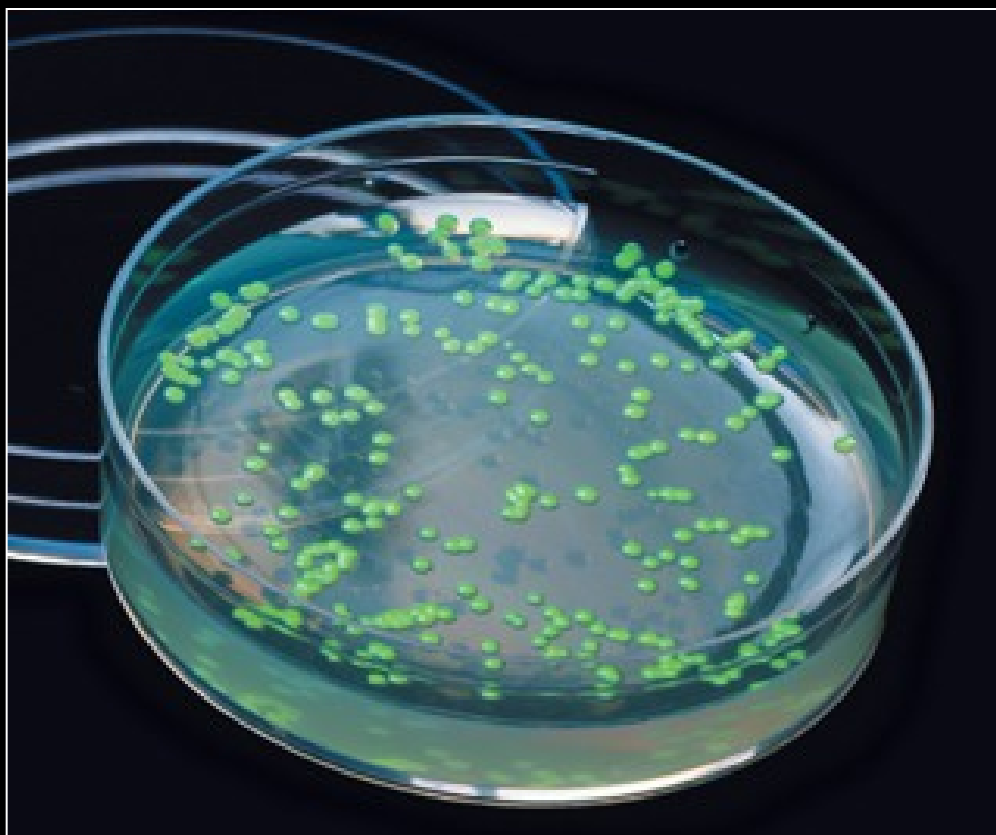




pGLO Bacterial Transformation Practical





What is transformation?

- Genetic transformation literally means change caused by genes.
- It occurs when a cell takes up (takes inside) and expresses a new piece of genetic material—DNA.
- This new genetic information often provides the organism with a new trait.
- Genetic transformation is used in many areas of biotechnology.
- In agriculture, genes coding for traits such as frost, pest, or drought resistance can be genetically transformed into plants.
- In bioremediation, bacteria can be genetically transformed with genes enabling them to digest oil spills.
- In medicine, diseases caused by defective genes are beginning to be treated by gene therapy; that is, by genetically transforming a sick person's cells with healthy copies of the defective gene that causes their disease.



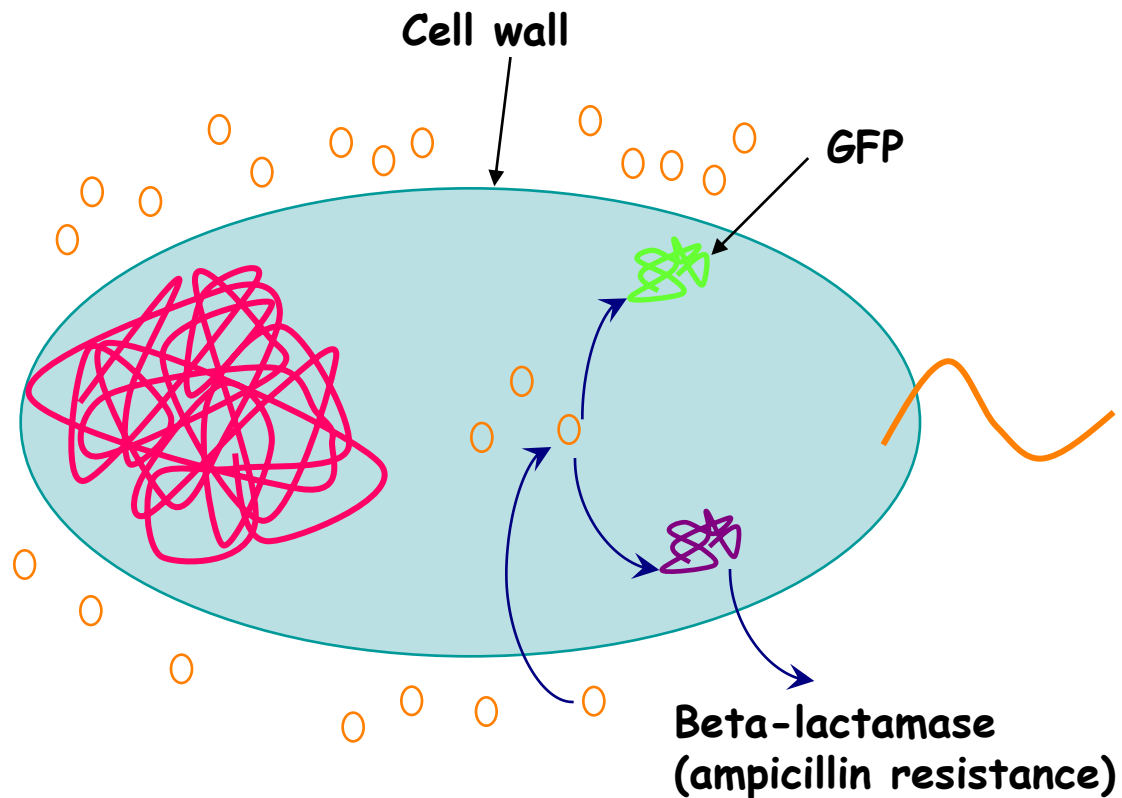
Bacteria

- **Why bacteria?**

- To genetically transform an entire organism, you must insert the new gene(s) into every cell in the organism. Which organism is better suited for total genetic transformation—one composed of many cells, or one composed of a single cell?
- Scientists often want to know if the genetically transformed organism can pass its new traits on to its offspring and future generations. To get this information, which would be a better candidate for your investigation, an organism in which each new generation develops and reproduces quickly, or one which does this more slowly?
- Safety is another important consideration in choosing an experimental organism.
- **Note:** The bacterium *Escherichia coli* (*E. coli*) strain HB101 K-12, best fits the requirements described above: it is made of only one cell, it reproduces every 20 minutes, it does not make people sick, and it cannot survive outside the laboratory

Bacteria

- One large chromosome
- Naturally contain one or more small circular pieces of DNA called plasmids.
- Plasmid DNA usually contains genes for one or more traits that may be beneficial to bacterial survival.
- In nature, bacteria can transfer plasmids back and forth, allowing them to share these beneficial genes.
- This natural mechanism allows bacteria to adapt to new environments.
- The recent occurrence of bacterial resistance to antibiotics is due to the transmission of plasmids.



