 min. An overnight culture from an isolated colony can yield about 2 billion cells per milliliter in liquid culture. From these two strains (there are other less commonly used strains) many new, related bacterial strains have been generated by mutating genomic genes and or adding additional new genes.

Nomenclature - Each strain of *E. coli* has a unique genotype associated with an observed behavior (phenotype). The various strains are named by a standard nomenclature. Genes are given three-letter, lowercase, italicized names that are often mnemonics. An example is the *ara* mutation. This mutation leaves a blockage in arabinose (a carbohydrate) metabolism.

Properties of Common Genotypes of <i>E. coli</i> Host Strains		
<i>Amy</i>	Expresses amylase	Allows amylose utilization
<i>dam</i>	Blocks adenine methylation at GATC sequences	Makes DNA susceptible to cleavage by some restriction sites
(DE3)	λ lysogen carrying the gene for T7 RNA polymerase	Used for T7 promoter-based expression systems
<i>dnaJ</i>	Inactivation of a specific chaperon	Stabilizes expression of some recombinant proteins
<i>endA1</i>	Activity of nonspecific endonuclease I is abolished	Improves yield and quality of isolated plasmid DNA
<i>lacZ</i>	Lactose permease activity is abolished	Blocks lactose uptake; improves IPTG-induced control of <i>lac</i> promoters
<i>lon</i>	Inactivation of Lon protease	Increased yield of protein expression
<i>mutS</i>	Deficient in mismatch repair	Stabilizes DNA heteroduplexes during site-directed mutagenesis
<i>ompT</i>	Mutation in outer-membrane protease	Improves yield of some recombinant proteins

From: *Methods in Molecular Biology*, Vol. 235: *E. coli* Plasmid Vectors. Ed. Casali and Preson. Humana press Inc.

DE3. Bacterial cells, usually BL21 strains that carry an extra gene notated DE3. Lambda (λ) DE3 gene expresses the T7 RNA polymerase. Some scientists use plasmids that are under the control of the T7 expression system. The plasmid does not include the T7 RNA polymerase and thus expression requires the DE3 gene already in the host cell. BL21(DE3) strain is often used because in addition to the T7 RNA polymerase, the strain is deficient in several proteases (OmpT and others) but is RecA positive making it a poor choice for DNA fidelity as the recombinase will likely alter plasmids fiving a poor quality DNA prep.

General Cloning Host Strains - There are a number of *E. coli* strains that carry mutations that improve the quality and stability of DNA. Simply put, these strains are less likely to damage, mutate or degrade the plasmid DNA as they are missing or have mutated recombinases, endo- and exonucleases and other important proteins. These strains also carry mutations that limit their ability to create all of the metabolites needed for cell growth and therefore are unable to grow in the wild or in their original source, the intestine. There are several additional safety features that include a way to stop the plasmids (vectors) from moving into the wild-type *E. coli* strains generating mutant bugs with antibiotic resistance. One mechanism to prevent the transfer of plasmids to wild bacteria are plasmid vectors that include "suppressor mutations" leading to termination with amber (UAG) or

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ochre (UAA) mutations. Most host *E. coli* strains include suppressors against these nonsense chain-terminating mutations. Wild-type cells do not and thus the plasmids will not express in these cells.

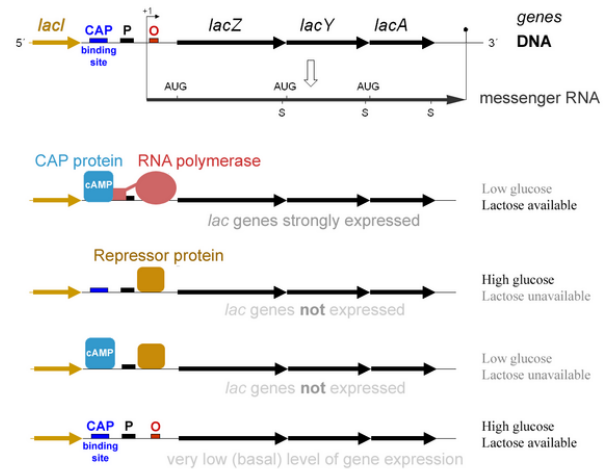
Blue-White Screening - Using the *lac* operon, molecular biologists can use a technique called "blue-white screen" to rapidly and easily determine if a piece of DNA is inserted (ligated) into a plasmid vector. After transfection (see below) cells are grown in the presence of a synthetic carbohydrate lactose mimic called X-gal (5-bromo-4-chloro-3-indoyl- β -D galactopyranoside). X-gal can be degraded by the enzyme β galactosidase coded by the *LacZ* gene. The product of the enzyme then dimerizes and oxidizes into an insoluble blue colored precipitate.

