# **Chapter 1: Welcome**

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# **Chapter 1: SEA-GENES Overview**

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| **content** | Welcome to the Science Education Alliance (SEA), a network of undergraduate researchers and instructors who collaborate on the PHAGES and GENES research projects. In the PHAGES project, researchers isolate novel bacteriophages from the environment by employing various techniques of microbiology, then fully annotate the bacteriophage genomes using an array of bioinformatic tools. Here, in the GENES project, researchers build on the discoveries of the PHAGES project, utilizing techniques of molecular biology and genetics to investigate the functions of genes encoded by these bacteriophages.   Where are we headed?   The number of isolated phages with annotated genomes has increased by the thousands in recent years; this is made possible in part by undergraduate students who have contributed to the research enterprise through the SEA-PHAGES project. Their contributions have not only led to advances in our understanding of how genes are organized within phage genomes and distributed across phage populations, but has also revealed the incredible diversity of genes present within the phage population. When these genes are grouped into families based on the sequence similarities of the proteins they encode, to date, over twenty thousand unique families can be identified! Strikingly, the large majority of these families do not resemble any previously characterized proteins. Indeed, it is this remarkable and unexplored diversity that has earned bacteriophages the reputation as the “dark matter” of the biological world. These thousands of families of unknown functions thus represent a treasure trove from which to uncover novel biological functions. It is the aim of the SEA-GENES research project to experimentally explore this vast unknown.   What might we find?   To consider this, it is important to revisit the details of a phage infection cycle, described in [Phage Infection Cycle Overview](#_11xvktswh9am) , taking into account the myriad interactions that occur between the phage and its bacterial host. Phages have been co-evolving with the diversity of bacteria across the planet for eons, where each phage infection is a battle between the phage and its bacterial host for vital resources. Engaged in this high stakes arms race, both players have defense and counter-defense strategies that are continually evolving. The pursuit of gene-function elucidation therefore promises to uncover an arsenal of functions that reflect this ancient and on-going competition and shed new light on the intricacies of phage-host interactions. Such discoveries also hold the potential to greatly impact the fields of medicine and biotechnology.    In medicine, particularly in an era of rampant antibiotic resistance, the phage gene pool is an invaluable resource for discovering novel strategies for controlling bacteria. Phage lysins, for example, are being developed as a strategy to treat bacterial infections, and recent studies of phage-host interactions are uncovering novel bacterial targets for the development of antimicrobials. In molecular biology, phage recombinases, as well as bacterial defense systems that target phage DNA, have enabled researchers to manipulate DNA with precision and make genetic modifications of cells in all domains of life.   How do we elucidate gene-function?   ‘Gene function’ refers to the specific role that a gene plays in an organism, which is determined by the structure of the final product it encodes, whether that be tRNA or, as in most cases, protein. This function can be complex, and is defined by all the work that a gene product performs. For example, a gene product may interact with an enzyme to catalyze a reaction that results in the production of a small molecule. A clear understanding of the role that a specific gene product performs cannot be gained from any one experiment, but instead requires the integration of multiple experimental approaches.    There are several approaches that are routinely used to study the function of a gene product. These include bioinformatic inferences, phenotypic assays, and protein-protein interaction assays. Each of these approaches, described below, provides clues and preliminary insight that, when taken together, can be used to build a case for assigning function to a particular gene. Bioinformatic inferences have already been used, as part of the SEA-PHAGES project, to uncover phage gene function. Here, as part of the SEA-GENES project, phenotypic and protein-protein interaction assays will be used to gain experimental insights into phage gene function.     * **Bioinformatic Inference.** Gene-function studies often begin with bioinformatic inferences, where computer algorithms can be used to search for similarities between a gene of interest and other genes stored in databases. When strong similarities are identified to a gene with a known function, a gene of unknown function can be preliminarily assigned the same function. In the SEA-PHAGES project, phage genes were assigned functions based on similarity in sequence to genes and proteins of known function, using search tools such as BLAST, as well as on the order of genes within genomes, using tools such as Phamerator. The latter, called synteny, is based on a characteristic of phage and bacterial genomes in which genes that function in the same biological process often cluster together and maintain their relative positions on the genome. For a review of assigning functions using bioinformatic approaches, see the [SEA-PHAGES Bioinformatics Guide.](https://seaphagesbioinformatics.helpdocsonline.com/home)      * **Phenotypic Assays.** The total and coordinated activity of all the genes expressed in an organism determines the observable traits and behavior, or phenotype**,** of that organism. As gene expression changes, so can its phenotype. The connection between gene expression and phenotypes is not only a fundamental tenet of biology; it is also central in the quest to study gene function. Phenotypic assays are a common strategy that involves altering gene expression in some way, either by turning it off completely, turning it on when it would not normally be expressed, or increasing or decreasing the amount of expression, and observing for a change in phenotype. An observable phenotypic change in response to the altered gene expression can provide important clues as to the role that gene plays. For example, expression of a phage gene in a bacterium may result in inhibition of bacterial growth, which suggests its involvement in a process essential for bacterial growth. This approach will be employed as part of the SEA-GENES project, and is described further in Chapter 3.      * **Protein-Protein Interaction (PPI) Assays.** PPI assays are employed to identify proteins that interact with one another. As proteins often do not function in isolation, but instead typically interact with other proteins as part of biological processes, PPI assays can be used to infer the function of one protein if the function of the interacting partner identified is known. For example, identifying a transcription factor as one interacting partner suggests that other proteins may also be involved in transcription. This approach will be employed as part of the SEA-GENES project, and is described further in Chapter 4.    SEA-GENES Research Workflow    |  |  |  | | --- | --- | --- | | Sequence | Description of Research | | | 1 | |  | | --- | |  | | |  |  | | --- | --- | | Alt: | molecular cloning | | | Molecular cloning techniques will be used to engineer a system that enables each gene of a mycobacteriophage to be expressed, individually, in *M. smegmatis* | | 2 | |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Phenotypic assay | | | Each gene of a mycobacteriophage will be expressed, individually, in *M. smegmatis* to identify those that are able to modify the behavior, or phenotype, of the bacterium. | | 3 | |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Molecular cloning | | | Molecular cloning techniques will be used to engineer a second system that enables specific mycobacteriophage genes identified by phenotypic assays to be expressed, individually, in *E. coli.* | | 4 | |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Interaction assay | | | Mycobacteriophage genes will be expressed in an *E. coli*  protein-protein interaction assay to identify potential interacting partners from the *M. smegmatis* proteome. | |

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# **Chapter 1: Phage Infection Cycle**

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| **content** | A phage infection cycle can be broken down into 6 stages. A clear understanding of the strategies employed by phages to complete each infection stage and to counter bacterial defence systems is key to the study of phage-gene function. It provides a framework for anticipating functions that are likely to be encoded in the phage genome. For example, understanding that phages must degrade the bacterial cell wall at the end of the infection cycle then suggests that its genome is likely to encode for enzymes to accomplish that function. Described below are the stages of an infection cycle, many with examples of defense and counter defense strategies employed by bacteria and phages.   1. Adsorption The first step of phage infection is attachment of a phage particle to the surface of its bacterial host, a process called adsorption. Adsorption is accomplished by phage-encoded **R**eceptor **B**inding **P**roteins (RBPs). On tailed phages, the RBPs are typically a component of the phage tail. These RBPs recognize specific structures of the host cell surface, enabling phages to not only attach to its hosts, but to also distinguish its hosts from other bacteria.    However, accessing these host cell surface receptors is not always trivial. For instance, the bacterial cell surface may be decorated with polysaccharides that can occlude the cell surface receptor. In fact, this appears to be a strategy employed by bacteria to evade phage infection. In turn, phages can encode polysaccharide degrading enzymes, often also as part of the phage tail, to unmask its cell surface receptor. 2. Genome Delivery Successful adsorption then sets in motion the delivery of phage DNA from the capsid into the host cell. To achieve this, phages must clear a path and assemble a channel across the cell envelope. Phages encode enzymes such as peptidases and glycosidases to break down the rigid peptidoglycan cell wall, and specialized proteins to form a channel for DNA delivery. For myoviruses with contractile tails, this channel is formed by a preassembled tail-tube that emerges upon contraction of the tail. For podoviruses with short non-contractile tails, specialized tail proteins likely assemble *de novo* to form a channel across the cell envelope.    Once delivered into the host cell, phage DNA must evade degradation by host nucleases. For example, commonly encoded bacterial exonucleases target the double-stranded ends of linear phage genomes. To protect against these exonucleases, phages encode recombinases that join the two ends of its genome to form a circular DNA molecule. Phages that do not circularize their genomes encode proteins that either bind to and protect the ends of its linear DNA, or bind to the exonucleases and prevent their recognition of its DNA ends. Bacteria also encode at least two defense systems that utilize endonucleases to target phage DNA - the restriction-modification (RM) system and the CRISPR system. In both of these systems, bacterial endonucleases survey the cell for specific “target” DNA sequences that, upon encountering, cleaves to expose double-stranded DNA ends that can then be degraded by exonucleases. In the RM system, the endonucleases are called restriction enzymes. Because the host DNA may also contain these target sequences, the host encodes modification enzymes, such as methyl-transferases, to modify and thereby mask target sequences that may occur on its own DNA. Phages can similarly evade restriction enzymes by encoding their own modification enzymes, as well as proteins that stimulate the activity of host modification enzymes, to rapidly modify and thereby protect phage DNA. Phages can also encode proteins that, when co-delivered into the host cell with its DNA, either bind to and protect target sequences that occur on its genome, or act like decoys by binding to and sequestering the host restriction enzymes. In the CRISPR system, the endonucleases are called **C**RISPR-**as**sociated (Cas) proteins. Cas proteins target DNA sequences that are specified by a region of host DNA called CRISPR. Phages can inactivate CRISPR-Cas systems by encoding **a**nti-**CR**ISPR (**acr**) proteins that inhibit the ability of Cas proteins to target or cleave DNA, or their own CRISPR-Cas system that targets and cleaves host CRISPR DNA.   3. Macromolecular Synthesis Immediately upon delivery of phage DNA into the cell, and while evading nucleases, phage gene expression and genome replication begins.    **3a. Gene Expression.** Transcription of phage genes is often initiated by host RNA polymerase and completed by either host or phage-encoded RNA polymerase, while translation is primarily executed by host translation machinery. For a productive infection, phage gene expression must be tightly regulated, and phages encode a myriad of proteins to regulate gene expression.    At the level of transcription, phages encode transcription regulators that, by enabling, promoting, or repressing transcription from given promoters, can not only modulate the levels of expression, but can also exert temporal control over gene expression. These transcription regulators can also completely inhibit the activity of host RNA polymerase, a strategy employed by phages to prevent transcription of host genes, thereby maximizing host recourses for transcription of phage genes.    Phage-encoded proteins are also able to modulate gene expression at the level of translation. For example, phages can encode mRNA-binding proteins to stabilize transcripts or occlude ribosome binding, or ribonucleases that target transcripts for degradation. Phages can also encode their own tRNAs, or proteins that modify tRNAs, which alter the cellular pool of these molecules and may facilitate translation of phage genes.    In defense, host-encoded proteins can also inhibit phage gene expression, with phages often equipped to counter these bacterial defense strategies. For example, when host gene expression is inhibited by an infecting phage, the relative amounts of a host-encoded transcript-cleaving toxin, which is more stable than the co-expressed host-encoded anti-toxin, increases. These “free” toxins can then promote the degradation of transcripts to shutdown phage gene expression. To counter such **T**oxin-**A**nti-toxin (TA) systems, phages can encode their own anti-toxins, which can bind to and neutralize the host toxins. As another means of defense, bacterial proteins can cleave tRNAs to inhibit translation. In turn, phages can restore translation by encoding polynucleotide kinase, phosphatase, and RNA ligase to repair cleaved tRNA.    **3b. Genome Replication.** Replication of phage genomes is often performed by both host and phage-encoded replication proteins such as DNA polymerase, helicase, and primase. As with transcription, phages can also encode proteins that specifically inhibit replication of the host genome as means to maximize reserves for replication of its own genome. Indeed, phages can also encode nucleases that degrade the host genome as well as enzymes that catalyze nucleotide formation to increase the pool of nucleotides available for phage genome replication. Phages that degrade host genomes also encode proteins that modify and protects its own DNA from degradation.    **3c. Lysogeny.** At an early stage of macromolecular synthesis, temperate phages have the unique ability to pause their infection cycle and adopt a different pattern of gene expression and genome replication. This state, called lysogeny, is achieved when the phage-encoded immunity repressor is expressed and able to continually inhibit transcription from promoters that are critical for a productive infection. The decision to enter lysogeny, and indeed the duration of lysogeny, is determine by host and phage-encoded proteins that modulate the expression or activity of the immunity repressor in response to environmental stimuli. These include host-encoded proteins that sense DNA damage, and phage-encoded receptors that can detect the amount of host bacteria or active infections in the immediate environment.  During lysogeny, phages must also ensure that their genomes are faithfully replicated and stably segregated as the host cells continue to grow and divide. To achieve this, phages typically insert their genomes into the host chromosome. This integration is performed by phage-encoded recombinases, called integrases. When phages resume a productive infection, the same integrases acts in concert with phage-encoded excisionases to excise phage genomes from the host chromosome. Non-integrating phages, on the other hand, maintain their genomes extra-chromosomally, like a very large plasmid, during lysogeny. These phages encode partitioning proteins that help ensure their genomes, once replicated, are properly partitioned as the host cell divides. These partitioning proteins include centromere proteins and actin- or tubulin-like polymerizing proteins that bind to and actively segregate phage DNA, respectively.   4. Host Immunity During the course on an infection, phages may promote survival of the infected host. For example, immunity repressor proteins expressed during lysogeny are able to recognize and similarly repress gene expression of identical or similar superinfecting phages. Indeed, phages can employ a variety of strategies to immunize its host from superinfection. For example, phage-encoded proteins can prevent superinfection by identical or similar phages by occluding cell surface receptors or by inhibiting cell wall degrading enzymes required for invasion. Phages can also provide immunity against more distantly related phages, for example, by encoding restriction enzymes that target successfully delivered phage DNA for degradation. 5. Assembly Towards the end of a productive infection cycle, the components of new phage particles begin to accumulate in the host. In stages, these components begin assembling into new phage particles; scaffolding proteins and chaperones guide assembly of capsid proteins into capsids, and the numerous tail proteins into tail tubes and tail fibers, while packaging proteins such as terminase and portal proteins package phage genomes into capsids. Finally, connector and adapter proteins bridge these preassembled parts to form mature phage particles.   6. Release As new phage particles accumulate in the host, so do phage-encoded proteins that are important for the final act of an infection cycle – phage release from the host. Accumulating holin proteins associate with the host cell membrane to form holes, through which lysins, with various hydrolase activities, can access and degrade the cell wall. This combined activity results in cell lysis and the release of phage particles that can begin new infection cycles. |

# **Chapter 1: Lab Safety**

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| **content** | This document offers an introduction to lab safety, and is a good starting point for a conversation about working responsibly in the lab.While every laboratory has its own specific rules and regulations, there are some universal guidelines that you should always follow. They are designed to keep you and your lab mates safe and to comply with state and federal regulations. Please read these rules and guidelines carefully and talk to your instructor about any additional rules that must be adhered to in your workspace. General Lab Safety Practices  * No eating or drinking. * No open-toe shoes. * Never work alone in a lab. * Tie back long hair. * Avoid wearing baggy clothing. * Never leave a lit Bunsen burner unattended. * Keep workspace clear of clutter. * Minimize loud talking and distractions. * Know the location of fire extinguishers, emergency eye wash stations, and emergency showers. * Properly dispose of waste generated from an experiment. Not everything can go down the drain or in a garbage can. * No mouth pipetting! * Wear safety glasses at all times. * Wear gloves when working with bacteria. * Wash your hands before leaving lab. * If you are not sure, ask first!  Guidelines for Working with Bacteria The following are recommendations that should be used to supplement general lab practices and established safety rules at your institution when working with bacteria. Before working with bacteria:  1. Ensure that all participants are aware of lab practices and safety rules, which should include the recommendations listed in this document. 2. Make sure that all participants are aware that:    * The bacteria recommended for use in this course are safe for those with a normal immune system. If an individual has any health concerns related to exposure to bacteria, including being immuno-compromised or pregnant, the individual should consult a physician to determine the appropriate level of participation in lab activities.    * The primary hazards of working with the bacteria used in this course are from exposure via mucosal membrane routes (eyes, mouth, and nose), parenteral inoculation (broken skin), or ingestion.  While working with bacteria:  1. Gloves and lab coats should be used. Change gloves when contaminated or when integrity is compromised. Do not touch face, apply cosmetics, adjust contact lenses, bite nails, etc. 2. Care should be taken to avoid generating aerosols of cultures or suspensions of bacteria; do not sonicate or vortex bacterial cultures or suspensions; transfer bacterial cultures and suspensions with care and deliberation. Safety glasses and surgical masks, or a biosafety cabinet, are recommended if there is risk of generating aerosols. 3. Label cultures, tubes and plates containing bacteria appropriately. 4. Lab work surfaces should be wiped-down with an appropriate phenolic disinfectant before and after use. An example of an appropriate disinfectant for Mycobacteria is CiDecon Detergent Disinfectant Concentrate, available from multiple vendors including Fisher Scientific and VWR. Disinfectants should be used according to the manufacturer instructions. Once cleaned, the work surface should then be wiped-down with 70 % ethanol to remove residual disinfectant from the surface. Note: As a disinfectant, 70 % EtOH is sufficient to kill most bacterial contaminants on surfaces. However, it is not effective on Mycobacteria because of the mycolic acid wall. Use of a disinfectant like CiDecon will remove mycobacterial contaminants. 5. Materials that have come in contact with bacteria and that are intended for reuse (e.g. glass flasks) should be treated with CiDecon before washing. Spent CiDecon can be disposed of in sinks with tap-water running. 6. Materials for disposal that have come in contact with bacteria (e.g. microcentrifuge tubes or agar plates) should be disposed of in biohazard bins. 7. Sticks or loops used for streaking or inoculating bacteria should not be flamed after use. Instead, sticks should be disposed of in a biohazard bin, while metal loops should be treated with CiDecon followed by 70 % ethanol.  After working with bacteria:  1. Wash hands thoroughly. 2. Dispose of all unused bacterial cultures appropriately by mixing the contents with CiDecon, bleach, or autoclaving. 3. Materials for disposal that have come in contact with bacteria (e.g. microcentrifuge tubes or agar plates) should be disposed of in biohazard bins. 4. Waste collected in biohazard bins should be inactivated using an autoclave, or removed by a licensed waste removal service provider. 5. Materials that have come in contact with bacteria and that are intended for reuse (e.g. glass flasks) should be treated with bleach or CiDecon before washing. Spent bleach and CiDecon can be disposed of in sinks with tap water running.  Guidelines for Avoiding Contamination Aseptic technique is used to prevent contamination in microbiology experiments. The growth of contaminating bacteria, fungi, and viruses can be avoided by disinfecting your bench, creating an updraft with a Bunsen burner, and moving with care and deliberation. When working with DNA and molecular biology techniques, it is essential to disinfect your bench and to ALWAYS wear gloves and a lab coat in order to reduce the chances of introducing contaminating DNA or degrading nucleases into your sample.  The information provided in this document is adapted from the SEA-PHAGES Phage Discovery Guide and Phage Discovery Instructors Guide © 2018, Howard Hughes Medical Institute. |

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# **Chapter 2 : Molecular Cloning**

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# **Chapter 2: An Overview: Molecular Cloning**