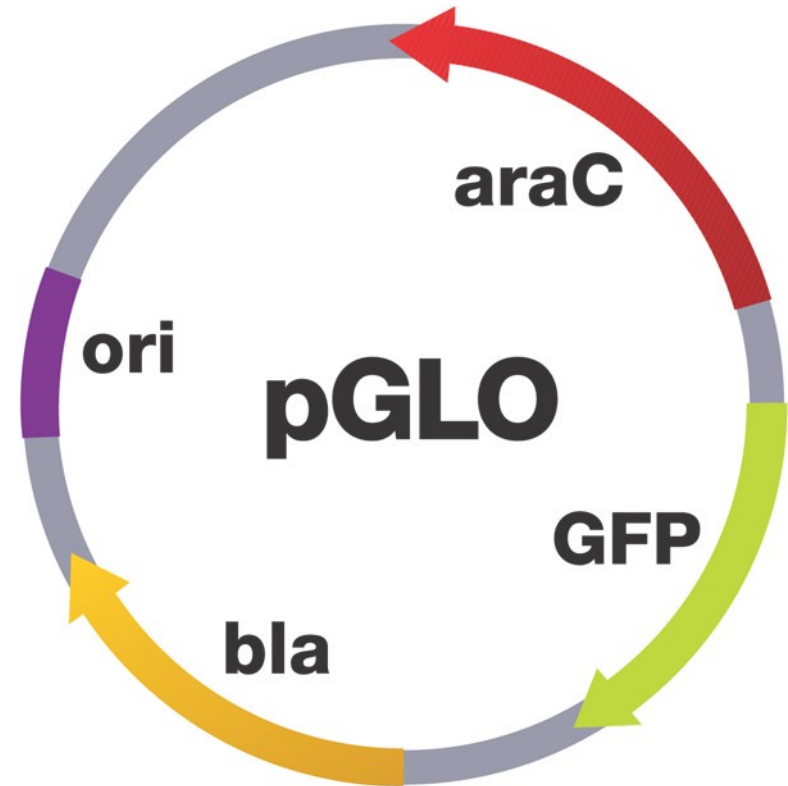


Our plasmid

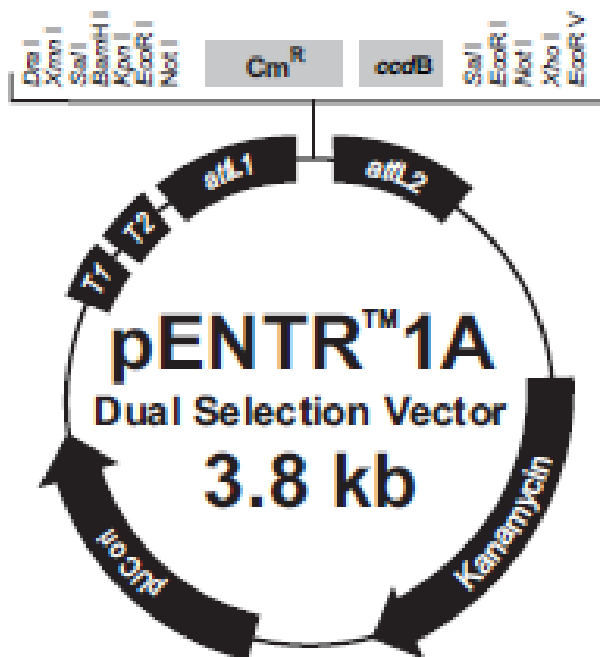
A circular DNA molecule, capable of self-replicating. Extra-chromosomal

- **Beta Lactamase**
 - Resistance to ampicillin
- **Green Fluorescent Protein (GFP)**
 - *Aequorea victoria* jellyfish gene
- **araC**
 - Regulates GFP transcription

DNA → RNA → PROTEIN → TRAIT



Plasmids



Comments for pENTR™ 1A
3754 nucleotides

rrnB T1 transcription termination sequence: bases 106-149

rrnB T2 transcription termination sequence: bases 281-308

attL1: bases 358-457 (complementary strand)

Chloramphenicol resistance gene (Cm^R): bases 608-1266

ccdB gene: bases 1608-1913

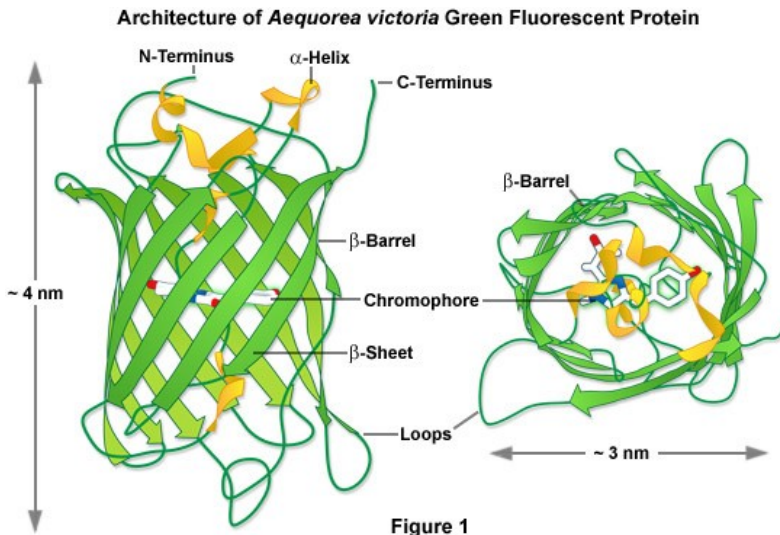
attL2: bases 1983-2082

Kanamycin resistance gene: bases 2205-3014

pUC origin: bases 3078-3751

Feature	Benefit
<i>rrnB</i> T1 and T2 transcription termination sequences	Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz <i>et al.</i> , 1991)
<i>attL1</i> and <i>attL2</i> sites	Allows site-specific recombination of the entry clone with a Gateway® destination vector (Landy, 1989)
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of expression clones
<i>ccdB</i> gene	Allows negative selection of expression clones
Kanamycin resistance gene	Allows selection of the plasmid in <i>E. coli</i>
pUC origin	Allows maintenance of the plasmid in <i>E. coli</i>

- The gene for GFP was originally isolated from the jellyfish, *Aequorea victoria*.
- The wild-type jellyfish gene has been modified by a biotech company; specific mutations were introduced into the DNA sequence, which greatly enhance fluorescence of the protein.
- This modified form of the GFP gene has been inserted into our pGLO plasmid.



Early work on GFP:
Douglas Prasher & Virginia Eckenrode (WHOI),
Roger Heim, Andrew Cubitt
S. James Remington (U. Or.)

CFP/YFP
Atsushi Miyawaki, Tullio Pozzan (Padova)
Jiang, Alice Ting, Brian Bacskai

FRET sensors:
Nathan Shaner, Varda Lev-Ram,
Mark Ellisman (UCSD)

RFPs, IFPs, miniSOG:
Frederick Y. C. Baird, Larry Gross,
Nathan Shaner, Varda Lev-Ram,
Mark Ellisman (UCSD)

Sunset with green flash as viewed from a C

iBiology.org



Aim

Explore mechanisms of genetic selection and gene regulation

- 1) The pGLO plasmid contains the gene for GFP and a gene for resistance to the antibiotic ampicillin.
- 2) pGLO also incorporates a special gene regulation system that can be used to control expression of the fluorescent protein in transformed cells. The gene for GFP can be switched on in transformed cells simply by adding the sugar arabinose to the cell's nutrient medium.



Important points

Antibiotic Selection

- The pGLO plasmid, which contains the GFP gene, also contains the gene for beta-lactamase, which provides resistance to the antibiotic ampicillin, a member of the penicillin family. The beta-lactamase protein is produced and secreted by bacteria that contain the plasmid.
- Beta-lactamase inactivates the ampicillin present in the LB nutrient agar to allow bacterial growth.
- Only transformed bacteria that contain the plasmid and express beta-lactamase can grow on plates that contain ampicillin. Only a very small percentage of the cells take up the plasmid DNA and are transformed. Untransformed cells cannot grow on the ampicillin selection plates.



Important points

Gene Regulation

What advantage would there be for an organism to be able to turn on or off particular genes in response to certain conditions?