But first we need to start at the beginning... The *lac* operon consists of several genes, three code for enzymes involved in lactose metabolism. In the absence of lactose, the suite (operon) of genes are relatively silent, except for the *LacZ* gene which will express a small amount of β galactosidase. Without lactose, a repressor protein *LacI*, binds to the *lac* operator and prevents transcription by blocking polymerase function. When lactose is present, it will be converted to allolactose by β galactosidase through an alternative pathway which will then in turn, bind to the repressor and allow the operon to then be expressed. IPTG is an allolactose analogue that will also bind to the repressor and is used to activate the *lac* operon gene expression.

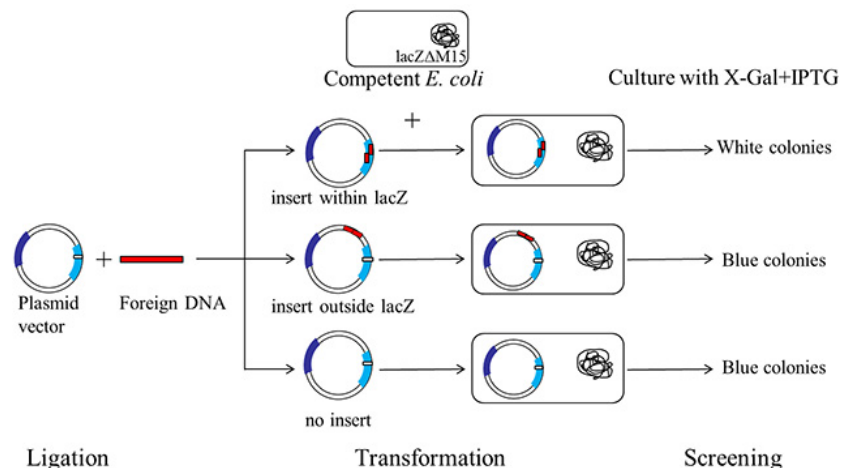
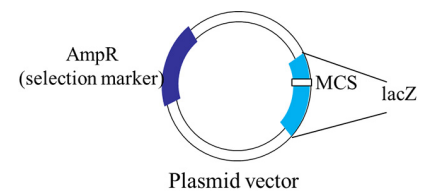
Thus the presence of IPTG or Lactose can induce the full expression of the *lac* operon. Remember that X-gal is a mimic of lactose. So addition of X-gal will induce the expression of β galactosidase causing the enzyme to degrade X-gal into the blue precipitate.

The selection of an appropriate host cell for blue-white screening is critical and based on alpha complementation. Cells bearing 5' deletion in *lacZ* produce an inactive C-terminal fragment of β galactosidase (also called the ω -fragment). Similarly cells with a 3' deletion in *LacZ* produce an N-terminal β galactosidase fragment (commonly labeled as the α -fragment). If these two fragments come together β galactosidase is restored. Together, having a host cell with the gene for one half of a protein and a plasmid vector with the other half of a protein is called alpha-complementation. The vector expresses the N terminal fragment while the cell produces the C-terminal fragment. Fragments of DNA are cloned into a vector in a multiple cloning site (MCS) that lies in the middle of the α -fragment. When a DNA fragment is ligated into the MCS the α -fragment is not expressed and β galactosidase is not functional. However, if the DNA is not ligated into

The *lac* Operon and its Control Elements



The *Lac* operon Wiki Commons



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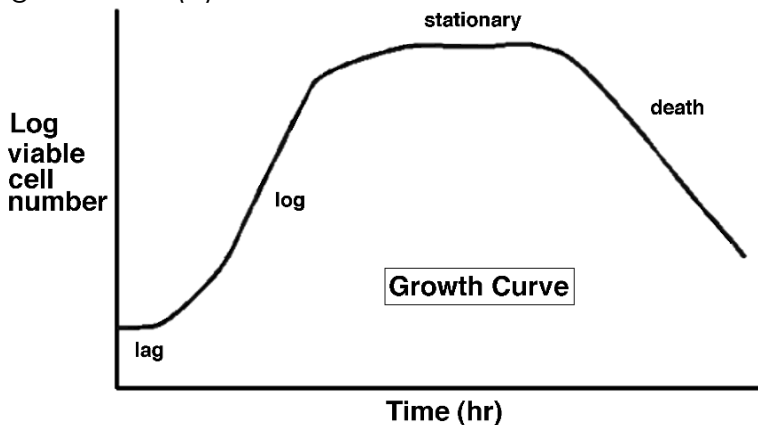
the MCS or into another site on the plasmid, the α -fragment remains intact and expressed to form a functional β galactosidase.