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| **content** | Plasmids and Gene Expression in Bacteria A typical bacterial genome is 5 million base pairs (5 Mbp) in length, though genomes as small as 0.1 Mbp and as large as 14 Mbp have been reported. In most bacteria, these millions of DNA base pairs are arranged as a single double-stranded, circular chromosome that is organized into genes encoding for several thousand different proteins. The *Mycobacterium smegmatis* genome contains ~ 7 Mbp, organized into 6,717 genes. As bacteria grow and divide, the chromosome is copied by the replication machinery and partitioned into the two resulting daughter cells, ensuring stable maintenance of all chromosomal genes.  Not all of the genes in a bacterial cell are necessarily found on this chromosome; bacteria can carry additional genes on DNA molecules known as plasmids, whichlike the chromosome, are also replicated and partitioned as bacteria grow and divide. Plasmids are small, circular molecules of dsDNA that are typically less than 10 kbp and contain just a handful of genes. Owing to its small size, plasmids are easily engineered and manipulated in the lab and can be made to contain almost any genetic sequence imaginable. The process of building a plasmid is known as molecular cloning and is described in the next section. For SEA-GENES, two plasmids have been engineered to facilitate the study of gene function, pExTra and p2Hα, which you will manipulate to contain your phage gene of interest (Figure 2.01).  All plasmids, including those that exist in nature or that have been created in a lab, contain two key features. The first, the origin of replication, contains all the necessary elements that enable replication of the plasmid, including the DNA sequence where replication is initiated by the bacterial machinery. The origin of replication for a plasmid thus determines the bacterial hosts in which the plasmid can be replicated and maintained. The pExTra plasmid has two origins of replication, enabling it to be replicated in *Escherichia coli* and *M. smegmatis,* whereas the p2Hα plasmid can only be replicated in *E. coli.* The second feature common to all plasmids is a gene that confers some type of advantage to the plasmid-containing cell, which in turn helps to ensure that the plasmid is maintained within a bacterial population. Oftentimes this advantageous gene encodes for antibiotic resistance, allowing the plasmid-containing cell to survive in the presence of a given antibiotic. In the lab, this feature can be used to identify and isolate, or select, bacteria that contain the specific plasmid from cells that do not. The pExTra and p2Hα plasmids confer resistance to the antibiotics Kanamycin and Spectinomycin, respectively.   |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure 2.01. pExTra and p2Hα plasmid maps | | | |  |  | | --- | --- | | Caption: | Figure 2.01. pExTra and p2Hα plasmid maps | |   In addition to these two features, the pExTra and p2Hα plasmids have been engineered to each contain a promoter, which is a DNA sequence that is recognized and bound by RNA polymerase. Once bound at the promoter, RNA polymerase can initiate transcription of the gene(s) immediately downstream of the promoter. In SEA-GENES research, and as part of Chapter 2, you will manipulate pExTra and p2Hα plasmids to contain your phage gene of interest immediately downstream of the promoter. By doing so, you will create a means to express your phage gene of interest in bacteria to begin exploring phage gene function as part of the research outlined in chapters 3 and 4. Molecular Cloning Techniques for the Construction of Plasmids The pExTra and p2Hα plasmids will be modified using a process known as molecular cloning, which is a set of experimental methods for the assembly, isolation, and propagation of recombinant DNA plasmids. 'Recombinant' simply means that these DNA molecules are composed of a mixture of sequences from different sources and would not naturally occur. For example, the recombinant pExTra plasmids we will build will contain sequences originally from mycobacteriophage, bacteria, naturally occurring plasmids, and sea anemones! Since the advent of molecular cloning in the 1970s, researchers have developed a number of cloning technologies and workflows that enable efficient engineering of DNA molecules such as plasmids. The workflows that we will use to clone phage genes into pExTra and p2Hα plasmids are described in detail in Protocol 2.1a and Protocol 2.1b, and are composed of the following 4 core steps.  **Step 1.** Copying and isolating a particular DNA fragment referred to as an 'insert'.  **Step 2.** Assembling the insert with a relevant plasmid, which in the context of cloning is referred to as a 'plasmid backbone.'  **Step 3.** Introducing the new recombinant plasmid into bacteria by a process known as 'transformation' and selecting bacteria carrying the plasmid.  **Step 4.** Purifying the recombinant plasmid and verifying that it contains the correct insert.  **Step 1. Copying and isolating a particular DNA fragment referred to as an 'insert'.**  Polymerase Chain Reaction, or PCR, is a powerful technique that allows a specific fragment of DNA to be copied exponentially, *in vitro*, generating large quantities of DNA from very little starting DNA (Figure 2.02).   |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure 2.02. A phage gene of interest is copied, or 'amplified', by PCR. | | | |  |  | | --- | --- | | Caption: | Figure 2.02. A phage gene of interest is copied, or 'amplified', by PCR. | |   PCR requires several key components:   |  |  | | --- | --- | | **Component** | **Description of Component** | | Template DNA | DNA sample that contains the sequence to be copied. This can be a purified DNA  sample such as extracted genomic DNA or plasmid DNA, or it can be a more complex  DNA-containing sample such as a phage lysate or bacterial colony. | | DNA Polymerase | An enzyme that synthesizes new DNA molecules. Polymerases from thermophilic  organisms are used due to their high heat tolerance. | | Nucleotides | The building blocks used by DNA polymerase to make new DNA molecules. | | Primers | Short pieces of single stranded DNA (15-60 nucleotides) that are complementary to a  portion of the template DNA sequence, marking the beginning and end of the sequence  to be copied. PCR primers are custom-built in a laboratory and can be engineered to  add specific sequences to the ends of a DNA fragment. |   The reaction consists of three steps (denaturation, annealing, and extension), each occurring at a specific temperature. Reaction components are mixed together and placed in a thermocycler machine, which cycles through these three temperatures, usually 20-40 times. During each cycle of denaturation, annealing, and extension, the specific segment of DNA is amplified exponentially. Exact temperatures and times for these steps are influenced by primer sequence, DNA length, and the polymerase used.   |  |  |  |  | | --- | --- | --- | --- | | **PCR Step** | **Temp.** | **Duration** | **Description of step** | | Denaturation | 94 - 98 °C | 5 -10 sec | High temperature denatures the two strands of the  template DNA. | | Annealing | 50 -72 °C | 5 sec | The temperature is lowered, allowing the primers to bind  to the complementary region of the template DNA strand. | | Extension | 68 - 72 °C | 10 - 30 sec per kb of amplified segment | DNA polymerase adds nucleotides to the end of each  annealed primer (in the 5' to 3' direction) to build a new  double stranded copy of the segment. |   The result of a PCR reaction can be assessed by gel electrophoresis, a technique in which DNA is passed through an agarose gel by an electric current. Negatively charged DNA molecules migrate through the sieve-like matrix according to their size, allowing for separation of DNA molecules based on their size. When visualized using a DNA dye such as ethidium bromide, DNA molecules will appear as bands whose size can be determined by comparison to a standard of known sizes, or 'ladder'. Usually, the desired result of a PCR reaction is a single band of the correct size; however, in some cases a PCR may yield multiple bands or no bands, requiring further optimization. It is necessary to purify the desired PCR product away from the other components of the reaction as they may interfere with downstream assembly steps. This can be done by washing the entire reaction over a commercially available silica column or first extracting a specific band of DNA from an agarose gel and washing that over a column.  Once an insert has been generated by PCR and purified, it can be assembled with a plasmid backbone using a variety of methods, each of which requires modification of the ends of the inserts. In SEA-GENES we will utilize two assembly strategies, which are described below. Step 2. Assembling the insert with a relevant plasmid, which in the context of cloning is referred to as a 'plasmid backbone.' There are two strategies that will be employed for the assembly reaction, either restriction enzyme digest and ligation, or isothermal assembly.  **Restriction enzyme digestion (RED) and ligation.** Assembling an insert and plasmid backbone by RED and ligation can essentially be thought of as a 'cut and paste' reaction that takes two linear DNA molecules and joins them to form a single circular molecule. The 'cut' is performed by restriction endonuclease enzymes, which recognize and cleave double-stranded DNA at specific 4–6 bp palindromic sequences called restriction sites. Some restriction enzymes cleave both strands at the same site, generating 'blunt ends', whereas other enzymes cleave the strands at different locations a few bp away from each other, generating single-stranded 'sticky ends.' These sticky ends allow for efficient binding of two pieces of DNA with complementary overhangs. To create compatible ends, the insert and plasmid should be digested with the same restriction enzyme and thus, must have the same restriction sites. If not already present, primers can be designed to introduce desired restriction sites to the ends of an insert sequence (flanking the gene) by PCR. Similarly, plasmids can be modified to contain necessary restriction sites, and often have several that can be used for cloning in a region known as the 'multiple cloning site.' Cleavage of a circular plasmid molecule by one or more restriction enzymes creates a linear plasmid backbone with appropriate ends for assembly. Digestion of the insert and plasmid with two different restriction enzymes (known as a double digest) ensures proper directional assembly. Some restriction sites occur naturally within gene sequences or plasmid sequences; when designing a cloning strategy, it is essential that restriction sites be unique within the insert and plasmid such that the right number of fragments of the correct size are generated upon digestion.  The 'paste' step is achieved by the enzyme DNA ligase, which covalently connects the sugar phosphate backbones of two DNA molecules with complementary sticky ends generated by restriction enzyme digest. Interestingly, the ligase enzyme used for this reaction is from the phage T4! Together, these two enzymatic steps produce a recombinant plasmid that is ready for transformation into bacteria.   |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure 2.03. 'Insert' DNA and prepared plasmid 'backbone' DNA are assembled into a new plasmid. | | | |  |  | | --- | --- | | Caption: | Figure 2.03. 'Insert' DNA and prepared plasmid 'backbone' DNA are assembled into a new plasmid. | |   **Isothermal Assembly.** Similar to RED and ligation, isothermal assembly 'cuts and pastes' together multiple linear DNA fragments. This method is an attractive alternative to RED and ligation cloning as it involves fewer individual steps and does not require DNA segments to have specific, unique restriction sites. However, it does require that the DNA segments have identical, overlapping sequences known as 'homology regions' at both ends; these homology regions should be at least 20 bp in length. As in the above strategy, primers can be engineered to introduce these homology regions into the insert or plasmid backbone by PCR, providing the necessary sequence match. Circular plasmids must be linearized for use in isothermal assembly; this is usually achieved by amplifying the plasmid by PCR, creating a linear plasmid backbone with the homology regions situated at the 5' and 3' ends.  In an isothermal assembly reaction, three enzymatic steps occur in a single tube during a single incubation step at 50 ºC:   1. An exonuclease chews back double stranded DNA at each 5' end, leaving 3' overhangs or 'sticky ends'. The exonuclease is quickly inactivated at 50 ºC. This ensures that only the DNA ends are digested, not the entire strand. Since the two DNA segments have overlapping sequences at their ends, the sticky ends that are generated can base pair to each other, allowing the two different DNA segments to anneal to each other. This base pairing is very efficient due to the extended length of the sticky ends—much more efficient than the base pairing of sticky ends generated by restriction enzyme digest, which are typically only a few bp long. 2. DNA Polymerase fills in the gaps on the annealed segments, and 3. DNA ligase covalently links the sugar phosphate backbones, creating a complete DNA molecule ready for bacterial transformation.  Step 3. Introducing the new recombinant plasmid into bacteria by a process known as 'transformation' and selecting bacteria carrying the plasmid. Transformation is the process of introducing a plasmid into bacterial cells. Cells that are capable of taking up a piece of foreign DNA are said to be 'competent.' Transformation is an essential step in molecular cloning of plasmids, as the bacterial cells are used to recover the cloned plasmid of interest from a ligation or isothermal assembly reaction. Transformed cells are also used to store and replicate the plasmid. Although many species of bacteria are or can be engineered to be competent, *E. coli* is the true workhorse of molecular cloning. *E. coli* grows quickly (optimally, doubling every~ 20 min) and is relatively easy to work with. As such, molecular biologists have modified the *E. coli* genome to facilitate its ability to take up and stably store recombinant plasmid DNA. For instance, many cloning strains have a mutation in the *endA* gene, disrupting the function of the encoded endonuclease and protecting plasmid molecules from degradation.  To transform bacteria, plasmid DNA is incubated with an ice-cold mixture of competent cells and glycerol. The plasmid molecules can be introduced into the cells by one of two methods: heat shock or electroporation. In the heat shock method, competent cells are treated with divalent cations (such as CaCl2) and briefly incubated at a high temperature (42 ºC); this creates pores in the membrane that the plasmid can travel through. Similarly, in the electroporation method, membrane pores are created by pulsing cells with an electric current. Although transformation is an efficient method, not all cells will take up the DNA. In both methods, transformed bacteria that have taken up the plasmid of interest must be 'selected' for based on the antibiotic resistance conferred by the plasmid. This is achieved by plating the transformation mixture on solid medium containing a selective concentration of antibiotic; only those cells that have successfully taken up and replicated the recombinant plasmid are able to grow.   |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure 2.04. During a transformation reaction, a fraction of bacteria will take up the DNA. These bacteria are said to be 'transformed'. | | | |  |  | | --- | --- | | Caption: | Figure 2.04. During a transformation reaction, a fraction of bacteria will take up the DNA. These bacteria are said to be 'transformed'. | |  Step 4. Purifying the recombinant plasmid and verifying that it contains the correct insert. Once the transformed *E. coli* cells harboring the plasmid clone have formed colonies on an antibiotic selection plate, it is necessary to purify the recombinant plasmid and verify that it was constructed correctly before it is used in any downstream experiments. To purify the plasmid, cells are grown to high density in selective liquid medium then lysed by the addition of SDS and NaOH, which denatures all DNA (chromosomal and plasmid) in the sample. This process, known as alkaline lysis, allows for separation of plasmid DNA from chromosomal DNA; upon neutralization of the alkaline solution, plasmid molecules are re-natured, whereas strands of chromosomal DNA are unable to re-anneal and become an insoluble aggregate that precipitates out of solution. Plasmid DNA can then be bound to a silica column, washed to remove salts, proteins and other contaminants, and eluted with sterile, nuclease-free water (ddH2O).   |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure 2.05. Bacteria containing a plasmid can be grown to high denstities, from which large quantities of the plasmid can be extracted for use in downsteam applications. | | | |  |  | | --- | --- | | Caption: | Figure 2.05. Bacteria containing a plasmid can be grown to high denstities, from which large quantities of the plasmid can be extracted for use in downsteam applications. | |   There are multiple methods that can be used to confirm that a recombinant plasmid contains the expected insert. The exact sequence of nucleotides in the insert-containing region of the plasmid can be precisely determined by a DNA sequencing technique known as Sanger sequencing. Alternatively, PCR and gel electrophoresis can be used diagnostically to determine whether a plasmid contains an insert of the expected size. One benefit of the latter method is that the plasmid does not need to be purified from cells before running the diagnostic; a transformed colony can be used directly as PCR template. In a typical cloning workflow multiple candidate clones are tested for a correct insert, and verifying a plasmid clone prior to going through the purification process can conserve time and reagents.  Once a plasmid has been purified and verified, it is ready for use in the experimental procedures outlined in Chapters 3 or 4! |

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# **Protocol 2.1a: Cloning into pExTra**

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| --- | --- |
| **templateId** | **chapterContent** |
| **name** | **Protocol 2.1a: Cloning into pExTra** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To construct a DNA molecule that will enable expression of a phage gene in *M. smegmatis.* Rationale: To express a phage gene in *M. smegmatis*, the gene must first be introduced into the bacterium, where it must then be stably maintained. These requirements can be achieved with the use of an expression plasmid, such as pExTra. Because pExTra contains a promoter (Ptet) that is activated in the presence of an 'inducer' molecule, expression of the phage gene can be turned on as needed. Here is presented a multi-step workflow for the cloning of a phage gene into pExTra. Briefly, a specific phage gene sequence will be copied from a phage lysate by PCR using a gene-specific primer that is complementary to the first ~20 bp of the gene (the forward primer) and one that is complementary to the last ~20 bp of the gene (the reverse primer). These primer sequences also introduce essential homology regions at the ends of the amplified gene sequence; these regions are a perfect sequence match to plasmid sequences upstream and downstream of the phage gene insertion site, enabling assembly of this insert and plasmid backbone by the isothermal assembly method. After isothermal assembly, recombinant plasmids are transformed into *E. coli* and selected clones are verified by diagnostic colony PCR. Correct plasmids will be propagated and purified from *E. coli* cells. This recombinant pExTra plasmid will then be ready for use in the host *M. smegmatis*. Procedure : Cloning a phage gene into the pExTra plasmid is a multi-step process, and requires knowledge about key features of the plasmid. The multi-step process is provided in the table below and includes:   * the sequence of steps, * a description of each step, * a general protocol for executing the step, and * specific information for cloning into pExTra that must be accounted for when utilising the general protocol.   Key features of the pExTra plasmid are presented with the plasmid diagram. Refer back to information provided within this protocol throughout the process of cloning genes into pExTra.   |  |  |  | | --- | --- | --- | | **Step** | **Step Description & Protocols** | **Protocol-Specific Information** | | 1 | Amplify Phage Gene(s)  [by Polymerase Chain Reaction - Protocol 2.2](#_agawv4ji2xgh) | **Template DNA:** Phage lysate or phage DNA  **2X Polymerase MasterMix:** Q5  **Primers:** Gene-specific | | 2 | Verify Size(s) of PCR Amplified DNA  [by Gel Electrophoresis - Protocol 2.3](#_reg54i7mfy66)  If Expected Fragment is Amplified,  Proceed to Step 3 Protocol 2.4  If Multiple Fragments are Amplified,  Proceed to Step 3 Protocol 2.5 or  Return to Step 1  If No Fragment is Amplified,  Return to Step 1 |  | | 3 | Purify Amplified DNA  [by Column Purification of DNA - Protocol 2.4](#_ui1k69asslld)  [by Gel Extraction of DNA - Protocol 2.5](#_4omr8mp0h1bq) |  | | 4 | Assemble Amplified DNA into pExTra  [by Isothermal Assembly - Protocol 2.7](#_1ht6r06vqd5j) | **Plasmid Size:** 6 kbp | | 5 | Transform E. coli with Assembled Plasmid DNA  [by Chemical Transformation of Bacteria - Protocol 2.9](#_a78ohaqyc10k)  If Colonies are Obtained,  Proceed to Step 6  If No Colonies are Obtained,  Return to Step 4 | **Medium:** LB agar + Kanamycin (50μg/ml)  **Incubation Temperature:** 37 °C | | 6 | Confirm Insert Size of Candidate Cloned  Plasmid(s)  [by Clone Verification - Protocol 2.10](#_4qdrx5kd7lab) | **Template DNA:** Colony of transformed cells  **2X Polymerase MasterMix:** Taq  **Primers:** pExTra\_universal F and pExTra\_universal R | | 7 | Propagate E. coli with Candidate Cloned  Plasmid(s)  [by Inoculating a Culture - Protocol 2.11](#_s1rnln94dbbm) | **Medium:** LB + Kanamycin (50μg/ml)  **Incubation Temperature:** 37 °C | | 8 | Extract Cloned Plasmid DNA  [by Preparing Plasmid DNA - Protocol 2.12](#_8jdwn4cy5g82) |  |  |  | | --- | |  | | |  |  | | --- | --- | | Alt: | pExTra plasmid map | | | |  |  | | --- | --- | | Caption: | pExTra plasmid map | |   **Helpful tips:** |

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# **Protocol 2.1b: Cloning into p2Hα**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.1b: Cloning into p2Hα** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To construct a DNA molecule that will enable expression of a phage gene in an *E. coli* protein-protien interaction (PPI) assay. Rationale: To express a phage gene in *E. coli* in order to look for interactions between the phage gene product and host protein fragments, the gene must first be introduced into an appropriate expression plasmid, p2Hα. The p2Hα plasmid contains all the elements necessary for stable propagation in *E. coli* cells, and expression of inserted phage genes is controlled by the Plac\* promoter, which is turned on in the presence of an inducer molecule. Here, a multi-step workflow for the cloning of a phage gene into p2Hα is presented. Briefly, a phage gene sequence will be amplified, not from a lysate, but using the pExTra recombinant plasmid as template DNA. Primers that anneal to the pExTra plasmid upstream and downstream of the inserted phage gene will be used, and the same primer pair can be used universally for all recombinant pExTra plasmids. Amplification of phage genes using these universal primers ensures that the appropriate restriction sites are found at the ends of the insert; these same sites are found on the p2Hα plasmid. Both plasmid and insert will be digested with appropriated restriction enzymes and ligated together, and recombinant plasmids are transformed into *E. coli*. Selected clones will be verified by diagnostic colony PCR, and correct plasmids will be propagated and purified from *E. coli* cells. This recombinant p2Hα plasmid will then be ready for use in the *E. coli* B2H selection assay presented in Chapter 4. Procedure : Cloning a phage gene into a the p2Hα plasmid is a multi-step process, and requires knowledge about key features of the plasmid. The multi-step process is provided in the table below and includes:   * the sequence of steps, * a description of each step, * a general protocol for executing the step, and * specific information for cloning into p2Hα that must be accounted for when utilizing the general protocol.   Key features of the p2Hα plasmid are presented with the plasmid diagram. Refer back to information provided within this protocol throughout the process of cloning genes into p2Hα.   |  |  |  | | --- | --- | --- | | **Step** | **Step Description & Protocols** | **Protocol-Specific Information** | | 1 | Amplify Phage Gene(s)  [by Polymerase Chain Reaction - Protocol 2.2](#_agawv4ji2xgh) | **Template DNA:** Recombinant pExTra with appropriate gene  insert **2X Polymerase MasterMix:** Q5  **Primers:** pExTra\_universal\_F and pExTra\_universal\_R | | 2 | Verify Size(s) of PCR Amplified DNA  [by Gel Electrophoresis - Protocol 2.3](#_reg54i7mfy66)  If Expected Fragment is Amplified,  Proceed to Step 3 Protocol 2.4  If Multiple Fragments are Amplified,  Proceed to Step 3 Protocol 2.5 or  Return to Step 1  If No Fragment is Amplified,  Return to Step 1 |  | | 3 | Purify Amplified DNA  [by Column Purification of DNA - Protocol 2.4](#_ui1k69asslld)  [by Gel Extraction of DNA - Protocol 2.5](#_4omr8mp0h1bq) |  | | 4 | Restriction Enzyme Double Digest of Insert  [by Restriction Enzyme Digest - Protocol 2.6](#_o8pw8kevvkv6) | **Enzymes:** NdeI and BamHI OR NdeI and SbfI | | 5 | Purify Digested Insert  [by Column Purification of DNA - Protocol 2.4](#_ui1k69asslld) |  | | 6 | Ligation of Insert and p2Hα backbone  [by DNA Ligation - Protocol 2.8](#_pl77g8b7ctd3) | **Plasmid size:** 5 kb | | 7 | Transform E. coli with Assembled Plasmid DNA  [by Chemical Transformation of Bacteria - Protocol 2.9](#_a78ohaqyc10k)  If Colonies are Obtained,  Proceed to Step 8  If No Colonies are Obtained,  Return to Step 4 | **Medium:** LB agar + Spectinomycin (50μg/ml)  **Incubation Temperature:** 30 °C or 37 °C | | 8 | Confirm Insert Size of Candidate Cloned  Plasmid(s)  [by Clone Verification - Protocol 2.10](#_4qdrx5kd7lab) | **Template DNA:** Colony of transformed cells  **2X Polymerase MasterMix:** Taq  **Primers:** o-p2HαF & o-p2HαR | | 9 | Propogate E. coli with Candidate Cloned  Plasmid(s)  [by Inoculating a Culture - Protocol 2.11](#_s1rnln94dbbm) | **Medium:** LB + Spectinomycin (50μg/ml)  **Incubation Temperature:** 30 °C or 37 °C | | 10 | Extract Cloned Plasmid DNA  [by Preparing Plasmid DNA - Protocol 2.12](#_8jdwn4cy5g82) |  |  |  | | --- | |  | | |  |  | | --- | --- | | Alt: | p2Hα plasmid map |  |  |  | | --- | --- | | Caption: | p2Hα plasmid map | | |

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# **Protocol 2.2: Polymerase Chain Reaction**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.2: Polymerase Chain Reaction** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To generate copies of a specific segment of DNA Rationale: Polymerase Chain Reaction (PCR) is a powerful technique that allows researchers to make large quantities of a specific segment of DNA. PCR is utilized in many scientific disciplines, including medicine, forensics, and genomics. In molecular biology, PCR is an essential step in many cloning strategies, including those we will employ for cloning phage genes into pExTra and p2Hα plasmids. Supplies :  * Template DNA\* * DNA Primers, forward and reverse (10 μM )\* * 2x Polymerase MasterMix\* * Sterile, nuclease-free water * Ice * Thermocycler * PCR tubes * Microcentrifuge tubes   \* Each PCR will require specific template DNA and DNA primers, as well as the 2X DNA polymerase MasterMix. Consult the protocol specific information provided in [Protocol 2.1a: Cloning into pExTra](#_h4smgykizv00) or [Protocol 2.1b: Cloning into p2Hα.](#_k2m5z4piw6o4) Procedure : **Important :** Gloves should always be worn when doing molecular cloning.   1. Set up the PCR:    1. Obtain and label a PCR tube for each PCR to be performed.    2. For each PCR, add all the following components to the tube. **Important :**Follow the order of addition as indicated in the table below, and use a new pipette tip for each addition.  |  |  |  | | --- | --- | --- | | **Order of Addition** | **PCR Component** | **Volume** | | 1 | sterile water | 9.0 μl | | 2 | forward primer | 1.25 μl | | 3 | reverse primer | 1.25 μl | | 4 | template DNA | 1.0 μl | | 5 | 2X polymerase MasterMix | 12.5 μl | |  | **Total volume** | **25 μl** |  1. Close the PCR tube cap(s) tightly and do a quick spin or gently tap on bench to settle all liquid to the bottom of the tube. 2. Place tube(s) in ice.   B. Set-up the thermocycle PCR program.   1. Set the heated lid temperature to 95 **°** C. 2. Set the thermocycler parameters based on the DNA polymerase being used. **Note :** Annealing temperatures may be adjusted based on the specific primer pairs. Extension times are based on the length of the DNA segment being amplified.  |  |  |  |  |  | | --- | --- | --- | --- | --- | |  | **Q5 DNA Polymerase** | | **Taq DNA Polymerase** | | | **PCR Step** | **Temperature** | **Duration** | **Temperature** | **Duration** | | a. Initial Denaturation | 95 **°** C | 5 min | 94 **°** C | 5 min | | b. Denaturation | 95 **°** C | 10 sec | 94 **°** C | 15 sec | | c. Annealing | 60 **°** C | 10 sec | 60 **°** C | 15 sec | | d. Extension | 72 **°** C | 30 sec/kb | 68 **°** C | 1 min/kb | | Repeat PCR Steps b -d for 29 cycles | | | | | | e. Final Extension | 72 **°** C | 5 min | 68 **°** C | 5 min | | f. Hold | 4 **°** C | infinite | 4 **°** C | infinite |  1. Place the PCR tube(s) in the thermocycler. 2. Run the PCR program. Once complete, store your reaction tubes at 4 °C until you are ready to proceed to[Protocol 2.3: Gel Electrophoresis.](#_reg54i7mfy66) |