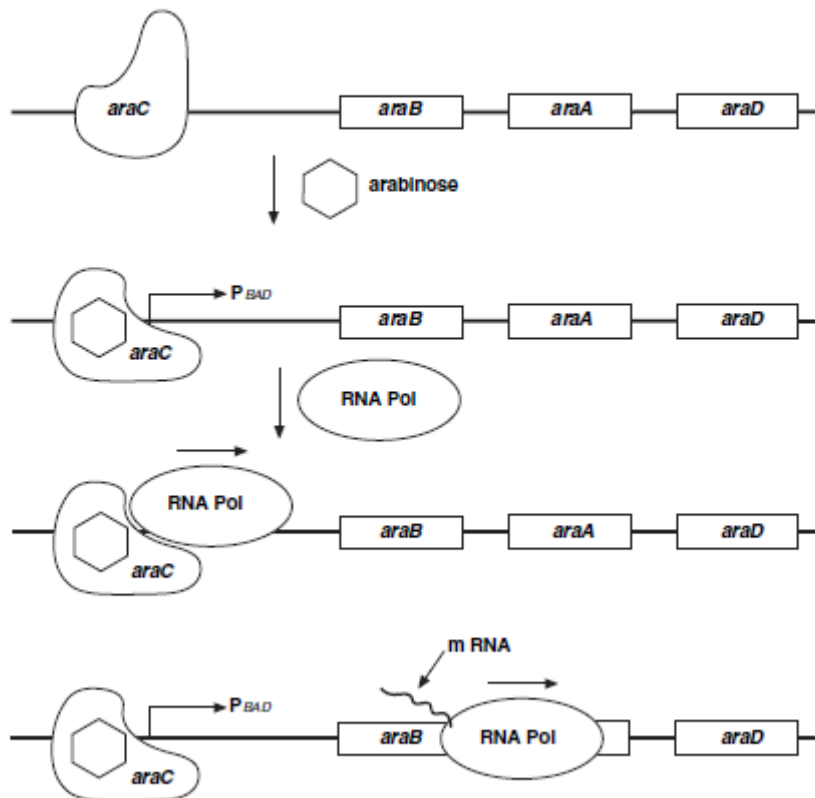
Important points

- Adaptation to differing conditions and prevention of wasteful overproduction of unneeded proteins.
- The genes involved in the breakdown of different food sources are good examples of highly regulated genes.
- For example, the simple plant sugar arabinose is a source of both energy and carbon for bacteria.
- The bacterial genes that make digestive enzymes to break down arabinose for food are not expressed when arabinose is not in the environment. But when arabinose is present, these genes are turned on. When the arabinose runs out, the genes are turned off again.
- Arabinose initiates transcription of these genes by promoting the binding of RNA polymerase.
- In the pGLO plasmid DNA, some of the genes involved in the breakdown of arabinose have been replaced by the jellyfish gene that codes for GFP. In the presence of arabinose, the GFP gene is turned on, and the bacteria glow brilliant green when exposed to UV light.

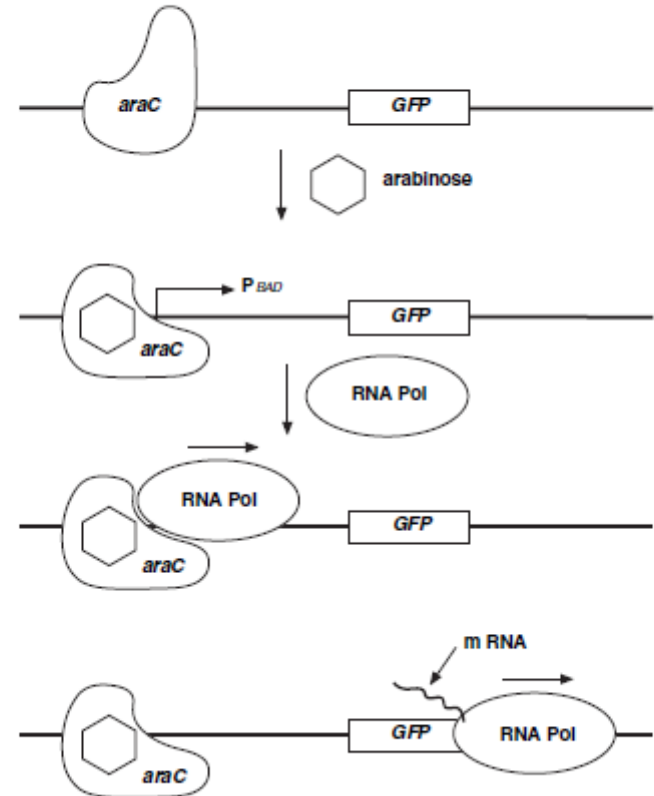
Gene regulation

an **operon** is a functioning unit of genomic DNA containing a cluster of genes under the control of a single promoter