To select for recombinant *E. coli*, bacilli are grown on media containing IPTG (to express the operon) and X-gal (for identification of active β galactosidase). Cells that have successfully cloned the insert will be white, while blue colonies (see figure for example) will not contain the properly ligated insert.



Image source: Wikimedia Commons; Accessed 6/3/15. Author: Stefan Walkowski

Bacterial Cell Growth and Storage - As discussed in the experimental handout, a typical growth curve for bacteria consists of several phases. A lag phase where newly inoculated cultures slowly increase in number until they reach a density and the cell cycle machinery kicks in when they double at a logarithmic rate (exponential or log phase). In the log phase, cells are growing by geometric progression. Dividing at a constant rate (cell dependent) $2^0, 2^1, 2^2 \dots 2^n$ where n = the number of generations. The doubling time also known as the generation time (G) is the time (t) per generation (n) where $G=t/n$.



Eventually the nutrients and density of cells (a phenomena called quorum sensing) is maintained as cells cease division. This is the stationary phase. At this point the ratio of plasmid DNA to RNA higher than in the stationary phase and is the preferred phase for DNA purification. Eventually the cells lose viability and begin to die, the death phase. Purifying DNA from cells in this phase will be poor as the DNA is being degraded.

Therefore, maintaining cells in a log phase is

critical for healthy cells, whether you are propagating cells to purify DNA or to express protein, knowing the density of the cells in terms of their phase is critical for successful experimental results.

There are a number of ways to measure cell density, including diluting a known volume onto an agar plate and counting the number of resulting colonies. Each colony will represent a single bacteria inoculated onto the plate. However a rapid and simpler approach is to measure the absorbance of a culture at 600 (sometimes 550) nm in a spectrophotometer. The measurement will not be a true absorbance (where a compound absorbs energy at a specific wavelength of light), but is instead considered the optical density (OD). The OD reading of a bacterial culture is a measure of the scattering of light of a cell suspension. As visible light passes through a suspension of cells, light is scattered (not absorbed) by the cells. The more cells the more scattering and less light is transmitted through the sample. The method to measure OD is straightforward. Blank the spectrophotometer with the broth/media, then read the "absorbance" of your cell culture. Depending on the instrument, 0.5 ml will be sufficient. The absorbance is the optical density value. A quick estimation of cell density can be roughly measured by determining the mass of a cell pellet. A 1-liter overnight culture with a cell density of $3-4 \times 10^9$ cells per ml will have a pellet mass of about 3 grams.

Bacterial cultures can be started (inoculated) from a number of sources, diluted from a liquid culture (formally expanding a culture), a thick growth on a stab (tube of agar), a colony can be picked from a plate, or a chip of frozen glycerol stock (see below). For plasmid and protein production, these should always be grown from a freshly streaked plate. Starting from mixed populations (glycerol stocks, old stabs) is considered poor microbiological practice as the expression or number of plasmids

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may of the cells may not be homogeneous. However, in a pinch it can be done, but shouldn't be a routine practice. For most *E. coli* strains, a single colony should be inoculated into 2-10 ml of medium and grown for 6-8 hours when they should be in the logarithmic growth phase. This culture is then used to inoculate larger volumes. Often diluting 1/500 to 1/1000 from the initial volume.

Long-term storage of cells can be maintained for several years at -80°C in what is commonly called a glycerol stock. A late log phase/stationary, overnight culture is diluted in equal parts with a 50% (v/v) sterile glycerol-water solution. It is critical to avoid freeze thaw cycles or even a significant warming of the frozen culture to maintain viability. To culture from a glycerol stock, simply use a pipette tip and scrape off a small bit of cells onto an agar plate before spreading. Remember, not to allow your glycerol stock thaw!