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# **Protocol 2.3: Gel Electrophoresis**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.3: Gel Electrophoresis** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To visualize and determine the size of DNA segments. Rationale: Gel electrophoresis is a technique that separates DNA molecules on the basis of size. DNA molecules are loaded onto an agarose gel and exposed to an electric current. The DNA molecules migrate through the sieve-like agarose gel due to their inherent negative charge; smaller DNA molecules move more easily, and therefore more quickly, than larger DNA molecules through the gel. The agarose gel contains a dye that binds to DNA and allows the DNA molecules that have now been separated by size to be visualized under ultraviolet light. The size of the DNA molecules can then be calculated by comparing to a ladder of DNA molecules of known sizes. Supplies:  * Pre-poured agarose gel (0.8%) * DNA loading dye (6X) * Electrophoresis apparatus and power supply * 1X TBE or TAE running buffer * DNA samples * DNA ladder * Gel imaging equipment  Procedure : **Important :** Gloves should always be worn when doing molecular cloning.   1. Prepare your DNA samples for gel electrophoresis.    1. Determine the volume of DNA sample to be loaded of the gel. **Important :** For verification of PCR products, 5 - 10 μl of sample is sufficient. For gel purification of PCR products, the entire PCR reaction volume (25 μl) should be used.    2. For each DNA sample to be loaded onto the gel, add the approproate volume of DNA sample to a clean microcentrifuge tube. Then add 1 μl of concentrated 6X DNA loading dye for every 5 μl of DNA sample. Pipet up and down gently to mix. 2. Load the DNA sample and loading dye mixture onto the agarose gel according to your instructor's directions and following the instructions below.    1. In the first well of the gel, load an appropriate volume of DNA ladder. Each manufacturer is different, so follow the manufacturer’s guidelines. **Important :** If gel electrophoresis is being performed as part of Protocol 2.5: Gel Extraction of DNA, skip a lane in the gel between each sample as well as the DNA ladder to avoid cross-contamination of samples.    2. Using a fresh tip on your micropipettor for each sample, pipette each DNA sample and loading dye mixture into a new well in the gel.       1. Holding the pipette in both hands, place your elbows on either side of the gel apparatus.       2. Situate your eyes directly above the wells to make the wells easier to see.       3. Place the pipette tip directly above the well, just below the surface of the buffer. Do not try to get the pipette tip into the well, or you might puncture the bottom of the gel.       4. Slowly depress the pipette plunger, allowing the solution to slowly sink into the well.       5. Remove the pipette from the gel before releasing the plunger.    3. Draw a picture of the gel in your lab notebook, making note of where your samples are relative to your classmates’ samples. (Once the gels are loaded, everyone’s samples look alike!)    4. Plug the electrodes into the appropriate locations on the power supply. Turn on the power supply and set the voltage to 100 V. **Important :** Remember, DNA runs toward the RED electrode!    5. Run the gel until the visible loading dye front has migrated two thirds the length of the gel. This will take between 30 - 60 minutes depending on the size of gel being used. **Important :** It is important to monitor the migration of the dye front in your gel, which migrates at the same speed as DNA molecules of approximately 300 bp. Running the gel for too short a duration will not allow for good separation of different sized DNA moecules. Running the gel for too long may result in small DNA molecules to run off the gel and into the buffer.    6. Turn off the power supply. 3. Gel extraction    1. If extracting DNA of a particular size from your gel, proceed directly to Step B of Protocol 2.5: Gel Extraction of DNA. Otherwise, continue to Step D, below. 4. Photograph the gel.    1. Using gloves, carefully remove the gel from the electrophoresis chamber.    2. Photograph your gel, following your instructor’s directions.    3. Include a copy of the gel photograph in your laboratory notebook. 5. Clean up your work area.    1. Dispose of your gel as directed by your instructor.    2. If another gel isn’t going to be run, empty the buffer into the sink, rinse out the gel apparatus, and set it aside to dry. The buffer can be reused, so check to see if anyone else will need to run a gel in the next few days.   **Helpful tips:** |

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# **Protocol 2.4: Column Purification of DNA**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.4: Column Purification of DNA** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To clean DNA samples after enzymatic reactions. Rationale: Molecular cloning involves multiple reaction steps in which DNA molecules are mixed with different enzymes and buffers. Often, the components from one reaction are incompatible with the conditions for a second or downstream reaction. As such, the DNA must be purified away from the other components of a reaction before it can be used in a downstream reaction. A convenient way for purifying DNA is to use commercially-available column kits. Using these column kits, DNA from various samples are mixed with salts that denature residual proteins and allow DNA molecules larger than 50 bp to bind silica resin within the column, while other reaction are washed away. The DNA can then be eluted from the column using water. A separate strategy for purifying DNA is described in [Protocol 2.5: Gel Extraction of DNA.](#_4omr8mp0h1bq) Supplies:  * DNA sample * DNA Clean and Concentrator Kit (Zymo) * sterile, nucelase free water * microcentrifuge * micrcentrifuge tubes  Procedure : **Important :** Gloves should always be worn when doing molecular cloning.   1. Bind DNA to the column.    1. For each DNA sample being purified, transfer the entire volume of the DNA sample to a new and clean microcentrifuge tube. **Important :** Make note of the DNA sample volume.    2. Based on the volume of DNA sample transfered, add 5 times that volume of DNA Binding Buffer to the DNA sample. Pipette up and down gently to mix. **Example:** *To 25 μl of DNA sample, add 125 μl of DNA Binding Buffer.*    3. Label a spin column according to your DNA sample, place the spin column in a collection tube, and transfer the mixture of DNA sample and DNA Binding Buffer to the labeled spin column.    4. Centrifuge at the maximum speed for 30 sec.    5. Remove the spin column and discard the flow-through that accumulated in the collection tube into an appropriate liquid waste container. Then place the spin column back onto the same collection tube, and proceed to the next step. 2. Wash the DNA now bound within the column.    1. Add 200 μl of DNA Wash Buffer to the spin column.    2. Centrifuge at the maximum speed for 30 sec.    3. Remove the spin column and discard the flow-through from the collection tube. Then place the spin column back onto the same collection tube.    4. Repeat Steps B1-3 3. Elute the DNA    1. Transfer the spin column to a new and labelled microcentrifuge tube.    2. Add 25 μl of sterile water to the spin column. **Important :** To make sure that the water soaks the column matrix, pipette the water directly onto the 'white' matix in the center of the column.    3. Incubate at room temperature for 1 min.    4. Centrifuge at the maximum speed for 30 sec.    5. The flow-through in the microcentrifuge tube is your eluted and purified DNA. 4. Measure the concentration of your DNA.    1. Follow your instructor's directions to measure the concentration of your purified DNA.    2. Record the concentration of DNA, in ng/ μl, on the side of the microcentrifuge tube and in your notebook.    3. Take note of the A260/A280 (DNA/protein) ratio and A260/A230 (DNA/organic compounds) ratio for your purified DNA. A 'pure' DNA sample should have a A260/280 ratio of ~1.8 and an A260/A230 ratio of 2.0 - 2.2.   **Helpful tips:** |

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# **Protocol 2.5: Gel Extraction of DNA**

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| --- | --- |
| **templateId** | **chapterContent** |
| **name** | **Protocol 2.5: Gel Extraction of DNA** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To extract DNA segments of a certain size from an agarose gel. Rationale: DNA molecules can easily be purified away from other reaction components, such as enzymes and buffers, using [Protocol 2.4: Column Purification of DNA](#_ui1k69asslld). However, column purification indiscriminately purifies all DNA molecules longer than 50 bp. To purify DNA molecules of a particular sizes from a mixture that contains DNA molecules of multiple sizes, DNA molecules can first be separated by size using gel electrophoresis, then physically removing parts of the agarose gel that contain DNA fragments of the desired size, and finally, purifying that DNA away from the components of the gel using a column. Supplies:  * DNA sample * Pre-poured agarose gel * DNA loading dye (6X) * Electrophoresis apparatus and power supply * DNA ladder * Razor blade, spatula, or gel extraction tool * Gel DNA Recovery Kit (Zymo) * Sterile, nucelase-free water * Microcentrifuge * Microcentrifuge tubes * Thermocycler, heat block, or incubator set to a temperature within 37 - 55 **°** C.  Procedure : **Important :** Gloves should always be worn when doing molecular cloning.   1. Perform gel electrophoresis    1. For each DNA sample from which you wish to purify DNA of a particular size, perform gel electrophoresis using [Protocol 2.3: Gel Electrophoresis](#_reg54i7mfy66) . **Important :** Skip a lane in the gel between each sample as well as the DNA ladder to avoid cross-contamination of samples. 2. Excise the band of interest from the agarose gel.    1. For each band of agarose to be extracted from the gel, obtain and label a new microcentrifuge tube.    2. Weight the microcentrifuge tube and write this initial mass on the side of the tube.    3. Carefully place the agarose gel on the gel imaging apparatus or UV box.    4. Wearing appropriate personal protective equipment to protect from UV light (e.g. a face shield) and following your instructor's directions, turn on the UV light source to visualize the DNA bands within the gel.    5. Using a razor blade, spatula, or gel extraction tool, carefully cut out the DNA band of interest from the gel and place the extracted gel piece in the labeled microcentrifuge tube. **Important :** Take care to avoid other DNA bands in the same or neighbouring lanes. **Note :** The gel slice can be stored at -20 **°** C for multiple days.    6. Weight the microcentrifuge tube containing the gel piece. Write the final mass on the side of the tube.    7. Calculate the mass of the gel slice by subtracting the initial mass of the tube from the final mass of the tube. 3. Extract the DNA from the agarose gel.    1. To the microcentrifuge containing the gel slice, add 300 μl of Agarose Dissolving Buffer (ADB) for every 100 mg of gel slice. **Example :** *For a gel slice with a mass of 150 mg, add 450 μl of ADB*    2. Incubate the tube at 37 - 55 **°** C for 5 - 10 minutes, until the agarose is completely dissolved. **Important :** To check if the agarose has completely dissolved, invert the tube 2 - 3 times to mix the contents of the tube.    3. Label a spin column, place the spin column in a collection tube, and transfer the entire DNA/ADB mixture to the labeled spin column.    4. Centrifuge at the maximum speed for 30 sec.    5. Remove the spin column and discard the flow-through that accumulated in the collection tube into an appropriate liquid waste container. Then place the spin column back onto the same collection tube, and proceed to the next step. 4. Wash the DNA now bound within the column.    1. Add 200 μl of DNA Wash Buffer to the spin column.    2. Centrifuge at the maximum speed for 30 sec.    3. Remove the spin column and discard the flow-through from the collection tube. Then place the spin column back onto the same collection tube.    4. Repeats Steps D1-3. 5. Elute the DNA    1. Transfer the spin column to a new and labelled microcentrifuge tube.    2. Add 25 μl of sterile water to the spin column. **Important :** To make sure that the water soaks the column matrix, pipette the water directly onto the 'white' matrix in the center of the column.    3. Incubate at room temperature for 1 min.    4. Centrifuge at the maximum speed for 30 sec.    5. The flow-through in the microcentrifuge tube is your eluted and purified DNA. 6. Measure the concentration of your DNA    1. Follow your instructor's directions to measure the concentration of your purified DNA.    2. Record the concentration of DNA, in ng/ μl, on the side of the microcentrifuge tube and in your notebook.    3. Take note of the A260/A280 (DNA/protein) ratio and A260/A230 (DNA/organic compounds) ratio for your purified DNA. A 'pure' DNA sample should have a A260/280 ratio of ~1.8 and an A260/A230 ratio of 2.0 - 2.2.   **Helpful tips:**  1. UV light can be mutagenic to DNA . To limit the UV exposure of your DNA samples, use long-wavelength UV for as short as time as possible when extracting bands from a gel. |

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# **Protocol 2.6: Restriction Enzyme Digests**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.6: Restriction Enzyme Digests** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To prepare the ends of DNA fragments for molecular cloning. Rationale: Restriction endonuclease enzymes recognize and cleave double-stranded DNA at specific 4-6 bp palindromic sequences called restriction sites. Some restriction enzymes cleave both DNA strands at the same position, generating 'blunt ends', whereas other restriction enzymes cleave each DNA strand at slightly different positions, generating single-stranded overhangs called 'sticky-ends'. These sticky-ends are very useful for molecular cloning, as they allow for two pieces of DNA, for example a gene insert and a plasmid, that have similar overhangs to pair efficiently and specifically. Once paired, the enzyme DNA ligase can be used to covalently link the two pieces of the together. Supplies:  * Purified DNA sample * Restriction enzymes \* (NEB) * 10X restriction enzyme buffer (NEB) * Sterile, nuclease-free water * Thermocycler, heat block, or incubator set to 37 **°** C   \*For cloning genes into the p2H α plasmid, gene inserts and p2H α are both cleaved with NdeI and BamHI restriction enzymes. If the phage gene insert has a BamHI site within it, NdeI and SbfI enzymes should be used. If the phage gene insert has an NdeI site within it, do not proceed with this protocol. Procedure **Important :** Gloves should always ben worn when doing molecular cloning.   1. Determine the volume of DNA sample to be used.    1. F or each DNA sample to be cleaved, use the concentration of the DNA sample to calculate the volume needed to obtain 0.5-1 µg of DNA. **Important :** If you do not have enough sample to obtain 0.5 µg of DNA, check with your instructor if it is suitable to use the maximum volume of DNA sample allowed in the reaction. 2. Set up the restriction enzyme digest reaction.    1. For each DNA sample to be cleaved, obtain and label a new microcentrifuge tube.    2. Add the following components to the microcentrifuge tube for each restriction enzyme digest reaction. **Important :** Follow the order of addition as indicated in the table below, and use a new pipette tip for each addition.  |  |  | | --- | --- | | **Reaction Component** | **Volume** | | Sterile water | up to 50 µl | | 10X reaction buffer | 5 µl | | Restriction enzyme (each) | 1 µl | | DNA sample | predetermined volume | | **Total Volume** | **50 µl** |  * 1. Mix the contents of each tube gently and quick spin the tube in a microcentrifuge tube for less than 1 min to move all the liquid to the bottom of the tube.   2. Incubate the tube(s) at 37 **°** C for 1 hour, or follow your instructor's directions.   3. After the incubation period, quick spin the tube in a microcentrifuge tube for less than 1 min to move all the liquid to the bottom of the tube. Store at -20 **°** C until ready to use for downstream applications.  1. Purify DNA from the reaction.    1. To purify DNA from the digest reaction for downstream applications,use [Protocol 2.4: Column Purification of DNA](#_ui1k69asslld) .   **Helpful tips:** |

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# **Protocol 2.7: Isothermal Assembly**

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| --- | --- |
| **templateId** | **chapterContent** |
| **name** | **Protocol 2.7: Isothermal Assembly** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To construct a plasmid from a gene insert and plasmid backbone DNA Rationale: Isothermal assembly is a molecular cloning strategy that allows for the rapid assembly of multiple DNA fragments into a single DNA molecule. This strategy is quick, with multiple enzymatic reactions happening simultaneously and at one incubation temperature, but requires that the DNA segments to be assembled be linear and have overlapping sequences that are at least 20 bp in length. Isothermal assembly will be used to assemble PCR-amplified gene inserts with the pExTra backbone DNA (i.e. plasmid that has been made linear). Supplies:  * Purified gene-insert DNA * Plasmid backbone DNA\* * 2X NEBuilder HiFi DNA Assembly MasterMix (NEB) * Ice * Sterile, nuclease-free water * Thermocycler, heatblock, or incubator set to 50 **°** C * Microcentrifuge or PCR tubes.   \* Plasmid backbone DNA will be prepared and provided by your instructor. Procedure  1. **Important :** Gloves should always be worn when doing molecular cloning. 2. This isothermal assembly protocol is optimized for 50 ng of plasmid backbone DNA, and 2 molar excess of insert DNA. To setup the assembly reaction, the volume of plasmid backbone and insert DNA to be used must first be determined.    1. Determine the volume of plasmid backbone DNA to be used.       1. Based on the concetration of the plasmid DNA prepared by yor instructor, use the formula below to calculate the volume of sample needed to obtain 50 ng of plasmid backbone DNA. Formula: volume needed, µl = (amount needed, ng) / (concentration of stock, ng/µl) ***Example :*** *F or plasmid backbone DNA at 100 ng/µl, volume needed, µl = (50 ng) / (100 ng/µl) = 0.5 µl*       2. Record this volume in the table below. See helpful tip below.    2. Determine the volume of insert DNA to be used.       1. Based on the length and the concentration of the insert DNA, use the formula below to calculate the volume of sample needed to obtain a 2 molar excess of insert DNA to 50 ng of plasmid backbone DNA. Formula: volume needed, µl = 2 x 50 ng x (length of insert, bp / length of plasmid backbone, bp) / (concentration of insert DNA, ng/µl) ***Example :*** *F or insert DNA that is 450 bp in length and prepared at 25 ng/µl, and will be cloned into the 6 kbp pExTra plasmid backbone, volume needed, µl = 2 x 50 ng x (450 bp / 6000 bp) / (25 ng/µl) = 0.3 µl*       2. Record this volume in the table below. See helpful tip below. 3. Determine the volume of water to be used.    1. Sum the volume of plasmid backbone and insert DNA in the table below. **Important :** The sum volume should not exceed 5 µl.    2. Subtract this summed value from 5 µl. This subtracted value is the volume of water to be used.    3. Record this calculated volume for water in the table below. 4. Set up the isothermal assembly reaction. **Important :** All components of the assembly reaction should be kept cold, on ice, during the setup process.    1. Obtain an aliquot of 2X HiFi DNA Assembly MasterMix and place it on ice. Flick the tube with your finger a few times to mix prior to adding to the reaction.    2. Obtain and label a PCR tube for each assembly reaction to be setup.    3. Using the information recorded in the table below, setup the assembly reaction. Important : Follow the order of addition as indicated in the table below., and use a new pipette tip for each addition.  |  |  | | --- | --- | | Reaction Component | Volume, µl | | Plasmid backbone DNA (50 ng) |  | | DNA insert |  | | Sterile, nuclease-free water |  | | 2X HiFi DNA Assembly MasterMix | 5 µl | | Total Volume | 10 µl |  * 1. Once all the components have been added, gently pipette up and down 4 - 5 times to mix, and keep on ice.   2. When ready to begin the assembly reaction, place the tubes at 50 **°** C for 30 min.   3. Once the reaction is complete, place the reaction on ice. Then proceed to [Protocol 2.9 Transformation of Bacteria](#_a78ohaqyc10k), or store the reaction at -20 **°** C   **Helpful tips:**  - It is not recommended to pipette volumes less than 1µl. If the concentration of your insert or plasmid backbone sample requires less than 1 µl be added, make an appropriate dilution of the sample.  ***Example 1 :*** *F or plasmid backbone DNA at 100 ng/µl,*  *volume needed, µl = (50 ng) / (100 ng/µl) = 0.5 µl*  *A 1:2 dilution can be made, mixing equal volumes of the plasmid backbone and sterile nuclease-free water, for a final DNA concentration of 50 ng/µl.*  *volume needed, µl = (50 ng) / (50 ng/µl) = 1 µl*  ***Example 2:*** *For insert DNA that is 450 bp in length and prepared at 25 ng/µl, and will be cloned into the 6 kbp pExTra plasmid backbone,*  *volume needed, µl = 2 x 50 ng x (450 bp / 6000 bp) / (25 ng/µl) = 0.3 µl*  *A 1:5 dilution can be made, mixing four volumes of sterile nuclease-free water for every volume of insert, for a final DNA concentration of 5 ng/µl.*  *volume needed, µl = 2 x 50 ng x (450 bp / 6000 bp) / (5 ng/µl) = 1.5 µl* |

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# **Protocol 2.8: DNA Ligation**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.8: DNA Ligation** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To construct a plasmid from a gene insert and plasmid backbone DNA Rationale: DNA ligation is the process by which the ends of two DNA molecules can be covalently linked together. This linkage is formed by the enzyme DNA ligase. When constructing a plasmid, DNA ligase can be used to covalently link together the ends of a gene insert with that of plasmid backbone DNA, thereby regenerating a circular plasmid, now with the gene insert. DNA ligation will be used to assemble the p2Hα plasmid containing a phage gene of interest. Supplies:  * Purified gene-insert DNA * plasmid backbone DNA\* * 2X Instant Sticky-end Ligase MasterMix (NEB) * Ice * Sterile, nuclease-free water * Thermocycler, heatblock, or incubator set to 65 **°** C * Microcentrifuge or PCR tubes.   \* Linear plasmid backbone DNA will be prepared and provided by your instructor.  **Important :** Gloves should always ben worn when doing molecular cloning.   1. This DNA ligation protocol is optimized for a 3:1 molar ratio of insert DNA to plasmid backbone DNA. To setup the ligation reaction, the volume of insert and plasmid backbone DNA to be used must first be determined.    1. Determine the volume of plasmid backbone DNA to be used.       1. Based on the concetration of the plasmid DNA prepared by your instructor, use the formula below to calculate the volume of sample needed to obtain 50 ng of plasmid backbone DNA. Formula: volume needed, µl = (amount needed, ng) / (concentration of stock, ng/µl) ***Example :*** *F or plasmid backbone DNA at 100 ng/µl, volume needed, µl = (50 ng) / (100 ng/µl) = 0.5 µl*       2. Record this volume in the table below.    2. Determine the volume of gene insert DNA to be used.       1. Based on the length and the concentration of the insert DNA, use the formula below to calculate the volume of sample needed to obtain a 3 molar excess of insert DNA to 50 ng of plasmid backbone DNA. Formula: volume needed, µl = 3 x 50 ng x (length of insert, bp / length of plasmid backbone, bp) / (concentration of insert DNA, ng/µl) ***Example :*** *F or insert DNA that is 750 bp in length and prepared at 25 ng/µl, and will be assembled with the 5 kbp p2Hα plasmid backbone, volume needed, µl = 3 x 50 ng x (750 bp / 5000 bp) / (25 ng/µl) = 0.9 µl*       2. Record this volume in the table below.    3. Determine the volume of water to be used.       1. Sum the volume of plasmid backbone and insert DNA in the table below. **Important :** The sum volume should not exceed 5 µl.       2. Subtract this summed value from 5 µl. This subtrated value is the volume of water to be used.       3. Record this calculated volume for water in the table below. 2. Set up the DNA ligation reaction.    1. Obtain and label a microcentrifuge or PCR tube for each DNA ligation reaction to be setup.    2. Using the information recorded in the table below, setup the reaction. **Important :** Follow the order of addition as indicated in the table below, and use a new pipette tip for each addition.  |  |  | | --- | --- | | Reaction Component | Volume, µl | | Plasmid backbone DNA (50 ng) |  | | DNA insert |  | | Sterile, nuclease-free water |  | | 2X Instant Sticky-end Ligase MasterMix | 5 µl | | Total Volume | 10 µl |  * 1. Once all the components have been added, gently pipette up and down 4 - 5 times to mix.   2. Incubate the reactions on the bench, at room temperature, for 10 min.   3. Once the reaction is complete, place the reaction on ice. Then proceed to [Protocol 2.9 Transformation of Bacteria](#_a78ohaqyc10k) , or store the reaction at -20 **°** C   **Helpful tips:**  - It is not recommended to pipette volumes less than 1µl. If the concentration of your insert or plasmid backbone sample requires less than 1 µl be added, make an appropriate dilution of the sample.  ***Example :*** *For plasmid backbone DNA at 100 ng/µl,*  *volume needed, µl (50 ng) / (100 ng/µl) = 0.5 µl*  *A 1:2 dilution can be made, mixing equal volumes of the plasmid backbone and sterile nuclease-free water, for a final DNA concentration of 50 ng/µl.*  *volume needed, µl = (50 ng) / (50 ng/µl) = 1 µl* |