The Arabinose Operon

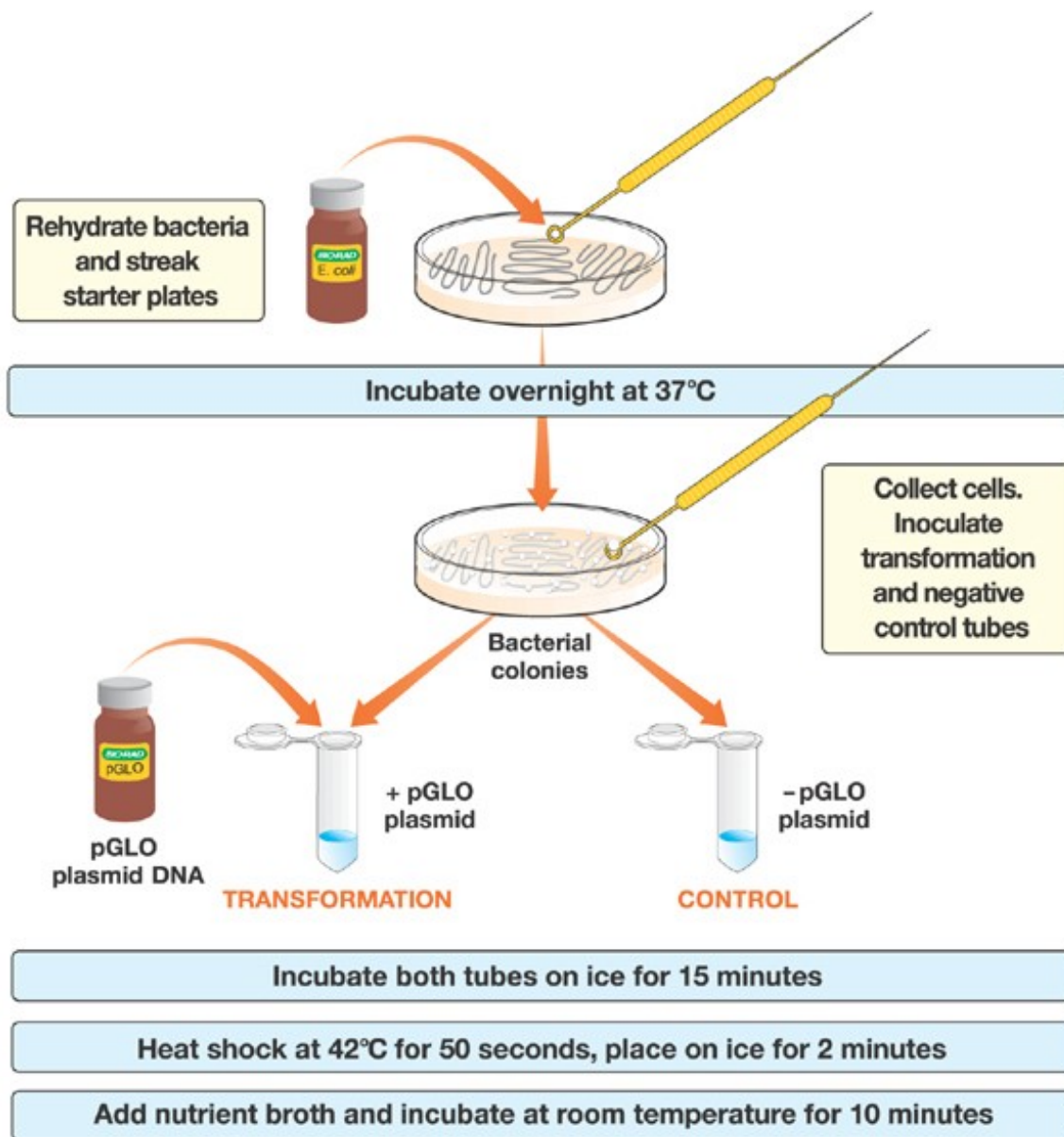


Expression of Green Fluorescent Protein



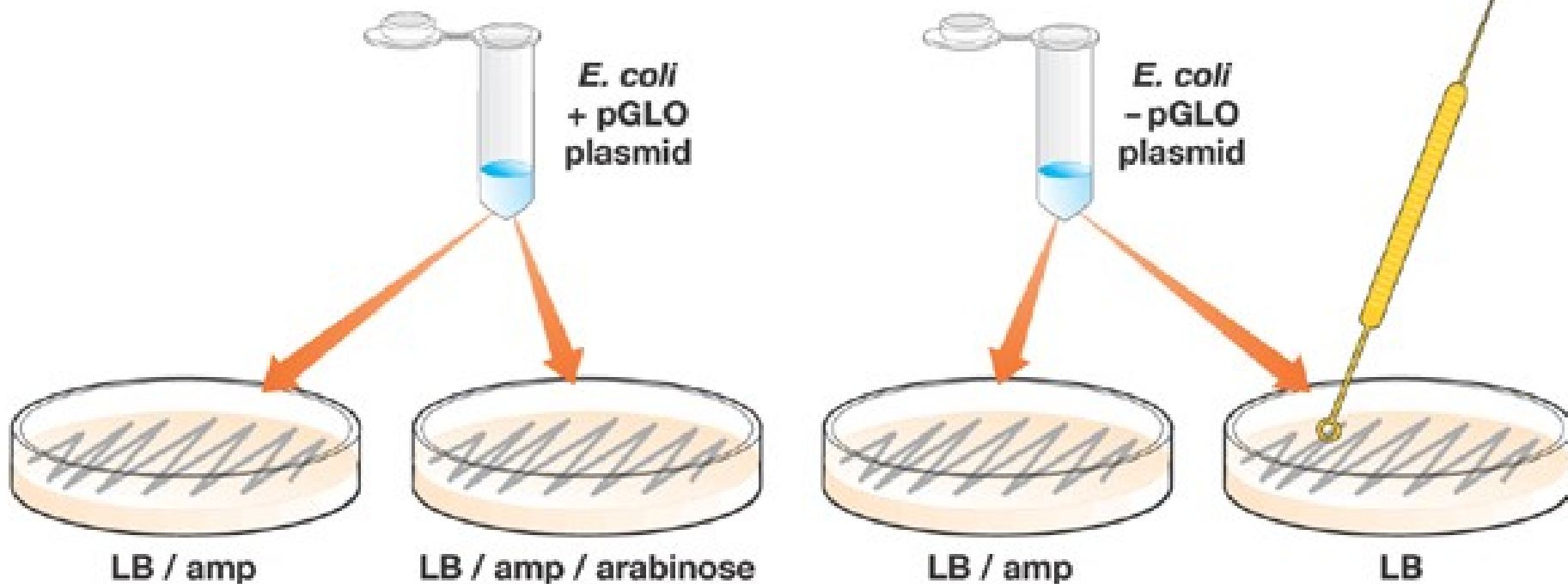


Experimental protocol



Experimental protocol

Spread bacterial suspensions onto plates



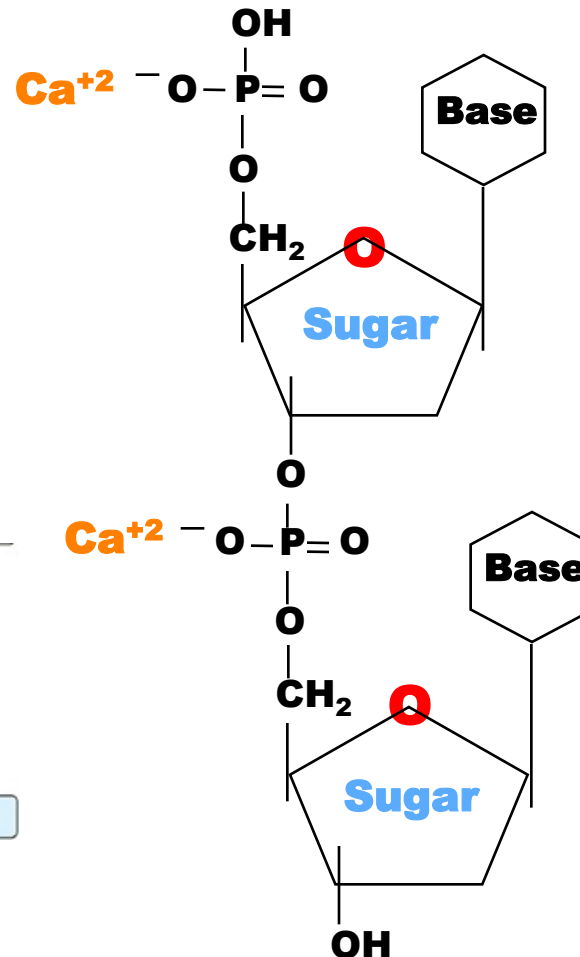
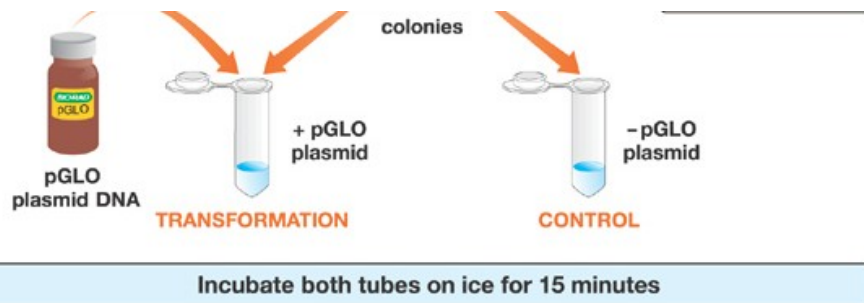
Incubate overnight at 37°C

Step by step-transformation solution

Why did we resuspend the bacterial colonies in transformation solution?

Transformation solution = CaCl_2

The positive charge of Ca^{+2} ions shields the negative charge of the phosphate groups

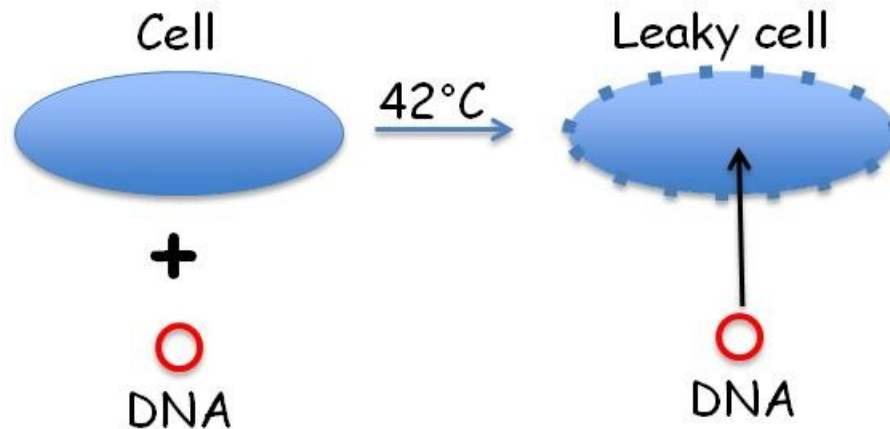




Step by step-heat shock

Heat shock at 42°C for 50 seconds, place on ice for 2 minutes

Chemically-competent cells uptake DNA after heat shock as it increases permeability of cell membrane





Step by step-LB nutrient broth

Add nutrient broth and incubate at room temperature for 10 minutes



- Luria-Bertani (LB) broth: made from an extract of yeast and an enzymatic digest of meat byproducts
- Medium that contains nutrients for bacterial growth and gene expression
 - Carbohydrates
 - Amino acids
 - Nucleotides
 - Salts
 - Vitamines
- Agar is derived from seaweed.
- It melts when heated, forms a solid gel when cooled (analogous to Jello-O), and functions to provide a solid support on which to culture bacteria.