Bacteria Media - Bacteria is grown in liquid media (sometimes called broths) or on solid (typically the same composition with addition of ~12g of agar per liter). There are several types of media used for *E. coli*. Bacteria are auxotrophs because they cannot provide all of the nutrients for growth on their own and thus be included in the medium. Most current media includes complex mixture of proteins degraded by proteases. A few common examples include beef protein extract (peptone), milk casein/whey proteins (tryptone), soybean meal (soytone), and yeast protein extract. Some bacterial strains may require blood serum or other components. For most *E. coli* strains used in molecular biology, there are only a few common types of media using a mixture of protein extract, salt, sugar source and are adjusted to a prescribed pH.

The most common *E. coli* medium types are: Luria Bertani (LB) used for growth and maintenance of *E. coli* strains; Terrific Broth (TB), a nutritionally rich media for high density growth; 2X-YT broth used for replication of M13 vectors; Super Optimal Broth (SOB) used as a rich medium for bacterial growth and sometimes in the preparation of competent cells; and Super Optimal broth with Catabolite repression (SOC), used after transformation as an outgrowth to support transfection and cell recovery.

As mentioned most common mediums include salts, one or more protein extract and various compounds (sugars, metals, vitamins). The most common, LB medium is prepared as shown below:

Tryptone	10g/liter
Yeast Extract	5 g/liter
NaCl	10 g/liter
pH adjusted to 7.0 before autoclaving.	

The pH of the medium should be adjusted before autoclaving, even when using a prepared packaged broth. When autoclaving to sterilize, it is important not to overheat or leave the medium in the autoclave longer than needed to finish the sterilization cycle. There are several components including sugars, proteins and other nutrients that can caramelize (react to form a dark brown Maillard compound) and will no longer serve as a quality growth medium for cells. Many antibiotics will not tolerate the high temperatures of autoclaving and will be hydrolyzed or react in another way with the compounds of the medium. The best practice is to prepare the medium and then when cooled between 60-40°C before adding. Care must be taken with agar medium. Mix well after adding antibiotic to avoid zones of high and low antibiotic concentration.

Antibiotics - Antibiotics should be used throughout all stages of cell growth/culture. Each plasmid must contain an antibiotic resistance. More appropriately stated, each plasmid will bear a gene for a protein that will specifically degrade an antibiotic. Only the cells with the plasmid will express this

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protein and thus can survive and/or divide in the presence of the antibiotic. Preparation and common usage of antibiotics is shown in the table below:

Antibiotic	Stock Concentration	Storage	Working Conc (dilution)
Ampicillin (Sodium Salt)	50 mg/ml in water (500X)	-20° C	100µg/ml (2 µl of stock/ml)
Chloramphenicol	34 mg/ml in EtOH (200X)	-20° C	170 µg/ml (5 µl of stock/ml)
Kanamycin	25 mg/ml in water (500x)	-20° C	50 µg/ml (2 µl of stock/ml)
Streptomycin	10 mg/ml in water (200X)	-20° C	50 µg/ml (5 µl of stock/ml)
Tetracycline HCl	5 mg/ml in EtOH (100X)	-20° C	50 µg/ml (10 µl of stock/ml)
Carbenicillin	50 mg/ml in EtOH (500X)	-20° C	100 µg/ml (2 µl of stock/ml)

Ampicillin and Kanamycin are two commonly used antibiotics in modern plasmids. Kanamycin is an aminoglycoside antibiotic which acts to bind and inhibit the 70S ribosomal subunit, blocking translocation and reducing protein production in cells. Kanamycin resistant plasmids code for the kanamycin B resistance (also known as neomycin phosphotransferase II; NEO) gene. The enzyme will phosphorylate kanamycin, rendering the drug inactive to bind and inhibit 70S ribosomal function. Ampicillin inhibits synthesis of the bacterial cell wall (in bacteria like *E. coli*, found between the inner and outer cell membranes), resulting in bacteria that are very structurally weak. In the hypotonic media in which these cells grow, cells exposed to ampicillin will swell and burst or not grow at all. For cells to survive, they must include a means to break down the ampicillin. The plasmid has an additional gene coding for an enzyme, β -lactamase, that is secreted by cells and in a local area will hydrolyze the ampicillin. Therefore, by adding ampicillin, only bacteria that contain the plasmid will survive. We also need to be sure not to allow our transformed *E. coli* to become overgrown. If the colonies on the LB plates are large they will break down enough ampicillin so that bacteria without the plasmid will survive and form satellite colonies in the surrounding region of inactivated antibiotic. Finally carbenicillin (Carb) is an ampicillin analog often used in place of Amp. Carbenicillin also inhibits in the same way as ampicillin but is much more slowly degraded than Amp. This reduces the growth of satellite colonies on plates and liquid media during long-term incubation.