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# **Protocol 2.9: Chemical Transformation of Bacteria**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.9: Chemical Transformation of Bacteria** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To introduce plasmid DNA into bacterial cells. Rationale: Transformation is the process of introducing a piece of DNA, typically a plasmid, into bacterial cells. Once transformed, bacterial cells store and replicate the plasmid as it does its own genetic material. In molecular cloning, plasmids that are assembled *in vitro*, for example by isothermal assembly or DNA ligation, are transformed into bacteria so that the plasmid can be replicated. Transformed bacteria, when grown to high densities, can therefore be used to generate large quantities of plasmid DNA, which can subsequently be extracted. Transformations are also an important technique for introducing specific genes into bacteria in order to study gene function.  In chemical transformation, plasmid DNA is mixed with bacterial cells, and this mixture is briefly incubated at a high temperature. This 'heat shock' allows plasmid DNA to enter the cells. During a transformation reaction, not all bacterial cells will be transformed with DNA. However, those bacteria that have been transformed with plasmid DNA can readily be selected based on antibiotic resistance conferred by the plasmid. To select transformed bacteria, bacterial cells from the transformation reaction are allowed to grow for an hour such that transformed cells begin expressing the antibiotic resistance gene, and all cells are then plated on agar supplemented with the appropriate antibiotic. Only cells that are transformed will survive, forming colonies on the agar plate. Supplies:  * Plasmid DNA * Ice * Microcentrifuge tubes * Microcentrifuge * Recovery broth\* * LB agar plates supplemented with the appropriate antibiotics * Rattler beads or spreader, with 100 % EtOH * Shaking incubator set to 37 **°** C * Chemical competent cells * Heat block set to 42 **°** C.   \*LB or SOC for *E. coli* cells Procedure: **Important :** Competent cells should be kept on ice throughout Steps A - C.   1. Retrieve chemical competent cells from -80 **°** C and place on ice, immediately, to thaw.    1. For each transformation reaction, 25 μl of competent cells will be needed. 2. Determine the volume of DNA to use for each reaction.    1. If transforming bacteria with purified plasmid, use 1 μl of plasmid DNA.    2. If transforming bacteria with the mix from an isothermal assembly or DNA ligation reaction, use 2 μl of the reaction mix. **Important :** The volume of DNA used in a chemical transformation should not exceed 10 % of the total transformation reaction volume, as it will decrease the efficiency of the transformation reaction. 3. Set up the transformation reaction.    1. For each tarsnformation reaction, obtain and label a clean microcentrifuge tube.    2. Add the volume of DNA, as determine in Step B, to the microcentrifuge tube.    3. Place the microcentrifuge tube with DNA on ice.    4. Add 25 μl of chemical competent cells from Step A to the microcentrifuge tube.    5. Incubate the mix on ice for 20 min. 4. Heat shock cells to allow for DNA uptake.    1. Transfer the microcentrifuge tube from ice directly to the 42 **°** C heat block, and incubate at 42 **°** C for 30 sec.    2. Transfer the microcentrifuge tube directly back onto ice, and incubate on ice for 3 min. 5. Allow cells to recover.    1. Using aseptic technique, add 1 ml of LB recovery broth to the microcentrifuge tube.    2. Incubate the miccrocentrifuge tube at 37 **°** C, with shaking, for 1 hr. 6. Prepare the appropriate selection plate(s)    1. Consult the information provided for the appropriate plasmid to determine the antibiotic resistance conferred by the plasmid. Obtain two plates for each transformation reaction.    2. Label the plates with your name, date, incubation temperature, and experimental information according to your instructor's direction. 7. Spread the transformation reaction onto selection plate(s).    1. For each transformation reaction:       1. Transfer 100 μl of the recovery mix to one of the two selection plates.       2. Spin the 900 μl of recovery mix remaining in the microcentrifuge tubes in a microcentrifuge at 2,000 x g for 5 min.       3. Remove 800 μl of the supernatant fraction, being careful to not disturb the pellet. Discard the supernatant in an appropriate liquid waste container.       4. Resuspend the pellet in the remaining liquid, approximately 100 μl, by pipetting up and down to mix.       5. Transfer the entire resuspension onto the second of the two selection plates.    2. Using Rattler beads or a glass spreader that has been decontaminated using EtOH, spread the mixture to even distribute the cells on the agar surface of each selection plate.    3. With the lids closed, allow the plates to sit on the bench until all the liquid has been fully absorbed into the agar.    4. Invert the plates and incubate the plates at the temperature suitable for propagation of the plasmid.   **Helpful tips:** |

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# **Protocol 2.10: Clone Verification**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.10: Clone Verification** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To determine if an assembled plasmid contains insert DNA of the correct size. Rationale: When plasmids are assembled, either by isothermal assembly or DNA ligation, and transformed into bacteria, it is important to verify that the assembly reaction occured as intended and that the assembled plasmid contains the correct insert. A strategy that is routinely employed for doing so involves verifying that the DNA insert is of the correct size. This can be achieved by amplifying the region of the plasmid that contains the insert by PCR, and analyzing the size of that amplified DNA by gel electrophoresis. To save on time and reagents, the plasmid within bacterial cells do not need to be purified for use as template DNA in a PCR reaction. Instead, whole bacterial cells containing the plasmid can be used directly in the PCR reaction. This is known as 'colony PCR'. Supplies: For colony PCR:   * *E. coli* colonies containing the plasmid to be analyzed * PCR tubes * 2X One Taq Polymerase Master Mix * DNA Primers (10 μM) * Ice * Sterile, nuclease-free water * Thermocycler   For gel electrophoresis   * Pre-poured agarose gel * DNA loading dye (6X) * Electrophoresis apparatus and power supply * DNA samples * DNA ladder * Gel imaging equipment  Procedure **Note:** This is not a new protocol, but instead a combination of two protocols: [Protocol 2.2: Polymerase Chain Reaction](#_agawv4ji2xgh) and [Protocol 2.3: Gel Eelctrophoresis](#_reg54i7mfy66) .   1. Observe your transformation plate and determine which colonies to test.    1. On the bottom of the plate, circle and label colonies to be tested. Typically, 3 - 4 colonies from a given transformation reaction are tested. **Important :** Select well-isolated colonies to minimize cross-contamination. 2. Set up the colony PCR.    1. Prepare template DNA.       1. Obtain and label a new microcentrifuge tube for each colony to be tested.       2. Add 100 μl of water into each microcentrifuge tube.       3. Gently touch a sterile pipette tip to a colony to be tested, removing only as much as a quarter of the colony. **Important :** Do not transfer the entire colony.       4. Swirl the pipette tip in the water within the microcentrifuge tube to transfer the bacterials cells. This is your template DNA.    2. Perform the PCR.       1. Consult the information for the appropriate plasmid to determine which primers and DNA polymerase to use for clone verification.       2. Follow instructions in [Protocol 2.2: Polymerase Chain Reaction.](#_agawv4ji2xgh) 3. Determine the size of PCR amplified DNA.    1. Follow instructions in [Protocol 2.3: Gel Electrophoresis](#_reg54i7mfy66) to determine the size of products for each colony PCR reaction.    2. Determine which colonies contain plasmid DNA with an insert of the correct size.    3. Record this information in your notebook and in a Clone Verification Data Card.    4. To prepare large quantities of plasmid with an insert of the correct size,proceed to [Protocol 2.11: Inoculating a Culture.](#_s1rnln94dbbm)   **Helpful tips:** |

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# **Protocol 2.11: Inoculating a Culture**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.11: Inoculating a Culture** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To inoculate a liquid culture from a single bacterial colony. Rationale: Culturing bacteria is a routine and important experimental step during molecular cloning. As a means to obtain large quantities of a given plasmid DNA, bacteria transformed with that plasmid DNA are cultured to high density, before being harvested and the plasmids extracted. To ensure that all bacteria in the culture maintain and propagate copies of the plasmid DNA, the antibiotics for which the plasmid confers resistance to is added the the culture. Culturing bacteria is also an important step for many microbiological experiments. Supplies:  * Sterile test tubes with lids * Liquid media supplemented with the appropriate antibiotic\* * Sterile inoculation or wooden sticks * Temperature-controlled shaking incubator or rotor * 5 ml serological pipettes * Pipette aid   \* *E. coli* should be cultured in LB liquid medium. Consult your instructor and the Plasmid Information Sheet to determine which antibiotic to use. Procedure  1. For each culture to be inoculated, obtain and label a test tube with a lid. 2. Using good aseptic technique and working close to a flame, with a serological pipette, transfer 5 ml of the appropriate liquid medium that has been supplemented with antibiotic to each test tube. 3. For each culture to be inoculated, identify a well-isolated bacterial colony. On the bottom of the plate, circle and label the colony. 4. Inoculate a culture. **Important :** Use aseptic technique, and work close to a flame, throughout Step D.    1. Using a sterile inoculation or wooden stick, gently touch the selected colony.    2. Remove the lid of the test tube, and swirl the tip of the wooden stick that touched the bacterial colony in the liquid medium.    3. Replace the lid of the test tube. 5. Incubate the test tube, with shaking or rotation, at the appropriate temperature for 16 - 24 hr.    1. Consult the information for the appropriate plasmid to determine the growth temperature.   **Helpful tips:** |

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# **Protocol 2.12: Preparing Plasmid DNA**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 2.12: Preparing Plasmid DNA** |
| **imgtxt** | **Molecular Cloning** |
| **img** | **molecular-cloning.svg** |
| **content** | Objective: To prepare a stock of plasmid DNA for use in downstream experiments. Rationale: As a means to obtain large quantities of a given plasmid, bacteria transformed with that plasmid are cultured to high density, from which the plasmid can be extracted and purified. A routine strategy for doing so is called alkaline lysis. Here, NaOH serves multiple purposes, including lysis of bacteria and denaturation of double-stranded DNA. When the alkaline solution is neutralized, small plasmid DNA readily renatures into double-stranded DNA, whereas large DNA molecules such as the bacterial chromosomal DNA becomes a tangled and insoluble aggregate. The renatured plasmid DNA can then be selectively bound to a silica column and purified away from contaminating salts and proteins. Once 'washed', the plasmid DNA can be eluted from the silica column with water. Supplies:  * High-density *E. coli* liquid culture * Microcentrifuge tubes * Microcentrifuge * Plasmid mini-prep Kit (Zymo) * Sterile, nuclease-free water  Procedure **Important :**Gloves should always be worn when doing molecular cloning.   1. For each plasmid to be purified, obtain and label a microcentrifuge tube. 2. Transfer 1 ml of the bacterial culture containing the plasmid to be purified into the microcentrifuge tube. 3. Lyse the bacterial culture.    1. Centrifuge the tubes in a microcentrifuge at maximum speed for 5 min. **Important :**Ensure that the centrifuge is properly balanced.    2. Remove and discard the supernatant, being careful not to disturb the pellet.    3. Recommended : For a higher yield, transfer an additional 1 ml of culture to the same tube and repeat Steps C1 - 2.    4. Resuspend the cell pellet in 600 μl of water.    5. Add 100 μl of 7X Lysis Buffer and carefully close the tube. The 7X Lysis Buffer is a viscous solution and contains a blue pH indicator.    6. Invert the tube 3 - 4 times or until the solution is uniformly blue. Important : Do not shake the tube too vigorously as this can shear the chromosomal DNA into smaller fragments that cannot be easily separated from plasmid DNA. 4. Neutralize the lysed culture.    1. Add 350 μl of chilled Neutralization Buffer.    2. Invert the tube 3 - 4 times to mix or until the solution turns uniformly yellow, indicating complete neutralization. A precipitate will form. Important : Do not shake the tube too vigorously as this can shear the chromosomal DNA into smaller fragments that cannot be easily separated from plasmid DNA.    3. Centrifuge at maximum speed for 4 min. Important : Ensure that the centrifuge is properly balanced. 5. Bind plasmid DNA to the column.    1. Label a spin column according to your DNA sample, and place the spin column in a collection tube.    2. Being careful not to disturb the large pellet, transfer the supernatant into the spin column. There should be ~ 900 μl.    3. Centrifuge at the maximum speed for 30 sec.    4. Remove the spin column and discard the flow-through that accumulated in the collection tube into an appropriate liquid waste container. Then place the spin column back onto the same collection tube, and proceed to the next step. 6. Wash the DNA now bound within the column.    1. Add 200 μl of Endo-Wash Buffer to the column    2. Centrifuge at the maximum speed for 30 sec.    3. Remove the spin column and discard the flow-through from the collection tube. Then place the spin column back onto the same collection tube.    4. Add 400 μl of Zyppy-Wash Buffer to the column.    5. Centrifuge at the maximum speed for 30 sec.    6. Remove the spin column and discard the flow-through from the collection tube. Then place the spin column back onto the same collection tube. 7. Elute the DNA    1. Transfer the spin column to a new and labeled microcentrifuge tube.    2. Add 30 μl of sterile water to the spin column. Important : To make sure that the water soaks the column matrix, pipette the water directly onto the 'white' matrix in the center of the column.    3. Incubate at room temperature for 1 min.    4. Centrifuge at the maximum speed for 30 sec.    5. The flow-through in the microcentrifuge tube is your eluted and purified DNA. This is your plasmid DNA stock. 8. Measure the concentration of your DNA.    1. Follow your instructor's directions to measure the concentration of your purified DNA.    2. Record the concentration of DNA, in ng/ μl, on the side of the microcentrifuge tube and in your notebook.    3. Take note of the A260/A280 (DNA/protein) ratio and A260/A230 (DNA/organic compounds) ratio for your purified DNA. A 'pure' DNA sample should have a A260/280 ratio of ~1.8 and an A260/A230 ratio of 2.0 - 2.2. 9. Plasmid DNA should be stored at -20 **°** C   **Helpful tips:** |

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# **Chapter 3: Phenotypic Assay**

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| **name** | **Protocol 3.1: Transformation of M. smegmatis by Electroporation** |
| **templateId** | **chapterContent** |
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# **Chapter3:An Overview**

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| **imgtxt** | **Phenotypic Assay** |
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| **content** | Chapter 2 guided you through the process of cloning a phage gene of interest into the plasmid, pExTra. In this chapter, the recombinant plasmid you built and purified will serve as an important tool in your research to investigate the function of this gene. Through the process of transformation by electroporation (Protocol 3.1), this plasmid will be introduced into *M. smegmatis*, the bacterial host of the mycobacteriophages encoding our genes of interest. pExTra was specifically engineered to enable the controlled expression of our phage genes in *M. smegmatis*, allowing us to ask the question: what happens when a particular phage gene is expressed in the host cell? The combined efforts of you and your fellow researchers will enable investigation of this research question on a genome-wide scale. From genes to phenotype As in all organisms, the expression of genes in bacteria, whether they are found on the bacterial chromosome, on a plasmid, or on an infecting phage genome, follows the central dogma: RNA polymerase transcribes the DNA gene sequence into an RNA molecule which is then translated into a peptide chain of amino acids by the ribosome. This amino acid sequence dictates the 3D structure that a peptide chain adopts; this in turn dictates that protein’s function, or the work that it performs in a cell. Together, the coordinated activities of the various proteins that are produced by an organism, determine the observable traits and behavior, or phenotype of that organism. In short, an organism’s phenotype reflects the set of genes being expressed. As the set of genes being expressed in an organism changes, so can the phenotype. For example, during phage infection of a bacterial cell, the expression of phage genes often changes the phenotype of the infected bacterial cell. Manipulating gene expression to study phage gene function The connection between gene expression and observable traits is not only a fundamental tenet of biology; it is also a valuable research tool in the quest to understand the function of a gene. By manipulating gene expression in some way and looking for an observable change in the organism, we can begin to associate the gene with processes it is involved in and thereby gather clues as to its function. The expression of a gene can be altered in various ways, either by turning it off completely, turning it on when it would not normally be expressed, or increasing or decreasing the amount of expression. In our investigation, we will express a given mycobacteriophage gene from the pExTra plasmid in the host cell and observe if and how it alters the host phenotype. To some extent, this approachmimics a typical phage infection of a mycobacterial cell, though with some key differences; for example, other phage gene products that may regulate or contribute to the function of our gene of interest will not be present in the cell. We know that many phage gene products manipulate host processes in some way in order to promote phage survival and propagation; consequently, we expect to observe some of the phenotypes that are typically observed during a phage infection, including growth inhibition of the host cell. Expression of the phage gene by itself allows us to ascribe any phenotypic change to the specific phage gene being studied  For controlled expression of the phage gene, and for careful assessment of gene expression, the pExTra plasmid was engineered with two features:  1. a promoter that allows gene expression to be turned on and off, and  2. a reporter gene that indicates when expression is occurring. 1. The pExTra inducible promoter A promoter is a DNA sequence that controls transcription of the gene(s) immediately downstream. Bacterial promoters are typically composed of two 6 bp long sequences, referred to as the -10 and -35 sites, to which RNA polymerase binds to initiate gene transcription. If RNA polymerase can access a given promoter at all times, and thereby initiate transcription continuously, the promoter is said to be constitutively ‘on’. However, many promoters are regulated by additional factors and are only ‘on’ under specific conditions. Such promoters are known as inducible promoters and can be either negatively or positively regulated.  In a negatively-regulated promoter, a repressor protein binds to a DNA sequence near or within the promoter DNA, known as an operator site. The bound repressor prevents access to the promoter by RNA polymerase, thereby maintaining the promoter in the ‘off’ state. pExTra plasmids contain a Tetracycline-inducible or ‘Tet’ promoter upstream of the phage gene sequence. This promoter is maintained in the “off” state by the tetracycline-repressor protein, TetR, and can be turned “on” by the addition of the molecule tetracycline. Tetracycline binds TetR, causing a conformational change that releases the repressor from the DNA, thereby enabling access to the promoter by RNA polymerase. Because tetracycline turns “on” the promoter and thereby “induces” gene expression, tetracycline is known as an inducer. In our experiments, we will use a chemically modified form of tetracycline as the inducer, known as anhydrotetracycline, aTc. As illustrated in the plasmid map in Protocol 2.1a, the gene encoding TetR is also found on pExTra, and the repressor is constitutively produced, ensuring that phage gene expression is tightly repressed until the aTc inducer is introduced into our experimental system.  As a side note, the plasmid that we will employ in Chapter 4 contains a different negatively-regulated promoter, the ‘Lac\*’ promoter, which is repressed by the transcriptional regulator LacI and induced by the chemical Isopropyl b-D-1-thiogalactopyranoside (IPTG). We will also utilize a positively regulated promoter in Chapter 4, in which ‘activator’ proteins recruit RNA polymerase to a promoter to initiate transcription. This positively-regulated promoter will be described in Chapter 4. 2. The pExTra reporter gene When we induce expression of a gene in order to investigate its effects, it is important to confirm that the gene is indeed being expressed. This is particularly important when the expression of a gene does not result in any observable change in phenotype. Typically, gene expression is confirmed by monitoring the levels of protein that accumulate in the cell by a technique known as Western blotting. This approach, however, is cumbersome and not suitable for the scope of our research, in which very many different genes will be expressed. Instead, a convenient strategy to monitor expression is by using a second gene, under the control of the same promoter, that produces an easily observable phenotype when expressed. Such genes are known as reporter genes, as they act to “report” on promoter activity. Commonly used reporter genes include genes encoding for fluorescent proteins that can change the color of the cell, or antibiotic resistance genes that allow cells to grow in the presence of antibiotics.  To monitor the expression of a phage gene, the pExTra plasmid has been engineered to contain a reporter gene encoding for the red fluorescent protein mCherry downstream of the phage gene. If transcription from the Tet promoter occurs, both genes will be co-transcribed as a single mRNA but will be translated as two separate proteins—the phage gene product and mCherry. Accumulation of the mCherry protein turns cells pink, providing a clear readout of whether or not the transcript is being produced and thus providing some indication as to whether or not the phage gene product of interest is being produced. Assessing the effects of phage gene expression on host phenotype The pExTra expression system allows us to both control and monitor expression of a particular phage gene in its native host cell environment, but how can we detect and measure a gene-mediated change in host phenotype? This requires the use of a phenotypic assay, or an experiment designed to evaluate a specific host characteristic. In SEA-GENES research, we will be performing two phenotypic assays on *M. smegmatis* transformed with a recombinant pExTra plasmid:  1. a Cytotoxicity Assay, and  2. a Superinfection Immunity Assay. 1. Cytotoxicity Assay The first phenotypic assay is a cytotoxicity assay, which measures the effect of phage gene expression on the ability of *M. smegmatis* to grow; a gene product that slows or prevents bacterial growth is said to be ‘toxic’ and likely interferes with some essential cellular process. Identifying toxic phage gene products is not only important for our understanding of how phages interact with their bacterial hosts but is also a key step in the scientific endeavor to develop new antibacterial therapeutics. We already know of some phage gene products that are toxic to bacteria, for example, the holin and lysin proteins that work in concert to lyse the cell; however, within the vast unexplored phage sequence space, there are certain to be many novel mechanisms by which phage gene products disrupt cellular function. 2. Defense Assay The second phenotypic assay is a defense test which determines whether expression of a phage gene in *M. smegmatis* can protect the cell from infection by the phage that encodes the gene. An example of defense, called superinfection immunity, typically occurs through the same mechanism that enables a phage to form a lysogen. During lysogeny, the phage immunity repressor gene is expressed, and the immunity repressor proteins bind to and represses expression of phage genes required for the lytic cycle. When a second or similar phage infects the lysogen, the repressor proteins in the cell can similarly repress expression from the second phage, thereby providing “immunity” to the second infection. Many immunity repressors can confer this immunity when expressed by themselves without other phage-encoded functions. Genes encoding for immunity repressor proteins have not yet been identified in many temperate mycobacteriophage genomes, and even those that have been bioinformatically predicted are poorly characterized. Performing a defense assay provides important experimental validation of this prediction and can be used to inform and improve genome annotation methods. Furthermore, not only immunity repressors are capable of protecting bacterial hosts from infection; other phage gene products have been shown to prevent infection by closely related phages by a variety of mechanisms, including blocking the phage receptor or inhibiting DNA entry. Thus, this phenotypic assay may reveal novel genes involved in such phage-phage interactions.  The recombinant pExTra plasmid you have constructed could be used in any number of phenotypic assays to observe gene-mediated effects on different host processes. Key to the interpretation of results from any of these assays, though, is the inclusion of appropriate experimental controls; it is essential to compare the behavior of *M. smegmatis* cells transformed with the pExTra plasmid of interest in these assays in the presence or absence of inducer, in order to draw any conclusions as to whether phage gene expression is responsible for an observed effect. Genome-wide analysis of gene-mediated phenotypic effects Combined, the recombinant pExTra plasmids built by you and your fellow researchers form a gene library, or a collection of individual genes cloned into an expression plasmid, that represents most or all of the genetic material in a particular phage genome. Together, you will perform phenotypic assays on the genes in this library, thereby screening the phage genome to identify the small subset of genes that have particular effects on the host phenotype. This collective analysis provides a genome-wide view of phage gene function, and as more and more of these phage gene libraries are built and screened, we can also begin to make comparisons between related genes within the broader phage population. Doing research at scale increases the chances of discovering promising leads to pursue in future research studies while using only a few experimental methods.  Gaining a thorough understanding of how a gene functions in the context of a phage infection, is a large undertaking, requiring the integration of ample data collected by many different methods. The data generated by you and your fellow researchers from the phenotypic assays outlined in this chapter provide important experimental observations that can lead to new hypotheses and get us a few steps closer to better understanding how these genes function in nature! |