Step by step-10 min incubation

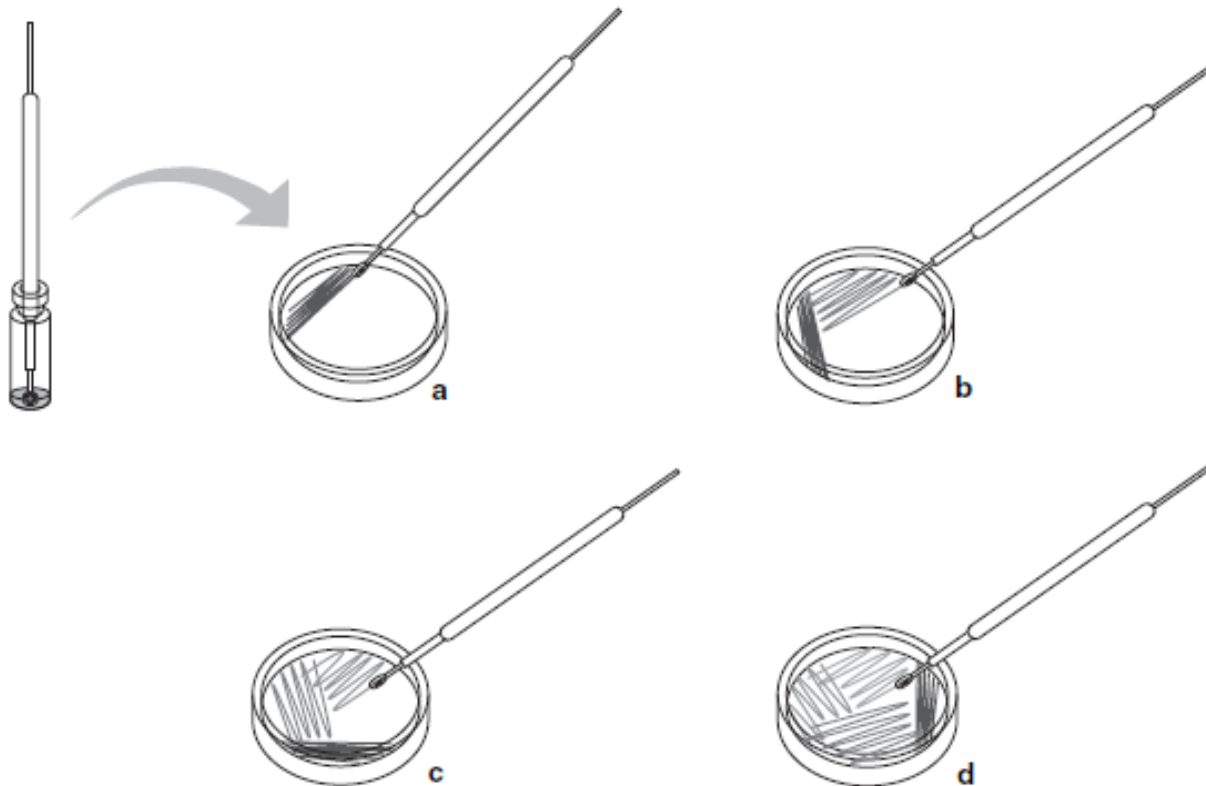
Add nutrient broth and incubate at room temperature for 10 minutes

The 10-min incubation period following the addition of LB nutrient broth allows the cells to recover and to express the ampicillin resistance protein beta-lactamase so that the transformed cells survive on the ampicillin selection plates.



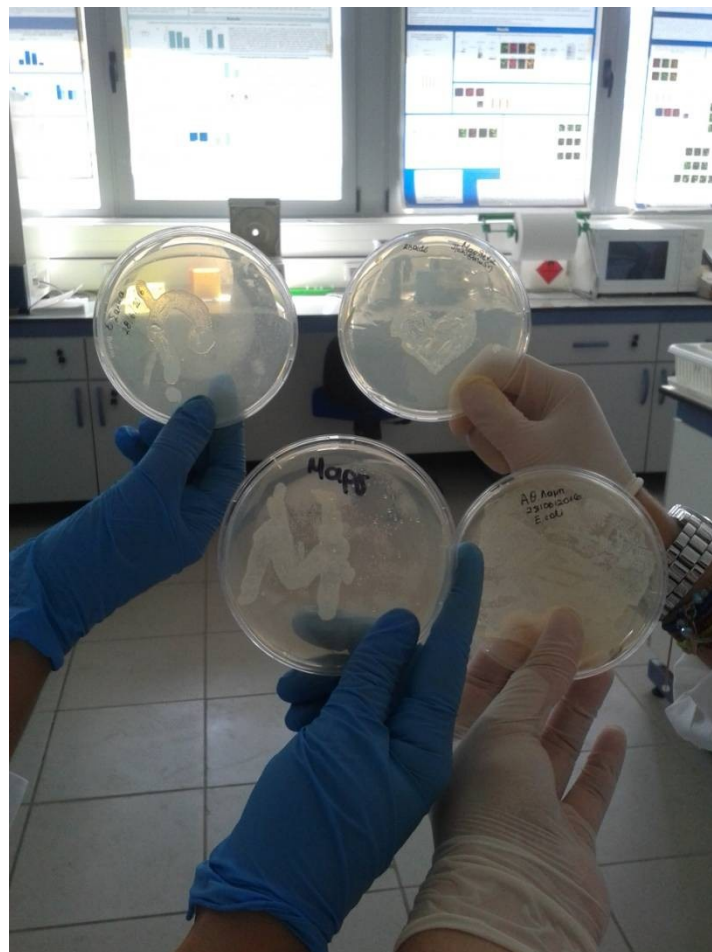
Step by step-streaking

The purpose of streaking is to generate single colonies from a concentrated suspension of bacteria. A minute amount of the bacterial suspension goes a long way. Under favorable conditions, one cell multiplies to become millions of genetically identical cells in just 24 hr.



A clump of genetically identical bacterial cells growing on an agar plate. Because all the cells in a single colony are genetically identical, they are called clones.