# **Protocol 3.1: Transformation of M. smegmatis**

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| **imgtxt** | **Phenotypic Assay** |
| **img** | **phenotypic-assay.svg** |
| **content** | Objective: To introduce plasmid DNA into bacterial cells. Rationale: Transformation is the process of introducing a piece of DNA, typically a plasmid, into bacterial cells. Once transformed, bacterial cells store and replicate the plasmid as it does its own genetic material. In molecular cloning, plasmids that are assembled in vitro, for example by isothermal assembly or DNA ligation, are transformed into bacteria so that the plasmid can be replicated. Transformed bacteria, when grown to high densities, can therefore be used to generate large quantities of plasmid DNA, which can subsequently be extracted. Transformations are also an important technique for introducing specific genes into bacteria in order to study gene function.  In electroporation, plasmid DNA is mixed with bacterial cells. This mixture is then placed in a special container, called an electroporation cuvette, before being pulsed with an electric current. This electric shock allows plasmid DNA to enter cells. When compared to chemical transformations, transformation by electroporation occurs at a much higher efficiency.  During a transformation reaction, not all bacterial cells will be transformed with DNA. However, those bacteria that have been transformed with plasmid DNA can readily be selected based on antibiotic resistance conferred by the plasmid. To select transformed bacteria, bacterial cells from the transformation reaction are allowed to grow for an hour such that transformed cells begin expressing the antibiotic resistance gene, and all cells are then plated on agar supplemented with the appropriate antibiotic. Only cells that are transformed will survive, forming colonies on the agar plate. Supplies:  * Plasmid DNA * Ice * Microcentrifuge tubes * Microcentrifuge * Recovery broth (7H9 neat) * 7H10 agar plates supplemented with 20 μg/ml Kanamycin * Rattler beads or spreader, with 100 % EtOH * Shaking incubator set to 37 **°** C. * Electrocompetent cells * Electroporation cuvettes (gap: 0.1 cm) * Electroporator (voltage: 1.8 kV) * Paper towel or KimWipe  Procedure : **Important :** Competent cells should be kept on ice throughout Steps A - B.   1. Retrieve electrocompetent cells from -80 **°** C and place on ice, immediately, to thaw.    1. For each transformation reaction, 25 - 50 μl of competent cells will be needed. Consult your instructor for the appropriate volume of cells to use. 2. Set up the transformation reaction.    1. For each transformation reaction, obtain and label a clean microcentrifuge tube and a electroporation cuvette. Place both vessels on ice.       1. Add 1 μl of purified plasmid DNA to the microcentrifuge tube. **Important :** Do not used unpurified DNA. The presence of salts from reactions such as isothermal assembly or DNA ligation can result in overheating and electric arcing when a current is applied, which will kill the bacterial cells.    2. Add the appropriate volume of electrocompetent cells from Step A to the microcentrifuge tube.    3. Pipette the mixture up and down gently, one time. **Important :** Act quickly to ensure that the mixture remains cold.    4. Transfer the mixture into the pre-chilled electroporation cuvette. **Important :** Pipette the mixture into the thin gap between two metal electrodes at the bottom of the cuvette. Gently tap the cuvette on the bench to evenly settle the mixture at the bottom of the cuvette. **Important :** Be careful not to introduce any air bubbles in the mixture. 3. Electroporate the cells to allow for DNA uptake.    1. Adjust the settings on the electroporator according to your instructor's directions.    2. Remove the cuvette from the ice, and using a paper towel, wipe off any moisture on the outside of the cuvette. Proceed quickly and carefully through the remaining steps.    3. Place the cuvette into the electroporator holder.    4. Pulse the cells, taking note of the pulse length value. It should be between 4 - 6 msec.       1. If a flash of light is seen or a loud popping sound is heard during the pulse, an electric arc has occured due to contaminating salts in the mixture. Repeat Procedure 2 from the beginning, this time using DNA that has been diluted in water to reduce the salt concentration in the final mixture. 4. Allow cells to recover.    1. Using aseptic technique, add 1 ml of the 7H9 recovery broth to the electroporation cuvette, then transfer the entire volume from the electroporation cuvette into a clean and labeled microcentrifuge tube.    2. Incubate the microcentrifuge tube at 37 **°** C, with shaking for 2 hr. 5. Prepare the appropriate selection plate(s)    1. Obtain two 7H10 agar plates supplemented with 20 μg/ml Kanamycin for each transformation reaction.    2. Label the plates with your name, date, incubation temperature, and experimental information according to your instructor's direction. 6. Spread the transformation reaction onto selection plate(s).    1. For each transformation reaction:       1. Transfer 100 μl of the recovery mix to one of the two selection plates.       2. Spin the 900 μl of recovery mix remaining in the microcentrifuge tubes in a microcentrifuge at 2,000 x g for 5 min.       3. Remove 800 μl of the supernatant fraction, being careful to not disturb the pellet. Discard the supernatant in an appropriate liquid waste container.       4. Resuspend the pellet in the remaining liquid, approximately 100 μl, by pipetting up and down to mix.       5. Transfer the entire resuspension onto the second of the two selection plates.    2. Using Rattler beads or a glass spreader that has been decontaminated using EtOH, spread the mixture to even distribute the cells on the agar surface of each selection plate.    3. With the lids closed, allow the plates to sit on the bench until all the liquid has been fully absorbed into the agar.    4. Invert the plates and incubate the plates at the temperature suitable for propagation of the plasmid.   **Helpful tips:** |

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# **Protocol 3.2: Cytotoxicity Assay**

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| **name** | **Protocol 3.2: Cytotoxicity Assay** |
| **imgtxt** | **Cytotoxicity Assay** |
| **img** | **cytotoxicity-assay.svg** |
| **content** | Objective: To determine whether expression of a given mycobacteriophage gene is toxic to the bacterial host*, M. smegmatis.* Rationale: To assess if expression of a particular phage gene has an effect on the growth of *M. smegmatis*, cells transformed with the appropriate pExTra recombinant plasmid are serially diluted and spotted onto solid growth medium containing antibiotic, to maintain selection of the plasmid, and the inducer molecule anhydro-tetracycline (aTc), to induce expression of the gene. The same dilutions are also spotted on plates lacking aTc, an essential control that allows changes in growth phenotype to be identified. Expression of non-toxic genes will cause no change in the amount of bacterial growth, whereas expression of toxic genes will result in less or no growth as compared to the control plate, or may result in smaller colonies. Plates should be observed over the course of several days and colony color should be monitored. Pink colony color is an indication that the phage gene is being transcribed. Supplies:  * *M. smegmatis* transformed with recombinant pExTra plasmid(s) encoding gene(s) of interest * *M. smegmatis* transformed with pExTra02 plasmid encoding positive control gene * *M. smegmatis* transformed with pExTra03 plasmid encoding negative control gene * Microcentrifuge tubes * 7H9 liquid medium * 7H10 Agar plates supplemented with Kanamycin (20 μg/ml) and aTc (10 ng/ml and 100 ng/ml) * Sterile wooden sticks * Incubator set to 37 **°** C * Vortex * Image documentation equipment * A printed plate template, available for download [here](https://dzf8vqv24eqhg.cloudfront.net/userfiles/11001/22461/ckfinder/files/Cytotoxicity%20Plate_Template.pdf) .  Procedure  1. Identify colonies for testing.    1. Observe your transformation plate(s) containing *M. smegmatis* transformed with your gene(s) of interest after incubation for 4-5 days at 37 ºC .    2. For each gene being tested, identify 3 well-isolated colonies.    3. On the bottom of the plate, circle and label the 3 colonies to be tested. **Note :** You will also need one positive control (pExTra02) and one negative control (pExTra03) colony, which should be obtained from your instructor. 2. Prepare colonies for testing.    1. Obtain and label a clean microcentrifuge tube for each of the 3 colonies to be tested, as well as for the positive and negative controls.    2. Fill each labeled microcentrifuge tube with 500 μl 7H9 liquid medium.    3. Using a sterile wooden stick, transfer each selected colony into the corresponding microcentrifuge tube. Shake the stick so transfer as much of the colony on the stick into the liquid medium.    4. Cap the microcentrifuge tube and vortex to disperse the colony. This is your undiluted cell suspension. **Note:** *M. smegmatis* cells should be properly suspended in the liquid medium before proceeding to the next step. Suspensions may be clumpy, even after vortexing. **Important :** Cells can settle in the tube when left on the bench, even after just a few minutes. Throughout the process of diluting and spotting your samples, it is critical to regularly mix the cell suspensions by pipetting up and down or vortexing for 1-2 seconds. 3. Set up 10-fold serial dilutions (Figure 3.2a).    1. For each of the cell suspensions, obtain 5 additional microcentrifuge tubes, arrange these microcentrifuge tubes in a rack, and label them 10-1, 10-2, 10-3, 10-4, and 10-5.    2. Add 900 μl of 7H9 liquid medium to each of the tubes    3. Perform Steps C 4 - 7 for each of your undiluted cell suspensions.    4. Transfer 100 μl of your undiluted cell suspension to the “10-1” tube. **Important :** Make sure that cell suspension is well mixed before transferring. Try to avoid transferring very large clumps of cells that were not properly suspended.    5. Cap the tube and vortex briefly to mix.       1. **Note :** This solution in this “10-1” tube contains 1/10th the number of cells as your undiluted sample. It is also referred to as a 1:10 dilution.    6. Using a new pipette tip, transfer 100 μl of the “10 -1” sample to the “10-2” tube and vortex well.       1. This solution contains 1/100th as many cells as your undiluted sample. It can also be referred to as your 1:100 dilution.    7. Continue each successive dilution until you get to your last tube, using a fresh tip for each dilution.  |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure showing serial dilutions. A volume of the undiluted cell suspension (100 μl) is transferred into 900 μl of 7H9 liquid media in the 10-1 tube. After the tube is mixed, 100 μl of the 10-1 sample is transferred into the 10-2 tube, and the tube is mixed. This serial dilution continues until the desired dilutions are obtained. The last tube will contain a final volume of 1000 μl. | | | |  |  | | --- | --- | | Caption: | Figure 3.2a. Serial dilutions. A volume of the undiluted cell suspension (100 μl) is transferred into 900 μl of 7H9 liquid media in the 10-1 tube. After the tube is mixed, 100 μl of the 10-1 sample is transferred into the 10-2 tube, and the tube is mixed. This serial dilution continues until the desired dilutions are obtained. The last tube will contain a final volume of 1000 μl. | |  1. Spot dilutions and controls on inducer plates.    1. Obtain the following 3 plates plate for each gene being tested:       1. one 'no inducer' plate,       2. one 'aTc10 inducer plate', and       3. one 'aTc100 inducer plate'.    2. Label the bottom edge of each agar plate with your name, the date, inducer (aTc) concentration, and the gene being tested.    3. Place the agar plate on a printed copy of the plate template. **Note :** The printable plate template is a mirror image of Figure 3.2b. This is to ensure that, when viewed from the bottom of the plate, the negative control is placed in the leftmost column, as in Figure 3.2b.    4. One at a time, aseptically transfer 5 μl of each dilution onto the appropriate position on each of the three agar plates, using a new pipette tip for each spot. **Important :** To ensure that samples are well mixed, pipette up and down a few times before transferring each sample to the plate. **Note :** The pattern of spotting is identical on each of the 3 plates. 2. Allow the plates to sit on the bench for approximately 30 min for the liquid to be absorbed into the agar. 3. Once the liquid has been absorbed, invert the plates and incubate at 37 **°** C for a total of 5 days.  |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure showing the printable 'plate template' which serves as a guide for spotting the samples on the agar plate. | | | |  |  | | --- | --- | | Caption: | Figure 3.2b. The printable 'plate template' serves as a guide for spotting the samples on the agar plate, such that when plates are viewed from the bottom, the samples are distributed as above. | |  1. Observe for cytotoxicity.    1. Plates should be observed for phenotypes on days 2 or 3 and on days 4 or 5 of incubation. **Note :** All colonies should grow on the 'no inducer' plate, and may or may not grow in the presence of aTc inducer.    2. Photograph your plates, following your instructor’s directions.   **Helpful tips:**  When observing plates for cytotoxicity phenotypes, in addition to comparing the growth of the experimental samples on the three concentrations of aTc, it is also helpful to compare their growth to that of the pExTra03 control. If the pExTra03 control exhibits signs of cytotoxicity on aTc, it suggests there may be a technical issue and that the experiment should be repeated! |

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# **Protocol 3.3: Defense Assay**

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| **name** | **Protocol 3.3: Defense Assay** |
| **imgtxt** | **Defense Assay** |
| **img** | **defense-assay.svg** |
| **content** | Objective: To determine whether expression of a phage gene protects the bacterial host, *M. smegmatis* from infection by phages. Rationale: During an infection, the expression of certain phage genes can protect the infected host cell from infection by a second phage. For example, during lysogeny, temperate phages produce an immunity repressor to prevent the expression of its own genes that are involved in lytic replication; expression of this immunity repressor gene also protects the lysogen from infection by a second closely related phage. Other phage gene products have also been found to protect host cells from infection by closely or more distantly related phages. In order to test the ability of individual phage genes to confer protection, or defense, to the host cell, the efficiency of plaque formation by a given phage on bacterial lawns expressing each phage gene will be determined. Supplies:  * *M. smegmatis* transformed with recombinant pExTra plasmid(s) encoding gene(s) of interest * *M. smegmatis* transformed with pExTra01 control plasmid * 7H9 liquid medium (neat) * 7H9 liquid medium (Complete, with Tween80), supplemented with Kanamycin (10 μg/ml)\*\* * 1X 7H9 top agar supplemented with Kanamycin (20 μg/ml) * 1X 7H9 top agar supplemented with Kanamycin (20 μg/ml) and aTc (100 ng/ml) * 7H10 agar plate(s) supplemented with Kanamycin (20 μg/ml) * 7H10 agar plate(s) supplemented with Kanamycin (20 μg/ml) and aTc (100 ng/ml) * Sterile test tubes with lids * Microcentrifuge tubes * Tabletop centrifuge * Sterile inoculation or wooden sticks * Temperature-controlled shaking incubator or rotor set to 37 ºC * 5 ml serological pipettes * Pipette aid   \*\* It is recommended to grow cultures in 10 μg/ml Kanamycin as this yields more consistent growth. Procedure  1. Generate two identical liquid cultures, or replicates, of *M. smegmatis* transformed with a recombinant pExTra plasmid encoding a gene of interest.    1. Observe your transformation plate(s) containing *M. smegmatis* transformed with your gene(s) of interest after incubation for 4 - 5 days at 37 ºC .    2. For each gene to be tested, identify and circle two well-isolated colonies.    3. For each of the two colonies selected, follow [Protocol 2.11](#_s1rnln94dbbm) to inoculate a liquid culture in 7H9 liquid medium (Complete, with Tween80) supplemented with Kanamycin (20 μg/ml). Note : There should be two cultures inoculated for a given gene of interest. To differentiate between these two replicate cultures, add 'rep 1 ' to the label for one culture, and 'rep 2 ' to the other.    4. Incubate the replicates cultures, with shaking or rotation, at 37 ºC for 24 - 48 hours, until the cultures have saturated or have an optical density (O.D.) of 3. Avoid using liquid cultures that have been inoculated for more than 48 hours.    5. **Important :** Control cultures (two replicates per class) should be inoculated with colonies of *M. smegmatis* transformed with the pExTra01 plasmid, following steps A1-4. 2. Wash cells to remove Tween80.    1. Aseptically transfer 1 ml of each saturated liquid culture from Step A4 to a clean and labeled microcentrifuge tube.    2. Spin the microcentrifuge tubes in a tabletop centrifuge at 2,000 x g for 10 min to pellet cells.    3. Discard the supernatant.    4. Aseptically transfer 1 ml of 7H9 liquid medium (neat) to each microcentrifuge tube and gently pipette up and down to resuspend each pellet.    5. For each resuspended cell pellet, aseptically transfer 250 μl of the cell resuspension to two sterile and labeled test tubes. Note : There should be four test tubes (two for each replicate) for each gene being tested. Per class, there should also be four pExTra01 control test tubes. 3. Prepare bacterial lawns on plates*without* inducer (aTc).    1. For each gene being tested, obtain two 7H10 agar plates supplemented with Kanamycin (20 μg/ml).       1. Label the bottom of the plates with the following information (see Figure 3.3a):          1. your initials, date, and incubation temperature,          2. no aTc          3. the phage gene being tested,          4. 'rep 1 ' on one plate and 'rep 2 ' on the other,          5. information about the phage lysate(s) that will be used to infect cells of your bacterial lawn. Note : You and your instructor should decide on the phage lysate(s) that will be used.          6. Divide the plate into rows for each phage lysate to be tested, with a maximum of 4 rows per plate.          7. Divide each row into sections for each lysate dilution to be spotted, with a maximum of 6 sections per row.    2. Obtain the 'rep 1 ' and 'rep 2 ' test tubes with host bacteria from step B5.    3. Obtain 1X 7H9 top agar supplemented with Kanamycin (20 μg/ml).    4. Using a sterile 5 ml pipette, transfer 3.5 ml of molten top agar to the 'rep 1 ' test tube containing host bacteria and then immediately draw the solution back into the same pipette. Important : Try to avoid making or withdrawing bubbles, as they can interfere with interpretation of results.    5. Dispense the top agar-bacteria mixture onto the labeled agar plate.       1. The top agar should not sit in the pipette for more than a few seconds because the agar will begin to solidify.       2. Gently, but quickly, tilt the plate in multiple directions until the top agar mixture evenly coats agar plate    6. Allow the plate to sit *undisturbed* for 20 minutes or until the top agar solidifies completely.    7. Repeat Steps C4 - C6 for the 'rep 2 ' test tube containing host bacteria prepared in step B5.    8. Important: Bacterial lawns should also be prepared for both replicates of the pExTra01 control, generating two class control plates without aTc. 4. Prepare bacterial lawns on plates *with* inducer (aTc).    1. For each gene being tested, obtain two 7H10 agar plates supplemented with Kanamycin (20 μg/ml) and aTc (100 ng/ml).       1. Label the bottom of the plates with the following information (see Figure 3.3a):          1. your initials, date, and incubation temperature,          2. aTc (100 ng/ml)          3. the phage gene being tested,          4. 'rep 1 ' on one plate and 'rep 2 ' on the other,          5. information about the phage lysate(s) that will be used to infect cells of your bacterial lawn. Note : You and your instructor should decide on the phage lysate(s) that will be used.          6. Divide the plate into rows for each phage lysate to be tested, with a maximum of 4 rows per plate.          7. Divide each row into sections for each lysate dilution to be spotted, with a maximum of 6 sections per row.    2. Obtain the 'rep 1 ' and 'rep 2 ' test tubes with host bacteria from step B5.    3. Obtain 1X 7H9 top agar supplemented with Kanamycin (20 μg/ml) and aTc (100 ng/ml).    4. Using a sterile 5 ml pipette, transfer 3.5 ml of molten top agar to the 'rep 1 ' test tube containing host bacteria and then immediately draw the solution back into the same pipette. Important : Try to avoid making or withdrawing bubbles, as they can interfere with interpretation of results.    5. Dispense the top agar-bacteria mixture onto the labeled agar plate.       1. The top agar should not sit in the pipette for more than a few seconds because the agar will begin to solidify.       2. Gently, but quickly, tilt the plate in multiple directions until the top agar mixture evenly coats agar plate    6. Allow the plate and to sit *undisturbed* for 20 minutes or until the top agar solidifies completely.    7. Repeat Steps D4 - D6 for the 'rep 2 ' test tube containing host bacteria prepared in step B5.    8. Important: Bacterial lawns should also be prepared for both replicates of the pExTra01 control, generating two class control plates with aTc.  |  | | --- | |  | | |  |  | | --- | --- | | Alt: | Figure showing plates and labels for a Defense Assay. | | | |  |  | | --- | --- | | Caption: | Figure 3.3a. Plates and labels for a Defense Assay. In the example provided, X, Y, and Z refer to 3 different phages that will be tested for their ability to infect M. smegmatis plated without inducer or expressing a particular phage gene (Gene #) and mcherry or mcherry by itself in the presence of aTc inducer. The assay is performed in replicate, labeled as 'rep 1 ' and 'rep 2 '. | |  1. Spot a dilution series of each phage lysate to be tested on the solidified bacterial lawns prepared in Steps C and D .    1. Your instructor will provide appropriately diluted phage lysate(s) or will provide instructions for you to prepare your own lysate dilutions.    2. One at a time, aseptically transfer 3 μl of all dilutions onto the proper location on each of the four bacterial lawns.    3. Spot 3 μl of sterile 7H9 liquid medium (neat) onto a free section of each lawn. This is your negative control.    4. Allow the liquid from the spots to absorb into the agar for 30 minutes or longer.    5. Important: The same diluted samples should be spotted on the pExTra01 class control lawns.    6. Incubate plates (right-side up, not inverted) at 37 ºC for 24– 48 hours. 2. Observe for defense.    1. Plates for each gene of interest and class control plates should be observed after 24 - 48 hours of incubation. For each plate,       1. The bacterial lawn should be visible and confluent.       2. Phage clearing or plaque formation within spots should be visible.       3. Make note of the color of the lawn. On aTc, lawns may or may not appear pink in color.       4. Note : If expression of a gene is toxic to the host, lawn growth on aTc may be sparse or no growth may be visible even after 48 hours at 37 ºC.    2. Photograph your plates, following your instructor’s directions. 3. Interpret your defense assay results    1. First, calculate the titer of each phage lysate tested based on the number of plaques that formed on the 'rep 1 ' and 'rep 2 ' bacterial lawns, in both the presence and absence of aTc inducer. Calculate the titers on 'rep 1 ' and 'rep 2 ' separately.    2. Next, calculate the titer of the same phage lysate based on the number of plaques that formed on the control bacterial lawns in the presence and absence of inducer (aTc). Note : The control bacterial lawn contains host bacteria transformed with the pExTra01 plasmid encoding the *mcherry* reporter gene only.    3. For each gene, compare the titers of each phage lysate in the presence or absence of aTc.       1. For the control, the titers should be similar on lawns with or without aTc.       2. For a gene of interest, the titers may or may not be similar on lawns with or without aTc.    4. Then, determine the efficiency of plating (e.o.p.), using the following formula for each phage and replicate. Titers should be comparable on both control plates, thus, titers from just one representative replicate can be used for this calculation. e.o.p. = titer of phage on bacterial lawn expressing your gene of interest with aTc/ titer of phage on the control bacterial lawn with aTc       1. If the e.o.p. is 1, the phage is equally able to infect the bacterial host regardless of whether or not the phage gene of interest is expressed. An e.o.p. of 1 therefore indicates that the phage gene of interests does not provide the bacterial host with protection to the phage tested.       2. If the e.o.p. is smaller than 0.1, the phage has a clear reduced ablity to infect the bacterial host when the phage gene of interest is expressed. An e.o.p. below 1 therefore indicates that the phage gene of interest provides the bacterial host some protection to the phage tested. The smaller the e.o.p., the more effective the protection.       3. If the e.o.p. is between 0.1 - 1, any protection provided by the phage gene of interest is minimal, and the experiment should be repeated to confirm the result.    5. In some cases, leaky gene expression from the repressed pExTra promoter may be sufficient to confer protection to the host. If expression of a gene of interest is toxic, inhibiting growth of the lawn on aTc, the e.o.p. can be calculated by comparing the titers on plates without aTc inducer, using the following formula for each phage and replicate: e.o.p. = titer of phage on bacterial lawn encoding your gene of interest without aTc / titer of phage on control bacterial lawn without aTc   **Helpful tips:**  For a review on spot titers, see Protocol 6.4 in the Phage Discovery Guide. |