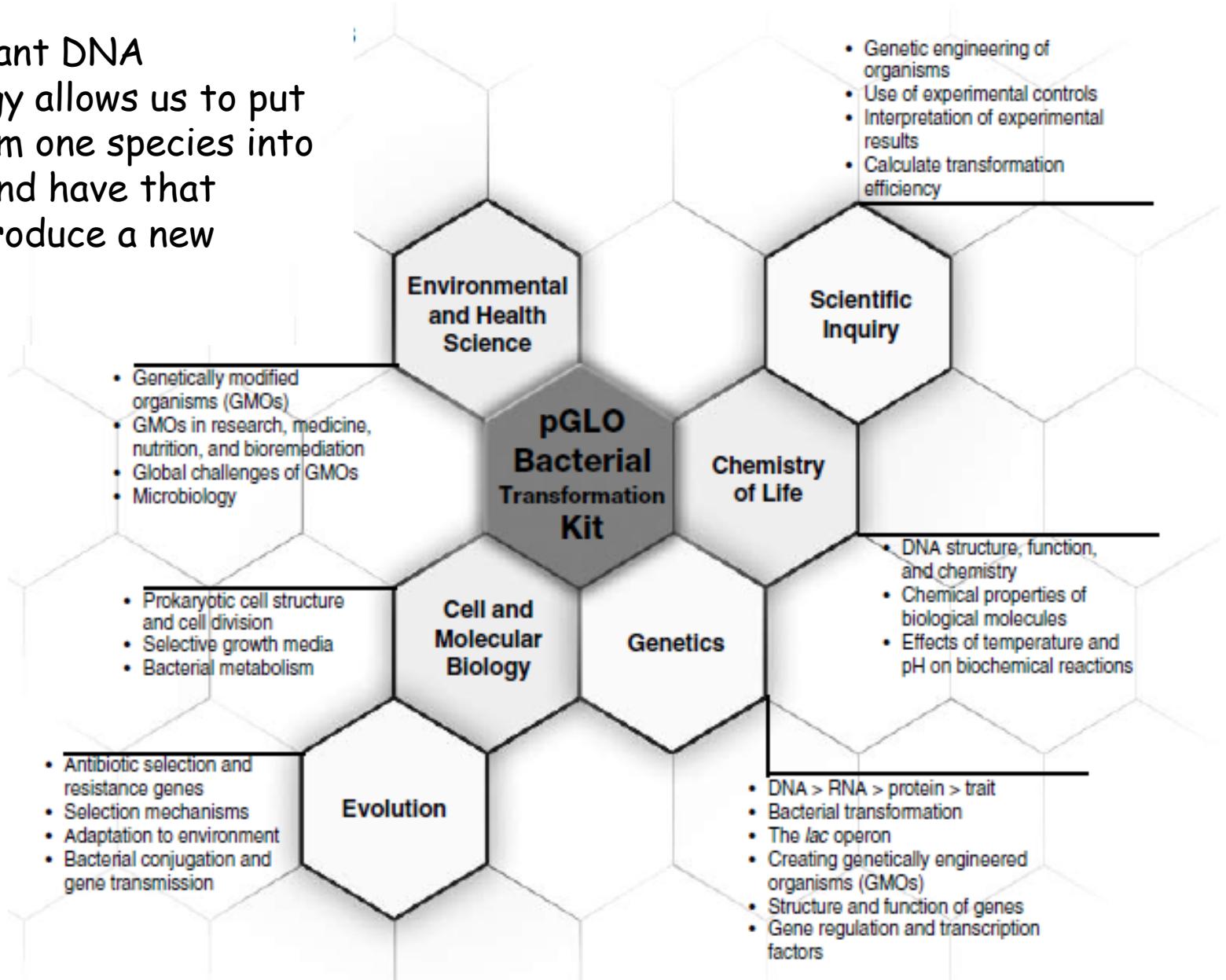
Step by step-streaking





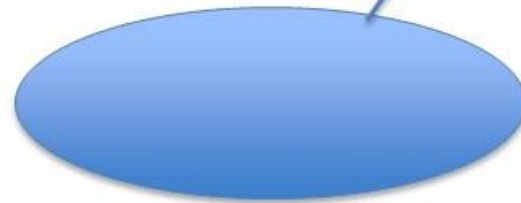
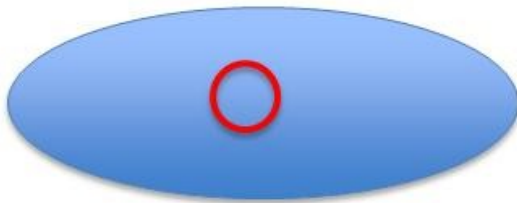
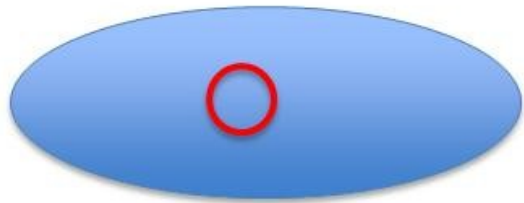
Biotechnology

Recombinant DNA Technology allows us to put genes from one species into another and have that species produce a new protein.



Quiz

Transformed cell



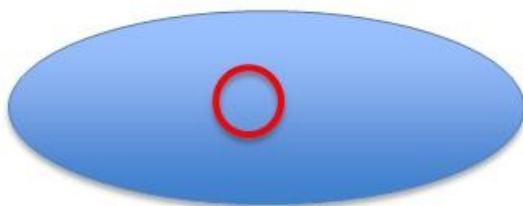
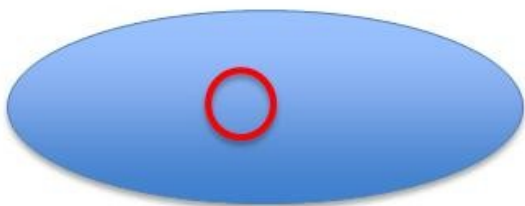
Untransformed cells

Growth plate

Include an antibiotic

Quiz

Transformed cell



Untransformed cells die

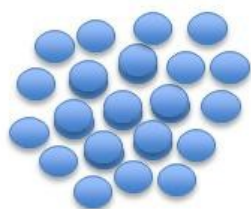
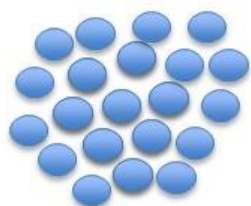
Growth plate

Include an antibiotic
(ampicillin)

Quiz

Include initiator (Arabinose sugar)

Transformed cells



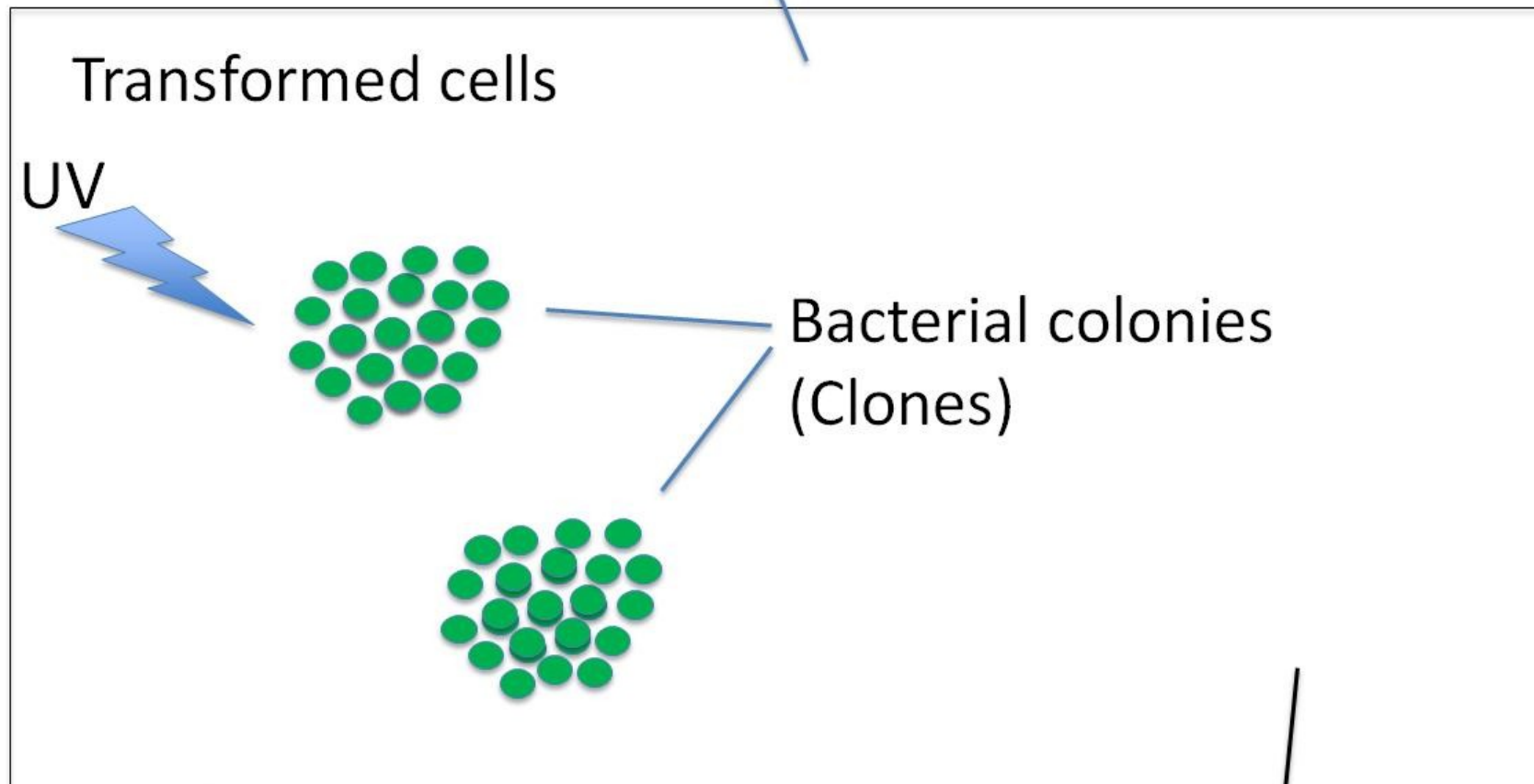
Bacterial colonies
(Clones)