# **Chapter4-TOC**

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# **Chapter4:An Overview**

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| **content** | Gene products rarely function in isolation, and the vast majority of biological processes depend upon physical contact between protein molecules. Cellular proteins can work together in pairs or may act as part of larger complexes involving many different proteins. Uncovering the protein partners a given protein is able to bind to, or ‘interact’ with, can provide valuable insight into the biological function of that protein, linking that protein to known cellular processes and proteins with well-defined functions. In this chapter, you will further explore the functions of a subset of phage genes, investigating the ability of their products to interact with proteins found in the M. smegmatis proteome. Since many phage proteins are expected to interact with host proteins as part of the phage infection cycle, the identification of an interaction between a phage protein and a host protein may provide further insight into the role of that gene in the phage life cycle and generate new hypotheses for future study.  There are several experimental approaches available to study the protein-protein interactions (PPIs), either in vivo (inside the cell) or in vitro (outside the cell, often using purified proteins). Each approach has its own benefits and pitfalls, but all of these techniques require an observable readout that allows one to detect that an interaction has occurred. A widely used experimental system to study PPIs in vivo is the 2-hybrid assay, in which a physical interaction between two specific proteins produced in a cell activates a specific reporter gene. This reporter gene confers a specific characteristic, or phenotype, to the cell and thus allows the PPI to be monitored. Many different 2-hybrid systems are available to study PPIs, most of which use fast-growing microorganisms such as yeast or E. coli and a variety of reporter gene readouts. The version of the 2-hybrid assay that will be utilized as part of SEA-GENES research is a bacterial 2-hybrid (B2H) assay, which was developed for use in E. coli cells. There are several key features of this B2H system, which are described below. Reporter Genes The B2H system employs a specially engineered strain of E. coli, called B2H SELECT, that encodes 2 reporter genes: bla, which encodes for beta-lactamase, an enzyme that breaks down beta-lactam antibiotics such as penicillin or carbenicillin, and lacZ, which encodes for beta-galactosidase, an enzyme that breaks down lactose. When these reporter genes are expressed in the bacterial cell, beta-lactamase enables that bacterial cell to grow on media supplemented with the antibiotic carbenicllin, while beta-galactosidase enables that bacterial cell to degrade a chemical analog of lactose, called X-gal, which then turns the bacterial cell blue. Thus, expression of the reporter genes in bacterial cells is easily detected because those cells are able to form colonies, which turn blue, on medium supplemented with carbenicllin and X-gal. On the other hand, cells in which the reporter genes are not expressed should not be able to form colonies due to their sensitivity to carbenicllin. Test Promoter for the Regulation of Reporter Genes In order for the B2H assay to act as a reliable indicator of protein-binding in the cell, expression of the reporter genes must be regulated such that it is only activated as a result of a PPI occurring in the cell. This is achieved through the use of a specially engineered promoter, known as a 'test' promoter, to control the transcription of both reporter genes. The test promoter is considered a ‘weak’ promoter because by itself, RNA polymerase is not able to stably bind and initiate transcription; thus, only negligible reporter gene expression can occur. However, this test promoter was engineered to contain an additional regulatory feature that can stabilize RNA polymerase at the test promoter, thereby allowing for transcription of the reporter genes to occur in a PPI-dependent manner. Regulating the Test Promoter The regulatory feature of the B2H test promoter is a binding site, or operator, for a specific DNA-binding protein, the CI (pronounced ‘see one’) immunity repressor protein of phage lambda. The CI operator is situated upstream of the test promoter, such that when CI is bound to its operator site, it is in close proximity to any RNA polymerase molecule that contacts the test promoter. If operator-bound CI and RNA polymerase are able to physically interact, RNA polymerase binding at the test promoter is stabilized, thereby activating transcription of the reporter genes. Importantly, though, at this test promoter, CI and RNA polymerase are not normally able to interact with one another. However, if CI and RNA polymerase are each fused to proteins that are able to interact with one another, then an interaction between the CI-fusion protein and RNA polymerase-fusion protein can bridge the gap between these proteins, stabilizing RNA polymerase at the test promoter, and turning on expression of bla and lacZ. Thus, to test if two proteins can interact with one another in the B2H assay, one protein is fused to CI and the other to RNA polymerase. If cells expressing these fusion proteins grow and turn blue in the presence of carbenicllin and X-gal, then the two proteins likely interact; if no growth is observed, it is likely that those two proteins do not interact with one another. Expressing Fusion Proteins To test for PPIs, the proteins of interests must first be expressed as fusions to CI or to a subunit of RNA polymerase. Each fusion protein is expressed as a single polypeptide encoded by two different genes that have been joined together. For the purposes of generating these fusion proteins, two plasmids were specifically engineered:   * p2Hα plasmid, cloned using the protocols outlined in Chapter 2, such that it can be expressed as a fusion to a portion of the α subunit of RNA polymerase, and * pCI plasmid, which encodes random fragments of M. smegmatis genes that will be produced as a fusion to the CI protein.   Both p2Hα and pCI plasmids contain inducible promoters to regulate the production of the fusion proteins they encode. Note that both plasmids contain the same negatively regulated inducible promoter, 'Lac\*'. Like the pExTra 'Tet' promoter, which is maintained in the 'off' state when bound by the Tetracycline-repressor protein, TetR, the Lac\* promoter, is maintained in the 'off' state when bound by the transcriptional regulator LacI. The Lac\* promoter is turned “on” by the addition of the inducer lactose or a lactose mimic known as Isopropyl b-D-1-thiogalactopyranoside, or IPTG. When either inducer binds LacI, it causes a conformational change that releases the regulator from the DNA and allows RNA polymerase to bind and activate transcription of the downstream fusion genes. The reporter strain B2H SELECT encodes the LacI regulator, which is constitutively produced, ensuring that fusion proteins under the control of the Lac\* promoter are not expressed until the IPTG inducer is introduced into the experimental system. Selecting for PPIs To perform the B2H assay, a p2Ha plasmid is used to transform B2H SELECT E. coli cells containing a pCI plasmid. Once transformed, IPTG is added to the cells, inducing expression of both fusion proteins encoded by these plasmids: a phage gene of interest is expressed from the p2Hα plasmid as a fusion to a portion of the α subunit of RNA polymerase (α-phage gene) and a M. smegmatis gene fragment fused to CI (CI-host gene fragment) is expressed from the pCI plasmid. If the phage protein of interest is able to interact with a particular M. smegmatis protein fragment, that interaction will activate expression of the reporter genes in that particular cell. In the absence of an interaction, no reporter gene expression occurs.  The M. smegmatis genome encodes ~ 9,000 different proteins. Assessing whether a phage gene product is able to interact with each of these host proteins, one-by-one, would be very labor and time-intensive. Instead, to determine which of these proteins, if any, interact with a phage protein of interest, the B2H assay has been developed to rapidly test for pairwise interactions between the phage protein of interest and a collection of ~ 1 million different M. smegmatis protein fragments cloned into a pCI gene fusion library. The B2H SELECT strain containing this pCI library is transformed with a p2Hα plasmid; in the presence of IPTG, each E. coli cell produces the same α-phage gene fusion but a different CI-host gene fragment fusion. The bla reporter gene used in the B2H assay enables us to perform a genetic selection; a million transformed cells can be plated on selective medium containing IPTG and carbenicillin, and only the subset of those cells in which bla is being expressed from the test promoter due to a phage-host PPI should be able to survive. Thus, we can quickly ‘select’ for candidate interaction partners from a large pool of host protein fragments. The second reporter gene, lacZ, acts as an additional indicator that expression is occurring from the test promoter in surviving colonies as these should turn blue on selection plates containing X-gal. Validating Hits Ideally, after performing a B2H selection assay we will isolate carbenicillin-resistant colonies in which a biologically relevant PPI triggers expression of the bla and lacZ reporter genes. However, 2-hybrid assays can produce both false negative and false positive results. Some proteins may not be properly folded or stable when produced in the non-native context of the E. coli cytoplasm, or fusion to another large protein domain may interfere with the ability of a protein to bind its normal interaction partner. Thus, even if a phage protein does have a biologically important binding partner in the M. smegmatis proteome, we may not be able to identify it in our system. Furthermore, some colonies isolated in the B2H selection may be resistant to carbenicillin for reasons other than the presence of a true PPI. Three possible types of false positive results are described below.   * Some B2H Select E. coli cells may have acquired a random mutation on the chromosome that confers resistance to carbenicillin. In most cases, this class of mutants should be easily identifiable since they will not be expressing the reporter genes and therefore form colonies that are white rather than blue. * Some B2H Select E. coli cells may have acquired a mutation in the test promoter, such that expression of the reporter genes is constitutive, and happens in the absence of a PPI. * Some B2H Select E. coli cells may express ‘sticky’ CI protein fusions that are able to interact non-specifically with the phage protein or even RNA polymerase itself.   After isolating a carbenicillin-resistant colony in our B2H selection, we must perform downstream analyses to try to distinguish between a true PPI candidate and potential false positives. We can use a diagnostic PCR to detect ‘sticky’ CI fusion fragments that appear repeatedly in selections with different phage proteins and eliminate them from our pool of candidate interaction partners. To further validate a PPI, the p2Hα and pCI plasmids can be extracted from a colony that survives the B2H selection. Doing so isolates the plasmids expressing the two candidate interacting proteins away from chromosomal or test promoter mutations that may be present in that particular carbenicillin-resistant cell. When a new batch of B2H SELECT cells is transformed with the isolated plasmids, a genuine PPI should result in all transformants being able to survive a B2H selection.  Once we have confirmed that growth on carbenicillin is due to an interaction between a phage gene product fusion and host protein fusion, we can sequence the pCI plasmid to identify the host interaction partner. Gaining a full understanding of the biological significance of this PPI and how it relates to the function of the phage gene product will take additional work, beyond the scope of one semester. Importantly, though, these interaction analyses will undoubtedly reveal important clues in our quest to understand gene function and will create new lines of research for phage biologists to explore! |

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# **Protocol 4.1: 2-Hybrid Selection Assay**

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| **content** | Objective: To perform a bacterial 2-hybrid selection in order to identify potential protein-protein interactions between a phage gene product of interest and host proteins. Rationale: In this protocol, a bacterial two-hybrid system is used to rapidly assay the pairwise interactions between a phage gene product of interest and a plasmid gene library encoding for roughly a million different fragments of the ~7,000 proteins in the *M. smegmatis* proteome. The 2-hybrid analysis is performed in a special *E. coli* reporter strain, B2H SELECT, containing a test promoter that controls the expression of two reporter genes, *bla*, which encodes for the carbenicillin-resistance factor beta-lactamase, and *lacZ* , which encodes for the enzyme beta-galactosidase; this strain is transformed with two plasmids encoding the two 'hybrid' fusion proteins. The phage gene is expressed from the p2Hα plasmid as a fusion to a subunit of *E. coli* RNA polymerase while each host protein fragment is expressed from a different pCI plasmid as a fusion to the CI DNA-binding protein. This library of pCI plasmids was previously transformed into the B2H SELECT reporter strain to generate the B2H SELECT/pCI-SMEG Library electrocompetent cells used in this protocol. In each aliquot of these cells there are about a billion different cells, each transformed with one of the million different pCI plasmids. You will transform these cells with the p2Hα plasmid of interest by electroporation and plate them on special selective medium containing antibiotics to maintain selection of the plasmids, IPTG to induce production of the fusion proteins, X-gal to monitor *lacZ* expression, and a high concentration of carbenicillin; out of the fraction of competent cells that took up the p2Hα plasmid, those that express two interacting fusion proteins will be able to activate expression of *bla* and survive in the presence of carbenicillin. These colonies should also be blue due to the activation of *lacZ* expression and the presence of the colored substrate X-gal in the selective medium; note, though, that the amount of blueness observed can vary from colony to colony. This 'selection' of colonies in which a potential protein-protein interaction is occurring works best when about a million transformed cells are grown on a plate, thus ensuring that many or most of the possible ~1 million different pairwise interactions have been tested. Supplies:  * B2H SELECT/pCI-SMEG Library electrocompetent cells * Plasmid DNA for the p2Hα plasmid expressing the phage gene fusion of interest * Ice * Microcentrifuge tubes * Recovery broth (LB broth without any antibiotics) * IPTG solution (10 mM) * B2H Selection plates * B2H Growth plates * Rattler beads or spreader, with 100 % EtOH * Shaking incubator set to 37 **°**C * Electroporation cuvettes (gap: 0.1 cm) * Electroporator (voltage: 1.8 kV) * Paper towel or KimWipe * Plate incubator set to 30 ºC  Procedure : **Important :**Competent cells should be kept on ice throughout Steps A and B.   1. Set up the transformation reaction. **Note :** The number of transformation reactions and the volume of competent cells used in the transformation reaction will depend on the competency of your bacterial cells, and should be discussed with your instructor.    1. Place the following three items on ice:       1. an aliquot of frozen B2H SELECT/pCI-SMEG Library electrocompetent cells       2. recombinant p2Hα plasmid DNA encoding your gene of interest on ice to chill       3. an electroporation cuvette    2. The transformation reaction is optimized for 100 ng of plasmid DNA. Based on the concentration of your p2Hα plasmid DNA, use the formula below to calculate the volume of sample needed to obtain 100 ng of plasmid DNA. Formula: volume needed, µl = (amount needed, ng) / (concentration of stock, ng/µl) *Example: For plasmid backbone DNA at 50 ng/µl, volume needed, µl = (100 ng) / (50 ng/µl) = 2 µl*    3. Add the volume of p2Hα plasmid DNA calculated in Step A3 to a pre-chilled microcentrifuge tube. **Important :** The maximum volume of p2Hα plasmid DNA that should be added to the competent cells is 3 µl.    4. Once the competent cells have thawed, transfer 50 µl of competent cells to the microcentrifuge tube containing the plasmid DNA, and keep on ice.    5. Pipette the mixture up and down gently, one time. **Important :** Act quickly to ensure that the mixture remains cold. Repeated pipetting can introduce bubbles, which may interfere with electroporation.    6. Transfer the mixture into the pre-chilled electroporation cuvette.    7. **Important :** Pipette the mixture into the thin gap between two metal electrodes at the bottom of the cuvette. Gently tap the cuvette on the bench to evenly settle the mixture at the bottom of the cuvette. **Important :** Be careful not to introduce any air bubbles in the mixture. 2. Electroporate the cells to allow for DNA uptake.    1. Adjust the settings on the electroporator according to your instructor's directions.    2. Remove the cuvette from the ice, and using a paper towel, wipe off any moisture on the outside of the cuvette. Proceed quickly and carefully through the remaining steps.    3. Place the cuvette into the electroporator holder.    4. Pulse the cells, taking note of the pulse length value. It should be between 4 - 6 msec.       1. If a flash of light is seen or a loud popping sound is heard during the pulse, an electric arc has occured due to contaminating salts in the mixture. Repeat the procedure from the beginning, this time using DNA that has been diluted in water to reduce the salt concentration in the final mixture.    5. Proceed immediately to Step C. 3. Allow cells to recover.    1. Using aseptic technique, immediately add 1 ml of the LB broth to the electroporation cuvette. Carefully pipette the liquid up and down 3-4 times to mix, then transfer the entire volume from the electroporation cuvette into a clean and labeled microcentrifuge tube.    2. Incubate the microcentrifuge tube at 37 **°** C, with shaking for 1 hour. 4. Prepare for plating.    1. After 1 hour of recovery at 37 **°** C, add 2 µl of inducer (IPTG) to the cells in the microcentrifuge tube.    2. Incubate the microcentrifuge tube at 37 **°** C, with shaking, for an additional 1 hour. During the 1 hour incubation period, perform Step D3.    3. Obtain three B2H Selection plates per transformation reaction, and label the plates with your name, date, incubation temperature, and experimental information according to your instructor's direction. Warm the selection plate(s) by placing them in a 30 **°** C incubator. 5. Spread the transformation reaction onto selection plate(s).    1. For each transformation reaction, transfer 25 μl of the recovery mix to one of the B2H Selection plates, 50 μl to the second plate, and 100 μl to the final plate. **Important :** Save the remaining recovery mix for Step F.    2. Using Rattler beads or a glass spreader that has been decontaminated using EtOH, spread the mixture to even distribute the cells on the agar surface of each selection plate.    3. With the lids closed, allow the plates to sit on the bench until all the liquid has been fully absorbed into the agar. 6. Setup and spot a dilution series of the recovery mix on plates without Carbenicillin.    1. Obtain 7 microcentrifuge tubes, and arrange these microcentrifuge tubes in a rack, and label them 10-1, 10-2, 10-3, 10-4, 10-5.10-6, and 10-7.    2. Add 180 μl of LB liquid medium to each of the tubes    3. Transfer 20 μl of the remaining recovery mix from Step E1 to the “10-1” tube.    4. Cap the tube and vortex briefly to mix.       1. **Note :** This solution in this “10-1” tube contains 1/10th the number of cells as your undiluted sample. It is also referred to as a 1:10 dilution.    5. Using a new pipette tip, transfer 20 μl of the “10 -1” sample to the “10-2” tube and vortex well.       1. This solution contains 1/100th as many cells as your undiluted sample. It can also be referred to as your 1:100 dilution.    6. Continue each successive dilution until you get to your last tube, using a fresh tip for each dilution.    7. For each transformation reaction, obtain four B2H Growth plates, and label the plate with your name, date, and incubation temperature. Divide the plate into 8 sections, and label the sections according to each dilution, including a section for the undiluted recovery mix.    8. One at a time, aseptically transfer 10 μl of each dilution onto the appropriate position on a labeled B2H Growth plate, using a new pipette tip for each spot.    9. On the remaining three B2H Growth plates, plate 100 μl of the 10-3, 10-4, and 10-5 dilutions, using a new pipette tip for each plate.    10. Using Rattler beads or a glass spreader that has been decontaminated using EtOH, spread the mixture to even distribute the cells on the agar surface of each selection plate.    11. With the lids closed, allow the four plates to sit on the bench for approximately 30 min for the liquid to be absorbed into the agar. 7. Place all the plates from Step E and Step F in a 30 **°** C incubator for 48 hours. 8. Evaluate outcomes of the 2-Hybrid selection.    1. After 48 hours, the B2H Selection plates should be photographed and evaluated for the number, color, and size of colonies formed. This information should be recorded in your lab notebook. **Note :** It is strongly recommended that plates be refrigerated for 2 - 24 hours prior to evaluating colony color. This low incubation temperature enhances the blue colony color on B2H Selection plates, making it easier to distinguish pale colonies from blue colonies.    2. Calculate the fraction of transformed cells that were able to grow on carbenicillin.       1. Evaluate the B2H Growth plates.          1. Of the plates from Step F9 on which 100 μl was plated, identify one that has a countable number of colonies (i.e. between 20 and 200 colonies).          2. Calculate the number of transformed cells in cfu/ml using the formula: (cfu/ml) = (# cfu/ volume used in μl) x (103 μl/ml) x dilution factor\* *\*For a 10-4 dilution, the dilution factor is 104* .  **Example**: If 60 colonies are observed on a plate with 100 μl of the 10-5 dilution plated, the calculation would look like this: cfu/ml = (60 cfu/100 μl) x (103 μl/ ml) x (105) = (6.0 x 103 x 105) cfu/ml = 6.0 x 107 cfu/ml          3. The cfu/ml represents the total number of transformed cells in your B2H transformation. Calculate the number of transformants plated on each selection plate by multiplying the cfu/ml by the fraction of the total 1 ml volume that was plated.  **Example**: 50 μl of the recovery mix represents 1/20th of the total 1 ml volume. Thus, on the B2H Selection plate on which 50 μl of recovery mix was plated, 1/20th of the total cfu were plated. cfu plated = (6.0 x 107 cfu/ml) x (1/20) = 3.0 x 106 cfu/ml       2. Evaluate the spot dilution plate.          1. Do the numbers of colonies in each spot make sense? For example, is there a 10-fold reduction in number of colonies as you move through the spots? If so, choose the dilution(s) that contain a countable number of colonies.          2. Do the colony numbers agree with the results from the plates on which 100 μl was plated? For example, is there a 10-fold increase in the number of colonies where 10-fold more volume was plated?          3. If none of the plates on which 100 μl was plated have a countable number of colonies, the cfu/ml can be approximated using this plate.  **Note :** B2H Selection plates on which fewer than 500,000 transformants or greater than 5 million transformants were plated should not be considered for candidate selection in Protocol 4.2.       3. The surviving fraction of transformed cells able to grow on Carbenicillin can be calculated by dividing the number of colonies on each B2H Selection plate by the number of cfu calculated in step H3. Typically this number is around 10-5 .  **Example:** If 25 colonies are observed on a plate on which 1.5 x 106 cfu were plated, the surviving fraction would be: fraction of surviving cells = 25 colonies/1.5 x 106 cfu = 1.7x 10-5 cfu 9. For B2H Selection plates containing colonies and on which 5 x 105 - 5 x 106 cfu were plated, proceed to Protocol 4.2 Selecting Candidate 2-Hybrid Hits.   **Helpful tips:** |