Growth plate

Include an antibiotic

Quiz

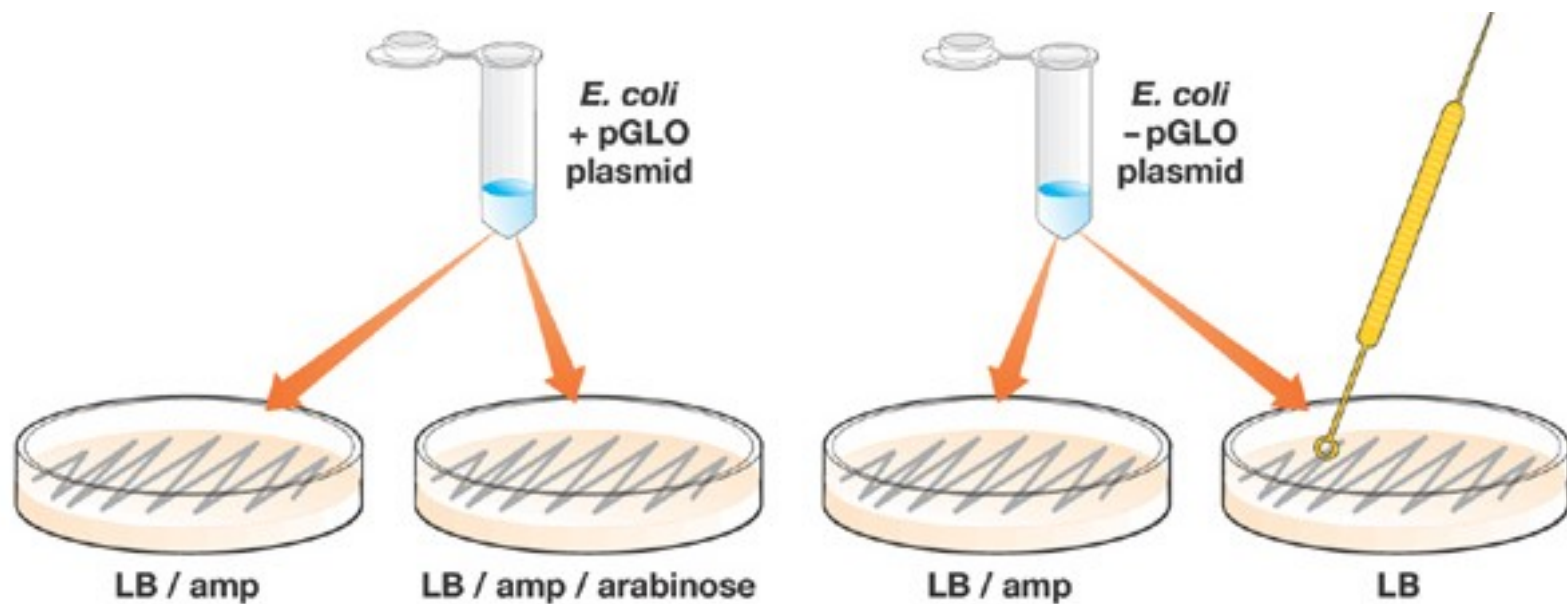
Include initiator (Arabinose sugar)



Growth plate

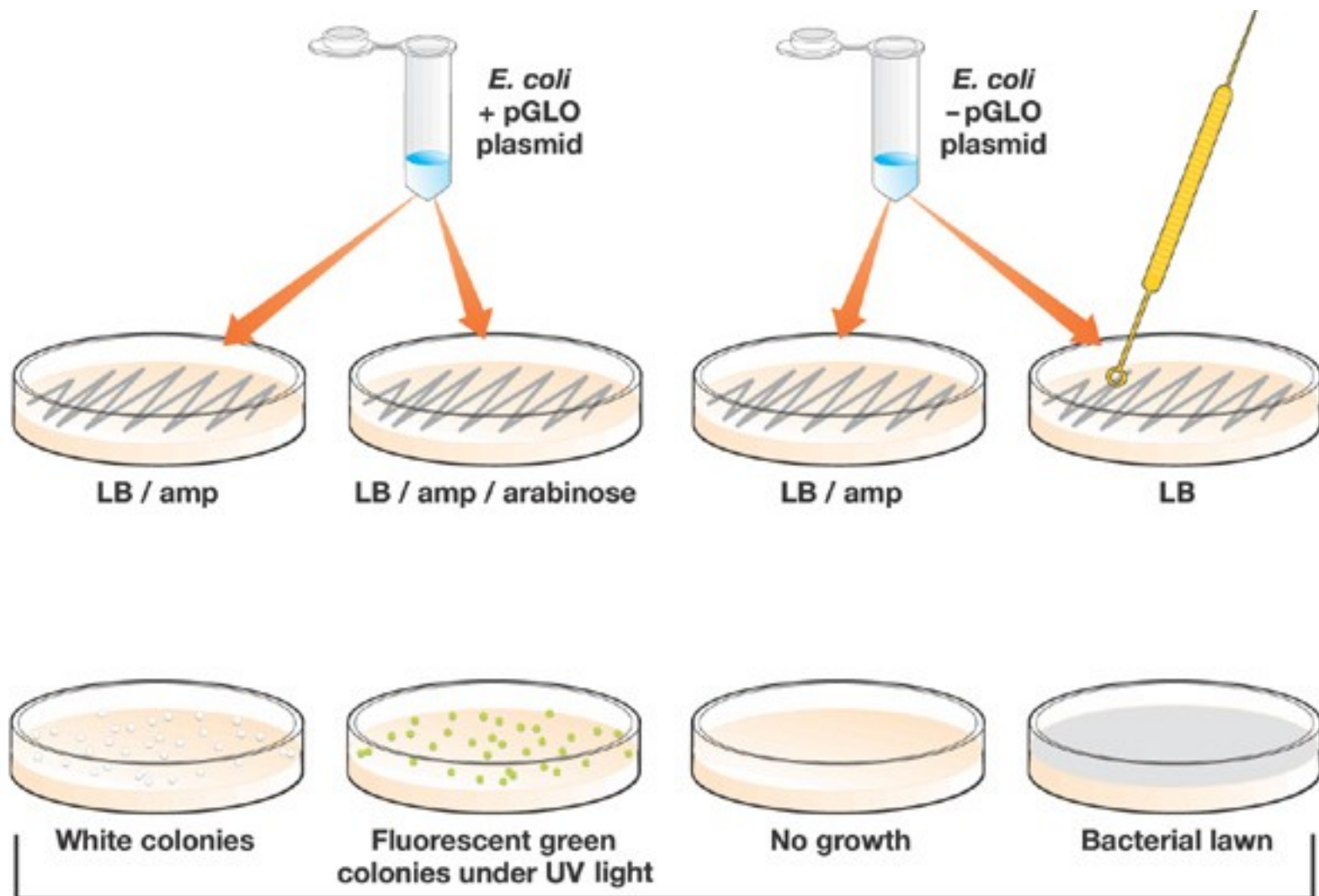
Include an antibiotic

Assessment of results





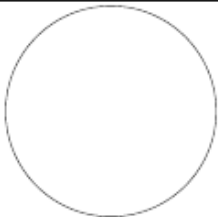
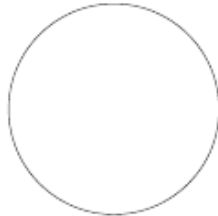
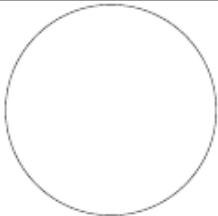
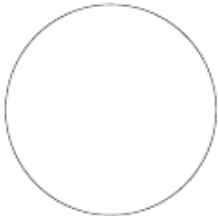
Assessment of results





Assessment of results

4. How many bacterial colonies are on each plate (count the spots you see).

		Observations
Transformation plates	+pGLO LB/amp	
	+pGLO LB/amp/ara	
Control plates	-pGLO LB/amp	
	-pGLO LB	



Historical links to Biotechnology

1865—Gregor Johann Mendel: Mendel presented his findings describing the principles by which genetic traits are passed from parent to offspring. From his work the concept of the gene as the basic unit of heredity was derived.

1928—Frederick Griffith: Griffith transformed nonpathogenic *Diplococcus pneumonia* into pathogenic bacteria using heat-killed virulent bacteria. He suggested that the transforming factor had something to do with the polysaccharide capsule synthesis. Griffith did not know of DNA, but knew the transformation was inheritable. Griffith's experiments in transformation can be seen as the birth of analytical genetic manipulation that has led to recombinant DNA technology and the prospects for human gene manipulation.



Historical links to Biotechnology

1944—Oswald Avery, Colin MacLeod: Avery and his colleagues announced that they had isolated the transforming factor to a high purity, and it was DNA. Since this classic experiment in molecular genetics, transformation, conjugation (bacterial mating), and transduction (viral DNA transfer) have been used to transfer genes between species of bacteria, *Drosophila*, mice, plants and animals, mammalian cells in culture, and for human gene therapy.

1972—Paul Berg, Janet Mertz: Berg used the newly discovered endonuclease enzyme, *EcoRI*, to cut SV40 DNA and bacteriophage P22 DNA, and then used terminal transferase enzyme and DNA ligase to rejoin these separate pieces into one piece of DNA. Creation of the first recombinant DNA molecule was the beginning of the age of biotechnology. The new molecule was not placed inside a mammalian cell because of concerns in the scientific community regarding genetic transfers.

1977—Genentech, Inc.: The first product of genetic engineering, the gene for human somatostatin (human growth hormone-releasing inhibitory factor), was expressed in bacteria and announced by Genentech.

1980—J. W. Gordon, Frank Ruddle: Gordon and Ruddle successfully microinjected normal genes into mouse germ-line cells.



Historical links to Biotechnology

1988—Steven Rosenberg: Rosenberg and his colleagues were given approval to perform the first gene transfer experiment in human patients suffering from metastatic melanoma. This experiment represented genetic tracking with the marker gene Neo^R and not gene therapy.

1990—W. French Anderson, Michael Blaese, Kenneth Culver: At 12:52 p.m. on Friday, September 14, 1990 at the National Cancer Institute, a four year old girl, Ashanthi De Silva from Cleveland, Ohio, became the first human gene-therapy patient. She was infused with her own white blood cells carrying the corrected version of the adenosine deaminase (ADA) gene. Drs. Anderson, Blaese and Culver did not expect meaningful results from the experiment for about 1 year. A second girl, Cynthia Cutshall, was similarly injected in 1990. Reports in June 1993 showed the two girls with smiles and childish energy, playing in a school yard. Both girls' immune systems were working effectively.

1994—Other gene therapy candidates include sickle cell anemia, hemophilia, diabetes, cancer, and heart disease patients. Germ line gene therapy is debated during meeting of the Recombinant DNA Advisory Committee. By 1996 a growing number of proposals await review by the Human Gene Therapy Subcommittee of the Recombinant DNA Advisory Committee.



Historical links to Biotechnology

- 1995**—Led by J. Craig Venter, a group at The Institute for Genomic Research (TIGR) in Maryland, published the full gene sequence of the bacterium *Hemophilus influenzae*, a landmark in microbiological research as the first free-living organism whose genetic “blueprint” was decoded.
- 1996**—A multinational collaboration including more than 100 laboratories from Europe, USA, Canada, and Japan was the first to unravel the entire genome sequence of a eukaryote, the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* is a commercially significant yeast commonly used in baking and in fermentation of alcoholic beverages and is widely used in the laboratory as a model organism for understanding cellular and molecular processes of eukaryotes.
- 1997**—Scientists led by Ian Wilmut at Scotland’s Roslin Institute reported the successful cloning of a sheep, named Dolly, from the cell of an adult udder cell. The cloning of Dolly sparked international debate about ethical and moral issues concerning cloning. Subsequently, scientists at Scotland’s Roslin Institute, in collaboration with Scotland-based PPL Therapeutics, successfully cloned two genetically modified lambs, named Polly and Molly, that were genetically modified with a human gene so that their milk contained a protein called factor IX, a blood-clotting protein that can be extracted and used in treating human hemophilia.



Historical links to Biotechnology

1998—Over **99% of the genome sequence** of the first multicellular organism, the tiny roundworm *Caenorhabditis elegans*, was reported. Although *C. elegans* is a primitive organism, it shares many of its essential genetic and biological characteristics with humans and may help scientists identify and characterize the genes involved in human biology and disease.

Scientists produced a detailed and accurate physical map, or location, for most of the 30,000 known human genes, a milestone for the Human Genome Project.



Historical links to Biotechnology

2000—A team led by Ingo Potrykus of the Swiss Federal Institute of Technology in Zurich and Peter Beyer of the University of Freiburg in Germany reported the creation of **genetically modified rice called “golden rice”**, which can produce large amounts of beta-carotene, a substance that human beings can turn into Vitamin A. “Golden rice” could alleviate blindness caused by vitamin A deficiency in millions of poverty-stricken people around the world.

The **genome sequence of the fruit fly *Drosophila melanogaster*** was published through a collaboration between a private company, Celera Genomics, and researchers worldwide studying the fruit fly. *D. melanogaster*, a model widely used in the laboratory, is the largest animal so far to have its genetic code deciphered.

A rough draft of the human genome was completed by a team of 16 international institutions that form the Human Genome Sequencing Consortium. Researchers at Celera Genomics also announced completion of their ‘first assembly’ of the genome.



Historical links to Biotechnology

2001—On February 12, 2001, Celera Genomics and the International Human Genome Sequencing Consortium jointly announced the publishing of the nearly complete sequence of the human genome - the genetic “blueprint” for a human being. This accomplishment took the international team almost twenty years and involved the collaboration of thousands of scientists from around the world. Celera Genomics reported completing the work in approximately nine months. The two groups differed in their estimates for the number of genes in the human genome, but the range predicted by both groups, between 25,000 and 40,000 genes, is far fewer than the previous estimate of 100,000 genes. This unexpected finding suggested that an organism as complex as a human being can be made of so few genes, only twice as many as found in the worm *C. elegans* or the fly *D. melanogaster*. The unveiling of the full sequence of the human genome makes it possible for researchers all over the world to begin developing treatments for many diseases.



Historical links to Biotechnology

President George Bush decided that only experiments involving the existing 64 embryonic stem cell lines would be eligible for possible federal funding. The president's decision was disappointing to many scientists who hoped to use embryonic stem cells to develop treatments for many ailments.

Advanced Cell Technology, a small company in Massachusetts, announced that it had successfully cloned human embryos for the purpose of extracting their stem cells. This method could ultimately be used to treat patients with a variety of diseases by making replacement cells, such as nerve and muscle cells, which can be transplanted back into same person without the risk of being rejected by the body.

PPL Therapeutics, the company that helped to clone Dolly the sheep, announced that it had cloned five genetically modified piglets with an inactivated, or "knocked out", gene that would make their organs much less likely to be rejected when transplanted into a human recipient. The success of PPL Therapeutics brings hope to the thousands of people who are waiting to receive donated organs such as hearts, lungs, kidneys, and livers.



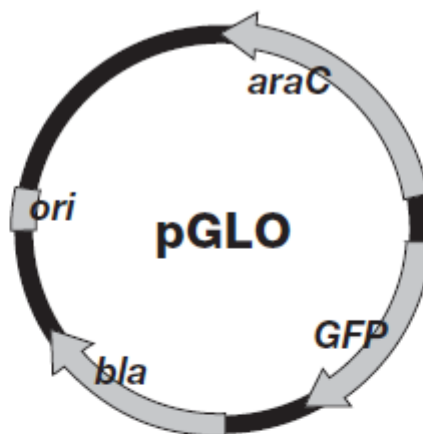
Historical links to Biotechnology

2002—Dolly the sheep, the first mammal to be cloned from an adult cell, developed arthritis at a relatively early age of five years. It is not clear whether Dolly's condition was the result of a genetic defect caused by cloning, or whether it was a mere coincidence. The news has renewed debates on whether cloned animals are susceptible to premature aging and health problems and has also been a setback for those who argue that cloning can be used to generate a supply of organs to help patients on the transplant list.

2008—Discovery and landmark developmental uses of GFP wins the Nobel Prize in Chemistry. Osamu Shimonura was the first to isolate GFP and found that it had fluorescent properties when exposed to UV light. Martin Chalfie used GFP as a luminous genetic tag. Roger Y. Tsien uncovered GFP's fluorescent mechanism.



References



**Biotechnology
Explorer™**

**pGLO™ Bacterial
Transformation Kit**

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See individual components for storage temperature.

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