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# **Protocol 4.2: Selecting Candidate 2-Hybrid Hits**

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| --- | --- |
| **templateId** | **chapterContent** |
| **name** | **Protocol 4.2: Selecting Candidate 2-Hybrid Hits** |
| **imgtxt** | **Interaction Assay** |
| **img** | **interaction-assay.svg** |
| **content** | Objective: To isolate and process candidate hits from a bacterial 2-hybrid selection assay for downstream verification. Rationale: After performing a bacterial 2-hybrid selection assay, surviving colonies should be isolated and cultured such that the plasmids they harbor can be purified for further analysis. In addition to the putative protein-protein interaction colonies, these selection plates may also contain colonies that are carbenicillin-resistant for less interesting reasons. For example, some cells may have randomly acquired a chromosomal mutation that confers resistance to carbenicillin; colonies that are completely white or very tiny may fall into this class. Furthermore, some surviving cells may harbor a mutation in the test promoter that allows for constitutive expression of the bla and lacZ reporter genes. Finally, some protein fragments may be 'sticky' and able to interact with many different phage proteins or even E. coli RNA polymerase itself. These last two classes of carbenicillin-surviving colonies are harder to distinguish as they will lead to healthy, blue colonies just like a true protein-protein interaction. In this protocol, colonies will be screened by colony PCR for known 'sticky' CI fusion proteins such that they can be eliminated from the pool of hits. In Protocol 4.3, the remaining candidates will be analyzed further to determine whether the ability to grow on carbenicillin is due to the expression of interacting partners or some type of mutation in the reporter strain. Supplies:  * B2H Selection plates on which 500,000 to 5 million transformed cells were plated and incubated at 30 ºC for 48 h * LB broth supplemented with 50 μg/ml Kanamycin, 25 μg/ml Chloramphenicol, 50 μg/ml Spectinomycin * Sterile test tubes with lids * Sterile inoculation or wooden sticks * Temperature-controlled shaking incubator or rotor * 5 ml serological pipettes * Pipette aid * Microcentrifuge tubes * Microcentrifuge * Plasmid mini-prep Kit (Zymo) * Sterile, nuclease-free water * PCR tubes * 2X OneTaq Polymerase Master Mix * DNA Primers (10 μM) * Ice * Sterile, nuclease-free water * Thermocycler  Procedure :  1. Inoculate liquid cultures.    1. After recording observations on the number, color, and size of colonies on the B2H Selection plate, follow Protocol 2.11, to inoculate a culture for each colony on the plate.       1. Colonies should be grown in LB broth supplemented with 50 μg/ml Kanamycin, 25 μg/ml Chloramphenicol, and 50 μg/ml Spectinomycin.    2. Cultures should be grown at 30 ºC, with shaking, for 16-24 h. 2. Screen candidates for known 'sticky' fragments by PCR    1. Prepare template DNA.       1. Obtain and label a new microcentrifuge tube for each sample to be tested.       2. Aseptically transfer 100 μl of each culture to the labeled tubes. These are your DNA templates.    2. Perform the PCR.       1. Consult your instructor to determine which primers to use for detection of 'sticky fragments' and the appropriate annealing temperature for each primer pair.       2. Follow the instructions in Protocol 2.2: Polymerase Chain Reaction, setting up a reaction for each culture to be tested.          1. 2X Taq Polymerase Master Mix should be used.    3. Determine whether amplification was successful.       1. Follow instructions in Protocol 2.3: Gel Electrophoresis to visualize and determine the size of any amplified products for each colony PCR reaction.       2. The presence of a band indicates the pCI plasmid encodes the 'sticky' fragment. Consult with the instructor to determine if the amplified product is the expected size.       3. Only proceed to Step C with those cultures that do not yield an amplified product.    4. Note: Cultures can be left on the bench for several hours while performing the PCR analysis before proceeding to Step C. If PCR analysis and plasmid prepping will be done on separate days, cultures can be pelleted by centrifugation for 5 min at *10,000 x g* and pellets stored at -20 ºC. 3. Purify p2Hα and pCI-SMEG plasmids.    1. Follow Protocol 2.12 to prepare plasmid from each culture.    2. This is a mixed plasmid prep, containing both the p2Hα and pCI-SMEG plasmids.   **Helpful tips:** |

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# **Protocol 4.3: Evaluating Candidate 2-Hybrid Hits**

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| **templateId** | **chapterContent** |
| **name** | **Protocol 4.3: Evaluating Candidate 2-Hybrid Hits** |
| **imgtxt** | **Interaction Assay** |
| **img** | **interaction-assay.svg** |
| **content** | Objective: To verify that growth on carbenicillin is due to the PPI of fusion proteins encoded by p2Hα and pCI-SMEG plasmids in the B2H SELECT strain. Rationale: In order to distinguish between a true PPI and a false positive colony that has acquired the ability to grow on carbenicillin due to some mutation in the original cell, the plasmids isolated from carbenicillin-resistant candidates in Protocol 4.2 must be transformed again into a new batch of B2H SELECT cells. These transformed cells will be incubated in the presence of IPTG in order to induce expression of the 2-hybrid fusion genes and plated again on both B2H Selection plates containing carbenicillin and B2H Growth plates without carbenicillin. If the original colony was isolated on carbenicillin due to the interaction of the two plasmid-encoded fusion proteins, then these new transformed cells should be able to grow on carbenicillin again. However, if the original colony was isolated on carbenicillin due to a resistance mutation, then these new transformed cells should not be able to grow on carbenicillin. Those candidates that are confirmed to grow on carbenicillin due to a plasmid-encoded PPI can be analyzed further to sequence and identify the M. smegmatis protein fragment that is able to interact with your phage gene product of interest. Supplies:  * B2H SELECT chemical competent cells * Plasmid DNA from isolated from each B2H Selection candidate. Note : Each sample will contain both p2Hα and pCI-SMEG plasmids * LB agar plates, supplemented with 50 μg/ml Kanamycin, 25 μg/ml Chloramphenicol, and 50 μg/ml Spectinomycin * LB broth, supplemented with 50 μg/ml Kanamycin, 25 μg/ml Chloramphenicol, 50 μg/ml Spectinomycin, and 20 μM IPTG * Recovery broth (LB broth with no antibiotics) * B2H Selection plates * B2H Growth plates * Ice * Microcentrifuge tubes * Microcentrifuge * Heat block set to 42 **°**C. * Shaking incubator set to 37 **°**C * Plate incubator set to 30 ºC * Rattler beads or spreader, with 100 % EtOH * Sterile inoculation or wooden sticks * A printed plate template, available for download [here](https://dzf8vqv24eqhg.cloudfront.net/userfiles/11001/22461/ckfinder/files/B2H%20re-test%20Plate_Template.pdf) .  Procedure :  1. Transform B2H SELECT cells with p2Hα and pCI-SMEG plasmids purified from B2H selection candidates.    1. Follow Protocol 2.9 to perform a chemical transformation of B2H SELECT chemical competent cells for each candidate plasmid prep generated in Protocol 4.2.       1. Transform 25 μl of competent cells with 1 μl of the mixed plasmid prep. This should be around 50 ng total of DNA.       2. After the recovery step, spin the 1 ml of recovery mix in a microcentrifuge at 2,000 x g for 5 min.       3. Remove 900 μl of the supernatant fraction, being careful to not disturb the pellet. Discard the supernatant in an appropriate liquid waste container.       4. Resuspend the pellet in the remaining liquid, approximately 100 μl, by pipetting up and down to mix.       5. Plate 20 μl of this resuspension on an LB agar plate supplemented with 50 μg/ml Kanamycin, 25 μg/ml Chloramphenicol, and 50 μg/ml Spectinomycin.       6. Incubate the plate at 30 ºC for 24 h. 2. Pick and resuspend two colonies for re-evaluation of plasmid-dependent growth on B2H Selection plates.    1. Observe the transformation plate(s) from step A.    2. On each transformation plate, identify 2, well-isolated colonies       1. On the bottom of the plate, circle and label the 2 colonies to be tested.       2. These 2 colonies represent replicates A and B for each candidate.    3. Obtain and label a clean microcentrifuge tube for each of the 2 colonies to be tested.       1. Fill each labeled microcentrifuge tube with 500 μl LB broth supplemented with 50 μg/ml Kanamycin, 25 μg/ml Chloramphenicol, 50 μg/ml Spectinomycin, and 20 μM IPTG.       2. Using a sterile wooden stick, transfer each selected colony into the corresponding microcentrifuge tube. Shake the stick so transfer as much of the colony on the stick into the liquid medium.       3. Cap the microcentrifuge tube, and vortex to disperse the colony. This is your undiluted cell suspension. 3. Induce expression of the α and CI fusion genes in each cell suspension.    1. Incubate the tubes from step B at 37 ºC with shaking for 1 h. 4. Set up 10-fold serial dilutions (Figure 4.3a)    1. For each of the cell suspensions, obtain 5 additional microcentrifuge tubes, arrange these microcentrifuge tubes in a rack, and label them 10-1, 10-2, 10-3, 10-4, and 10-5.    2. Add 900 μl of LB broth supplemented with 50 μg/ml Kanamycin, 25 μg/ml Chloramphenicol, 50 μg/ml Spectinomycin, and 20 μM IPTG to each of the tubes    3. Perform the Steps D 4 - 7 for each of your undiluted cell suspensions.    4. Transfer 100 μl of your undiluted cell suspension to the “10-1” tube.    5. Cap the tube and vortex briefly to mix. Note : This solution in this “10-1” tube contains 1/10th the number of cells as your undiluted sample. It is also referred to as a 1:10 dilution.    6. Using a new pipette tip, transfer 100 μl of the “10 -1” sample to the “10-2” tube and vortex well. Note : This solution contains 1/100th as many cells as your undiluted sample. It can also be referred to as your 1:100 dilution.    7. Continue each successive dilution until you get to your last tube, using a fresh tip for each dilution.  |  | | --- | |  | | |  |  | | --- | --- | | Alt: | An image showing A volume of the undiluted cell suspension (100 μl) is transferred into 900 μl of LB broth with the appropriate supplements in the 10-1 tube. After the tube is mixed, 100 μl of the 10-1 sample is transferred into the 10-2 tube, and the tube is mixed. This serial dilution continues until the desired dilutions are obtained. The last tube will contain a final volume of 1000 μl.Figure 3.2b. | | | |  |  | | --- | --- | | Caption: | Figure 4.3a. Serial dilutions. A volume of the undiluted cell suspension (100 μl) is transferred into 900 μl of LB broth with the appropriate supplements in the 10-1 tube. After the tube is mixed, 100 μl of the 10-1 sample is transferred into the 10-2 tube, and the tube is mixed. This serial dilution continues until the desired dilutions are obtained. The last tube will contain a final volume of 1000 μl.Figure 3.2b. The printable 'plate template' serves as a guide for spotting the samples on the agar plate, such that when plates are viewed from the bottom, the samples are distributed as above. | |   Figure 4.3a. Serial dilutions. A volume of the undiluted cell suspension (100 μl) is transferred into 900 μl of LB broth with the appropriate supplements in the 10-1 tube. After the tube is mixed, 100 μl of the 10-1 sample is transferred into the 10-2 tube, and the tube is mixed. This serial dilution continues until the desired dilutions are obtained. The last tube will contain a final volume of 1000 μl.Figure 3.2b. The printable 'plate template' serves as a guide for spotting the samples on the agar plate, such that when plates are viewed from the bottom, the samples are distrubuted as above.   1. Spot each dilution series on B2H Selection and B2H Growth plates.    1. Obtain B2H Selection and B2H Growth plates.       1. A total of 6 dilution series can be spotted on each plate (Figure 4.3b).          1. Dilution series for up to 3 transformed candidates can be tested on each plate.          2. For each candidate, the dilution series for the two replicate cell suspensions should be spotted next to each other on the same plate.          3. Each dilution will be spotted onto one B2H Selection plate and one B2H Growth plate. Note : The pattern of spotting is identical both plates.    2. Label the bottom edge of each agar plate with your name, the date, and whether or not carbenicillin is present.    3. Divide the plate into columns for each candidate to be tested, and label the candidate number above each column.    4. Place the agar plate on a printed copy of the plate template. Note : The printable plate template is a mirror image of Figure 4.3b. This is to ensure that, when viewed from the bottom of the plate, the negative control is placed in the leftmost column, as in Figure 4.3b.    5. One at a time, aseptically transfer 5 μl of each dilution onto the appropriate position on the agar plates, using a new pipette tip for each spot.    6. Allow the plates to sit on the bench for approximately 30 min for the liquid to be absorbed into the agar.    7. Once the liquid has been absorbed, invert the plates and incubate at 30 **°**C for a total of ~36-48 hrs.    8. Note : A positive and negative control should be spotted on B2H Selection and B2H Growth plates for each class. Consult your instructor for information on how these control plates should be prepared.  |  | | --- | |  | | |  |  | | --- | --- | | Alt | An image showing the printable plate template serves as a guide for spotting the samples on the agar plate, such that when plates are viewed from the bottom, the samples are distributed as above. | | | |  |  | | --- | --- | | Caption | Figure 3.2b. The printable 'plate template' serves as a guide for spotting the samples on the agar plate, such that when plates are viewed from the bottom, the samples are distrubuted as above. | |   Figure 3.2b. The printable 'plate template' serves as a guide for spotting the samples on the agar plate, such that when plates are viewed from the bottom, the samples are distributed as above.   1. Evaluate the ability of B2H SELECT cells transformed with candidate plasmids to grow on carbenicillin.    1. Note : It is strongly recommended that plates be refrigerated for 2 - 24 hours prior to evaluating colony color. This low incubation temperature enhances the blue colony color on B2H Selection plates, making it easier to distinguish pale colonies from blue colonies.    2. All colonies transformed with candidate plasmids should grow on B2H Growth plates, and may or may not grow on B2H Selection plates.       1. For each candidate, observe and record which, if any, dilutions on the B2H Selection plate have growth.          1. In those dilutions that have single colonies, observe the size and color of those colonies.          2. Does this colony morphology match the original candidate isolated in Protocol 4.1?    3. Photograph your plates, following your instructor’s directions.   **Helpful tips:** |

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# **Recipe Cards**

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| **name** | **Recipe Cards** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **child** | **false** |
| **appendix** | **true** |

# **Recipes for Solutions**

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| **child** | **true** |
| **appendix** | **false** |
| **templateId** | **chapterContent** |
| **name** | **AD Supplement** |
| **templateId** | **chapterContent** |
| **name** | **Calcium Chloride (1 M)** |
| **templateId** | **chapterContent** |
| **name** | **Calcium Nitrate (0.8 M )** |
| **templateId** | **chapterContent** |
| **name** | **Carbenicllin Stock Solution** |
| **templateId** | **chapterContent** |
| **name** | **Cycloheximide Stock Solution** |
| **templateId** | **chapterContent** |
| **name** | **Dextrose (40 %)** |
| **templateId** | **chapterContent** |
| **name** | **Enrichment Broth** |
| **templateId** | **chapterContent** |
| **name** | **EDTA** |
| **templateId** | **chapterContent** |
| **name** | **Glycerol (40 %)** |
| **templateId** | **chapterContent** |
| **name** | **Isopropanol (80 %)** |
| **templateId** | **chapterContent** |
| **name** | **Magnesium Chloride (1 M)** |
| **templateId** | **chapterContent** |
| **name** | **Magnesium Sulphate (1 M)** |
| **templateId** | **chapterContent** |
| **name** | **Nuclease Mix** |
| **templateId** | **chapterContent** |
| **name** | **Phage Buffer** |
| **templateId** | **chapterContent** |
| **name** | **Phage Precipitation Solution** |
| **templateId** | **chapterContent** |
| **name** | **SDS (10 %)** |
| **templateId** | **chapterContent** |
| **name** | **Tris Borate EDTA (TBE)** |
| **templateId** | **chapterContent** |
| **name** | **Tris, pH 7.5 (1 M)** |
| **templateId** | **chapterContent** |
| **name** | **Tween80 (20 %)** |
| **templateId** | **chapterContent** |
| **name** | **Uranyl Acetate Stain** |
| **templateId** | **chapterContent** |
| **name** | **Water** |
| **templateId** | **chapterContent** |
| **name** | **Zinc Chloride** |

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# **AD Supplement**

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| **templateId** | **chapterContent** |
| **name** | **AD Supplement** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **AD Supplement (0.5 L)** | | | | **For bacterial host** | *M. smegmatis* mc2 155 only | | | **Ingredients** | **Amount** | **Final Concentration** | | NaCl  Albumin (Fraction V)  ddH2O  Dextrose | 4.25 g  25 g  To 0.5 L  10 g | 145 mM  5.0 %    2.0 % | | **To Prepare** | 1. Add 400 mL of ddH2O to a 1-L Erlenmeyer flask. 2. Weigh out NaCl and albumin, and slowly, while stirring, add to the ddH2O in the Erlenmeyer flask. 3. Stir until dissolved. 4. Once dissolved, continue stirring and slowly add the dextrose. 5. Once the dextrose is dissolved, transfer the solution to a 1-L OR 0.5 L graduated cylinder, and bring the volume to 0.5 L with ddH2O. 6. Sterilize. | | | **To Sterilize** | Filter-sterilize; do not autoclave. | | | **To Store** | At 4 ˚C, indefinitely if not contaminated | | |  |  | | | **Usage** | In Recipe:   * 7H9 liquid medium complete | | |  |  | | | **Notes** | 1. Adding the dextrose last helps keep it from recrystallizing on the bottom of the flask. 2. Make sure the albumin is completely in solution before the filter-sterilization step. To dissolve the albumin, use a large stir bar spinning at a moderate to high speed. Very vigorous stirring will cause the albumin to denature and collect at foam/bubbles at the surface of the solution. 3. Standard medium for the growth of mycobacteria (Middlebrook) generally contains ADC (for albumin, dextrose and catalase) or OADC (ADC plus oleic acid). This guide uses the recipe for ADC but without catalase, hence the term “AD supplement”. 4. 0.5 L of AD supplement is require to prepare 5 L of 7H9 liquid media . 5. Approximately 2.5 L of 7H9 liquid media is used per semester for a class of 20 students. | | |

# **Calcium Chloride (1 M)**

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| **templateId** | **chapterContent** |
| **name** | **Calcium Chloride (1 M)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **1 M CaCl2 (100 mL)** | | | |  | | | | **For bacterial host** | General | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | CaCl2    ddH2O | 14.7 g of CaCl2.2H20 **or**  11.1 g of CaCl2 (anhydrous)  see instructions below | 1 M | |  | | | | **To Prepare** | Note: You will need stock solutions of CaCl2 at 1 M and 100 mM.    For 1 M CaCl2:   1. Add 90 mL of ddH2O to a large beaker. 2. Weigh out CaCl2 and slowly, while stirring, add to the ddH2O until the CaCl2 is dissolved. 3. Transfer the solution to a graduated cylinder, and add ddH2O to 100 mL. 4. Sterilize.     For 100 mM CaCl2:   1. Add 50 mL of 1 M CaCl2 to a large beaker. 2. Add ddH2O to 500 mL 3. Sterilize\*   \*if steps 1 & 2 are performed using sterile ddH2O and sterile 1M calcium chloride, as well as using aseptic technique, there is no need to sterilize the solution. | | |  | | | | **To Sterilize** | Filter-sterilize; do not autoclave. | | |  | | | | **To Store** | At room temperature, indefinitely. Do not refrigerate | | |  | | | | **Usage** | In Recipe:   * 7H9 liquid medium complete and top agar * PYCa liquid medium, agar, and top agar. * Phage buffer | | |  |  | | | **Notes** | 1. CaCl2 should not be autoclaved as it can precipitate out of solution at high temperatures. 2. Due to the high concentration of salts in Middlebrook 7H9 media, CaCl2 has a tendency to precipitate out of solution. As such, CaCl2 will ideally be added to 7H9 media immediately before the solution is used. 3. 100 mL of both 1 M CaCl2 and 100 mM CaCl2 should be sufficient for preparing all reagents requiring CaCl2 for ~ 20 students per semester. | | |

# **Calcium Nitrate (0.8 M )**

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| **templateId** | **chapterContent** |
| **name** | **Calcium Nitrate (0.8 M )** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **0.8 M Ca(NO3)2 (100 mL)** | | | | **For bacterial host** | *Streptomyces spp.* only | | | **Ingredients** | **Amount** | **Final Concentration** | | **Ca(NO3)2**  **ddH2O** | 18.89 g of Ca(NO3)2.4H20  To 100 mL | 0.8 M | | **To Prepare** | 1. Add 80 mL of ddH2O to a large beaker. 2. Weigh out Ca(NO3)2 and slowly, while stirring, add to the ddH2O until the Ca(NO3)2 is dissolved 3. Transfer the solution to a graduated cylinder, and add ddH2O to 100 mL. 4. Sterilize. | | | **To Sterilize** | Autoclave. | | | **To Store** | At room temperature, indefinitely. Do not refrigerate | | | **Usage** | In Recipe:   * Media Supplement for *Streptomyces* phage | | | **Notes** |  | | |

# **Carbenicllin Stock Solution**

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| **templateId** | **chapterContent** |
| **name** | **Carbenicllin Stock Solution** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Carbenicillin (CB) at 1000X** | | | |  | | | | **For bacterial host** | *M. smegmatis* mc2155 only | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | Carbenicillin (powder) | 50 mg/ml | stock=1000X | |  | | | | **To Prepare** | 1. Weigh out 100 to 600 mg of carbenicillin powder and place it in a 10-15 mL tube. 2. Divide the number of milligrams of carbenicillin by 50 to determine the volume (in milliliters) of ddH2O to add (e.g., for 477 mg of CB, add 9.5 mL ddH2O). 3. Stir or shake until dissolved. 4. Filter-sterilize (0.22µm pore size) into a fresh sterile tube. 5. Prepare 1 mL aliquots and freeze at -80 ˚C | | |  | | | | **To Sterilize** | Filter-sterilize (0.22µm pore size) into a fresh sterile tube. | | |  | | | | **To Store** | At -80 ˚C for 3 years.  At 4 ⁰C for ≤ 60 days. | | |  |  | | | **Usage** | In Recipe: 7H9 Liquid Medium | | |  |  | | | **Label Stock Solution** | CB, 1000X, date, initials | | |  |  | | | **To Prepare Final Solution** | 1. Remove a frozen aliquot, allow to thaw, and use as follows. Do not return thawed aliquot to -80 ˚C. Instead, store thawed aliquots at 4 ˚C 2. When using, add stock solution at 1:1000. For example, for 1 L of medium, add 1 mL of stock. | | |  |  | | | **Notes** | 1. If your host bacteria is resistant to CB, it can be added to prevent growth of other bacteria. 2. Mechanism of action: CB is a semi-synthetic penicillin that interferes with bacterial cell-wall synthesis. 3. 50 mL of carbenicillin should be sufficient per semester for a class of ~ 20 students. | | |

# **Cycloheximide Stock Solution**

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| **templateId** | **chapterContent** |
| **name** | **Cycloheximide Stock Solution** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Cycloheximide (CHX)** | | | |  | | | | **For bacterial host** | General | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | Cycloheximide (powder) | 10 mg/ml | stock=1000X | |  | | | | **To Prepare** | 1. Weigh out 50 to 100 mg of cycloheximide powder and place it in a 10-15 mL tube. 2. Divide the number of milligrams of cycloheximide by 10 to determine the volume (in milliliters) of ddH2O to add (e.g., for 51 mg of CHX, add 5.1 mL ddH2O). 3. Stir or shake until dissolved. 4. Filter-sterilize (0.22µm pore size) into a fresh sterile tube. 5. Prepare 1 mL aliquots and freeze at -80 ˚C. | | |  | | | | **To Sterilize** | Filter-sterilize (0.22µm pore size) into a fresh sterile tube. | | |  | | | | **To Store** | At -80 ˚C for 1 year.  At 4 ⁰C for ≤ 60 days. | | |  | | | | **Usage** | In Recipe:   * 7H9 Liquid Medium * PYCa Liquid Medium * Luria Agar * PYCa Agar | | |  |  | | | **Label Stock Solution** | CHX, 1000X, date, initials | | |  | | | | **To Prepare Final Solution** | 1. Remove a frozen aliquot, allow to thaw, and use as follows. Do not return thawed aliquot to -80 ˚C. Instead, store thawed aliquots at 4 ˚C. 2. When using, add stock solution at 1:1000. For example, for 1 L of medium, add 1 mL of stock. | | |  | | | | **Notes** | 1. CHX is added to media to prevent growth of fungi and yeast. 2. Mechanism of action: CHX inhibits protein biosynthesis in eukaryotic organisms. It interferes with the peptidyl transferase activity of the 60S ribosome, thus blocking translational elongation. 3. 50 mL of cycloheximide should be sufficient per semester for a class of ~ 20 students. | | |

# **Dextrose (40 %)**

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| **templateId** | **chapterContent** |
| **name** | **Dextrose (40 %)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **40 % Dextrose (200 mL)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | Dextrose anhydrous  ddH2O | 80 g  To 200 mL | 40 % | | **To Prepare** | 1. Add 100 mL of ddH2O to a beaker, and then add the dextrose while stirring.  2. Top up with ddH2O to 200 mL, and continue stirring until dextrose has dissolved. If the dextrose does not fully dissolve, gently warm the solution on the stir plate (or in a microwave).  3. Filter-sterilize the solution. Do not autoclave. | | | **To Sterilize** | Filter-sterilize. Do not autoclave. | | | **To Store** | At room temperature, indefinitely if not contaminated. | | | **Usage** | In Recipe:   * PYCa media * PYCa agar plates * PYCa top agar * Media Supplement for *Streptomyces* phage | | | **Notes** | 1. 100 mL of 40 % dextrose should be sufficient for preparing all reagents and media requiring dextrose, per semester for a class of ~ 20 students. | | |

# **Enrichment Broth**

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| **templateId** | **chapterContent** |
| **name** | **Enrichment Broth** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Enrichment Broth (100 mL)** | | | | **For bacterial host** | *M. smegmatis* mc2155 only | | | **Ingredients** | **Amount** | **Final Concentration** | | **7H9 liquid media neat**  **100 mM CaCl2 stock** | 99 mL  1 mL | 1X  1 mM | | **To Prepare** | All ingredients must be added to a sterile bottle or flask using aseptic technique. The final medium can be filter-sterilized. | | | **To Sterilize** | All ingredients are sterile. The final medium can be filter-sterilized. | | | **To Store** | At room temperature, for up to 1 year. Discard if solution is discolored, contaminated or has precipitation. | | | **Usage** | In Protocol: 5.2, 5.5 | | | **Notes** | 1. If antibiotics or antimicrobials are added, the medium must be stored at 4 ⁰C. 2. A good rule of thumb is that CaCl2 should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because CaCl2 will precipitate out when cooling after autoclaving or when refrigerated. 3. 30 ml of Enrichment broth is required per student per soil sample tested. | | |

# **EDTA**

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| **templateId** | **chapterContent** |
| **name** | **EDTA** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **EDTA 0.5 M (100 mL)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | **EDTA disodium salt dihydrate**  **ddH2O** | 18.61 g    To 100 mL | 0.5 M | | **To Prepare** | 1. Weigh out EDTA and place in a large beaker. 2. Add approximately 80 mL of ddH2O and stir until dissolved. 3. Adjust the pH to 8.0 with NaOH. 4. Transfer the solution to a graduated cylinder and add ddH2O to 100 mL. | | | **To Sterilize** | Filter-sterilize or autoclave | | | **To Store** | At room temperature, indefinitely if not contaminated. | | | **Usage** | In Protocol: 9.1a, 9.1b, 9.2a, 9.2b | | | **Notes** | 1. For protocol 9.2b, prepare a 0.1 M EDTA solution by diluting the 0.5 M EDTA solution in water. | | |

# **Glycerol (40 %)**

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| **templateId** | **chapterContent** |
| **name** | **Glycerol (40 %)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **40 % Glycerol (500 mL)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | ddH2O  Glycerol (also known as glycerin or glycerine) | To 500 mL  200 ml | 40 % | | **To Prepare** | 1. Measure 250 mL ddH2O and place in a large beaker. 2. Slowly add 200 mL of glycerol while stirring. 3. When the glycerol has completely mixed with the water, transfer the solution to a graduated cylinder. 4. Bring the volume to 500 mL with ddH2O. Mix. 5. Sterilize | | | **To Sterilize** | Filter-sterilize or autoclave. | | | **To Store** | At room temperature, indefinitely if not contaminated. | | |  |  | | | **Usage** | In Recipe:   * 7H9 liquid medium   In Protocol:   * Making glycerol stocks | | |  |  | | | **Notes** | 100 mL of 40 % glycerol should be sufficient for all reagents and media requiring glycerol per semester. | | |

# **Isopropanol (80 %)**

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| **templateId** | **chapterContent** |
| **name** | **Isopropanol (80 %)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **80 % Isopropanol (50 ml)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | 99% Isopropanol  ddH2O | 40 ml  To 50 ml | 80 % | | **To Prepare** | 1. Measure 40 ml isopropanol and place in a sterile conical tub 2. Slowly add ddH2O to 50 ml. 3. Mix using a vortex or by inversion. | | | **To Sterilize** | Not required | | | **To Store** | Important: Do Not Store.  80 % isopropanol should be prepared and used within the same day. | | | **Usage** | In Protocol: 9.1, 9.2 | | | **Notes** | 1. Prepare 80 % isopropanol only as needed. Isopropanol will evaporate with time, resulting in a solution with reduced isopropanol concentration. Isopropanol solutions below 80 % will result in premature elution of DNA during phage DNA extraction. 2. 6 mL of isopropanol is used per attempt at DNA extraction from phage lysates. | | |

# **Magnesium Chloride (1 M)**

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| **templateId** | **chapterContent** |
| **name** | **Magnesium Chloride (1 M)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | **M)**   |  |  |  | | --- | --- | --- | | **1 M MgCl2 (100 mL)** | | | | **For bacterial host** | *Streptomyces spp.* only | | | **Ingredients** | **Amount** | **Final Concentration** | | **MgCl2** hexahydrate\*  **ddH2O**    *\*****MgCl2*** *hexahydrate can be substituted with 9.5 g of* ***MgCl2*** *anhydrous in this recipe* | 20.33 g  To 100 mL | 1 M | | **To Prepare** | 1. Weigh out MgCl2 and place in a large beaker containing approximately 75 mL of ddH2O, and stir until MgCl2 is dissolved. 2. Transfer the solution to a graduated cylinder, and add ddH2O to 100 mL. 3. Sterilize | | | **To Sterilize** | Autoclave or filter-sterilize | | | **To Store** | At room temperature, for a year. | | | **Usage** | In Recipe:   * Media Supplement for *Streptomyces* phage | | | **Notes** |  | | |

# **Magnesium Sulphate (1 M)**

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| **templateId** | **chapterContent** |
| **name** | **Magnesium Sulphate (1 M)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  |  | | --- | --- | --- | --- | | **1 M MgSO4 (100 mL)** | | | | |  | | | | | **For bacterial host** | | General | | |  | | | | | **Ingredients** | **Amount** | | **Final Concentration** | | MgSO4 (anhydrous)  ddH2O | 12.04 g  To 100 mL | | 1 M | |  | | | | | **To Prepare** | 1. Weigh out MgSO4 and place in a large beaker containing approximately 95 mL of ddH2O, and stir until MgSO4 is dissolved. 2. Transfer the solution to a graduated cylinder, and add ddH2O to 100 mL. 3. Sterilize. | | | |  | | | | | **To Sterilize** | Autoclave or filter-sterilize | | | |  | | | | | **To Store** | At room temperature, for a year. | | | |  | | | | | **Usage** | In Recipe:   * Phage buffer | | | |  | | | | | **Notes** | 1. 10 mL of MgSO4 is required to prepare 1 L of phage buffer. 2. 100 mL of MgSO4 is sufficient for 1 – 5 semesters for a class of ~ 20 students. | | | |

# **Nuclease Mix**

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| **templateId** | **chapterContent** |
| **name** | **Nuclease Mix** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Nuclease Mix (5 mL)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | NaCl  ddH2O  DNase 1  RNase A  Glycerol | 0.09 g  4.25 mL  250 µL of 5-mg/mL stock  125 µL of 10-mg/mL stock  2.5 mL | 150 mM    0.25 mg/mL or 500 KU/ml  0.25 mg/mL or 12.5 U/mg  50 % | | **To Prepare** | **Note**: Begin by preparing the RNase A and DNase 1 stock solutions according to the manufacturer’s protocol. RNAse A stock solutions are typically prepared in Tris-Cl (10 mM; pH 7.5) and NaCl (15 mM), and includes a 5-min heating step at 100 ˚C.   1. Dissolve the NaCl in 4.25 mL of ddH2O in a sterile 15-mL conical tube. 2. Transfer 2.1 mL of the NaCl solution into a new 15-mL conical tube. Note: the remaining 2.1 mL can be stored indefinitely and used at a later date to prepare more nuclease mix. 3. Add the glycerol to the NaCl solution, and mix with gentle inversion. 4. Add the RNase and DNase stock solutions, and mix with gentle inversion. 5. Add ddH2O to a final volume of 5 mL, and mix with gentle inversion until the solution is homogeneous. 6. Aliquot into microcentrifuge tubes. | | | **To Sterilize** | Not required | | | **To Store** | At -20 ⁰C, for up to 2 years. | | | **Usage** | In Protocol: 9.1 | | | **Notes** | 1. Be extra careful not to contaminate pipettes, benches, or other reagents with this nuclease mixture, because it will degrade any DNA samples. 2. Prepare DNase I and RNase A stock solutions according to the instructions provided by the manufacturer. 3. 5 µL of nuclease mix is used per attempt at DNA extraction from phage lysates. 4. Estimate 1-2 attempts at DNA extraction per student. | | |

# **Phage Buffer**

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| **templateId** | **chapterContent** |
| **name** | **Phage Buffer** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Phage Buffer (PB; 1 L)** | | | |  | | | | **For bacterial host** | General | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | 1 M Tris stock (pH 7.5)  1 M MgSO4 stock  NaCl  ddH2O  100 mM CaCl2 stock  Glycerol (100 %), if using\* | 10 mL  10 mL  4 g  up to 1 L  10 mL  100 mL | 10 mM  10 mM  68 mM    1 mM  10 % | |  | | | | **To Prepare** | 1. Add 800 mL of ddH2O to a large flask or beaker. 2. Add all ingredients to the water, while stirring. Glycerol should be added at this stage, if using (see notes\*). 3. Stir until all components have completely dissolved. 4. Add ddH2O to 1 L. 5. Filter-sterilize. 6. Aliquot into sterile bottles, flasks, or tubes in 50-mL or 100-mL (or other) portions as needed. | | |  | | | | **To Sterilize** | Filter-sterilize | | |  | | | | **To Store** | At room temperature, for up to 3 years. Discard if solution is discolored, contaminated or has precipitation. | | |  | | | | **Usage** | In Protocol:  5.3, 5.4, 5.6, 6.1, 6.2, 6.3, 6.4, 6.5, 7.1, 8.1a, 8.1b, 11.1, 11.4 | | |  | | | | **Notes** | 1. \*If your phage is particularly unstable in phage buffer, you may consider supplementing your phage buffer with glycerol to 10 %. 2. 1 L of phage buffer is sufficient per semester for a class of ~ 20 students. | | |

# **Phage Precipitation Solution**

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| **templateId** | **chapterContent** |
| **name** | **Phage Precipitation Solution** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Phage Precipitation Solution (100 mL)**  **[If using, prepare as necessary]** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | ddH2O  Polyethylene glycol 8000  (PEG 8000)  NaCl | To 100 mL  30 g    19.3 g | 30 %    3.3 M | | **To Prepare** | 1. Add 60 mL of ddH2O, followed by 19.3 g NaCl, to a glass bottle with a screw cap, and swirl until the NaCl is completely dissolved. 2. Slowly add 30 g of PEG 8000. Add only a few grams at a time, alternately swirling and gently heating in a microwave (e.g. by heating only for 10 sec at a time) until all the PEG is dissolved. 3. Add sterile water to 100 mL. 4. Add a clean magnetic stir bar and stir until homogeneous. 5. Filter-sterilize. 6. Dispense into sterile bottles or tubes. | | | **To Sterilize** | Filter-sterilize. | | | **To Store** | At room temperature, indefinitely. Discard if solution is deteriorating, discolored, contaminated or has precipitation. | | | **Usage** | In Protocol: 9.1 of Instructors Guide | | | **Notes** | 1. The NaCl and the PEG 8000 are difficult to dissolve. Several iterations of heating and stirring may be required. If there are precipitates in the solution, do not use it! Rather, attempt to dissolve particulates by alternately heating the solution in a microwave and swirling. Allow the solution to cool before using. Use this solution only at room temperature. 2. Up to 4 mL of phage precipitation buffer is used per attempt at DNA extraction using the PEG precipitation protocol. | | |

# **SDS (10 %)**

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| **templateId** | **chapterContent** |
| **name** | **SDS (10 %)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **For bacterial host** | General | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | SDS powder  ddH2O | 1 g  up to 10 mL | 10 % | |  | | | | **To Prepare** | Important: SDS may cause respiratory irritation if inhaled. Is using loose powder of SDS (instead of micropellets), a mask should be used when weighing out the powder.     1. Weigh out 1g of SDS powder and place it in a 50 mL tube. 2. Add ddH2O to 10 mL. 3. Invert the tube gently and repeated until the SDS is fully dissolved. Vigorous shaking will lead to excessive foaming. 4. Filter-sterilize (0.22µm pore size) into a fresh sterile tube. 5. Prepare 1 mL aliquots. | | |  | | | | **To Sterilize** | Filter-sterilize (0.22µm pore size) into a fresh sterile tube. | | |  | | | | **To Store** | At room temperature, indefinitely if not contaminated. | | |  |  | | | **Usage** | In Protocol: 9.1a, 9.1b, 9.2a, 9.2b | | |  |  | | | **Label Stock Solution** | 10 % SDS | | |  |  | | | **Notes** | 1. 2 mL of 10 % SDS should be sufficient per semester for a class of ~ 20 students. | | |

# **Tris Borate EDTA (TBE)**

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| **templateId** | **chapterContent** |
| **name** | **Tris Borate EDTA (TBE)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **1X TBE** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | 10X TBE  ddH2O | 100 mL  900 mL | 1X (89-mM Tris, 89-mM boric acid, and 2-mM EDTA) | | **To Prepare** | Dilute the 10X TBE to 1X with the volumes above (i.e., 100 and 900 mL). There is no need to check or adjust the pH of this solution, and it does not have to be sterilized before use. | | | **To Sterilize** | Not required | | | **To Store** | At room temperature, indefinitely. Discard is discolored, contaminated or has precipitation. | | | **Usage** | In Protocol: 10.2 | | | **Notes** | Volume of buffer will depend on size of gel and gel tank | | |

# **Tris, pH 7.5 (1 M)**

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| **templateId** | **chapterContent** |
| **name** | **Tris, pH 7.5 (1 M)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **1 M Tris (base), pH 7.5 (100 mL)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | Trizma base  ddH2O | 12.11 g  To 100 mL | 1 M | | **To Prepare** | 1. Add approximately 95 mL of ddH2O to a large beaker. 2. Add Trizma base to the breaker while stirring. 3. When the Trizma base is dissolved, bring the pH to 7.5 with HCl. 4. Transfer the solution to a graduated cylinder, and add ddH2O to 100 mL 5. Sterilize | | | **To Sterilize** | Autoclave or filter-sterilize | | | **To Store** | At room temperature, for up to a year. | | | **Usage** | In Recipe:   * Phage buffer | | | **Notes** | 1. 10 mL of 1M Tris is required to prepare 1 L of phage buffer. 2. 100 mL of 1M Tris is sufficient for 1 – 5 semesters for a class of ~ 20 students. | | |

# **Tween80 (20 %)**

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| **templateId** | **chapterContent** |
| **name** | **Tween80 (20 %)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **20 % Tween80 (100 ml)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | ddH2O  Tween80 | To 100 ml  20 ml | 20 % | | **To Prepare** | 1. Measure 60 mL of ddH2O and place in a medium-size beaker. 2. Slowly add 20 mL of Tween80 while stirring. 3. Once the Tween80 has completely dissolved, transfer the solution to a graduated cylinder and bring to 100 mL with ddH2O. 4. Sterilize | | | **To Sterilize** | Filter-sterilize. Do not autoclave | | | **To Store** | At room temperature, indefinitely if not contaminated | | | **Usage** | In Recipe:   * 7H9 liquid medium | | | **Notes** | 1. It may take a while for Tween80 to dissolve. Add Tween 80 slowly and alternate between stirring and warming the solution. For example, alternate the beaker between the stir plate and at 55 ⁰C water bath to dissolve the Tween80. 2. 100 mL of 20 % Tween80 is sufficient for 1 – 10 semesters for a class of ~ 20 students. | | |

# **Uranyl Acetate Stain**

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| **templateId** | **chapterContent** |
| **name** | **Uranyl Acetate Stain** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Uranyl Acetate Stain** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | Uranyl acetate  ddH2O | 0.1 g  10 mL | 1 % w/v | | **To Prepare** | 1. Weigh out the uranyl acetate and place in a conical containing approximately 9 mL of ddH2O, and stir 2. When the uranyl acetate is dissolved, bring the final volume up to 10 mL with ddH2O. | | | **To Sterilize** | Prepare a 0.22-µm filter by passing 3 mL of ddH2O through the filter. Discard the filtered ddH2O. Filter-sterilize the uranyl acetate solution and dispense in 1-mL aliquots. | | | **To Store** | Cover each aliquot in foil to protect from light. Store at room temperature, for up to 3 years. Discard appropriately if precipitation occurs. | | | **Usage** | In Protocol:  8.1a, 8.1b | | | **Notes** | 1. This is a highly toxic compound. Always wear gloves when preparing or working with it. 2. 5 µL of uranyl acetate stain is used per sample to be imaged. | | |

# **Water**

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| **templateId** | **chapterContent** |
| **name** | **Water** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | **Water, sterile (200 mL)** | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | ddH2O | 200 mL |  | | **To Prepare** | 1. Measure 200 mL ddH2O (ideally deionized water) and place bottle with a cap. 2. Sterilize | | | **To Sterilize** | Filter-sterilize or autoclave. | | | **To Store** | At room temperature, indefinitely if not contaminated. | | |  |  | | | **Usage** | In Recipe:   * 80 % Isopropanol   In Protocol:   * DNA Extraction * Restriction Enzymes Digests | | |  |  | | | **Notes** | 500 mL of sterilized water should be sufficient for all reagents and experiments per semester. | | |

# **Zinc Chloride**

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| **templateId** | **chapterContent** |
| **name** | **Zinc Chloride** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **Content** | |  |  |  | | --- | --- | --- | | Zinc Chloride (2 M) | | | | **For bacterial host** | General | | | **Ingredients** | **Amount** | **Final Concentration** | | ZnCl2  ddH2O | 10.9 g  To 40 mL | 2 M | | **To Prepare** | 1. Weigh out ZnCl2 and place in a conical tube containing approximately 35 mL of ddH2O, and vortex until ZnCl2 is dissolved. 2. Add ddH2O to 40 mL. 3. Sterilize. | | | **To Sterilize** | Filter-sterilize. | | | **To Store** | Important: Do Not Store.  \*Prepare immediately before use. | | | **Usage** | In Protocol:  9.2b | | | **Notes** | \* When prepared at this high concentration, ZnCl2 will precipitate after ~ 1 hour at room temperature. | | |

# **Recipes for Media**

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| **templateId** | **toc** |
| **name** | **Recipes for Media** |
| **child** | **true** |
| **appendix** | **false** |
| **templateId** | **chapterContent** |
| **name** | **7H9 Liquid Medium(Neat)** |
| **templateId** | **chapterContent** |
| **name** | **7H9 Liquid Medium(Complete)** |
| **templateId** | **chapterContent** |
| **name** | **7H9 Liquid Medium(Complete with Tween80)** |
| **templateId** | **chapterContent** |
| **name** | **Carbenicllin Stock Solution** |
| **templateId** | **chapterContent** |
| **name** | **7H9 Top Agar (1X)** |
| **templateId** | **chapterContent** |
| **name** | **7H9 Top Agar (2X)** |
| **templateId** | **chapterContent** |
| **name** | **Luria Agar (L-Agar) Plates** |
| **templateId** | **chapterContent** |
| **name** | **Media Supplement for Streptomyces phage** |
| **templateId** | **chapterContent** |
| **name** | **Nutrient Broth** |
| **templateId** | **chapterContent** |
| **name** | **Nutrient Broth(with Supplements for S.griseus)** |
| **templateId** | **chapterContent** |
| **name** | **Nutrient Agar with Streptomyces phage supplement (NA+) Plates (1 L)** |
| **templateId** | **chapterContent** |
| **name** | **PYCa Agar Plates** |
| **templateId** | **chapterContent** |
| **name** | **PYCa Liquid Medium (1 L)** |
| **templateId** | **chapterContent** |
| **name** | **PYCa Top Agar (PYCa TA at 1X concentration; 1 L)** |
| **templateId** | **chapterContent** |

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# **7H9 Liquid Medium(Neat)**

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| **templateId** | **chapterContent** |
| **name** | **7H9 Liquid Medium(Neat)** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **content** | **r**   |  |  |  | | --- | --- | --- | | **Middlebrook 7H9 Liquid Medium: Neat (900 mL)** | | | |  | | | | **For bacterial host** | *M. smegmatis* mc2155 only | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | 7H9 broth base  40% glycerol stock  ddH2O | 4.7 g  5 mL  To 900 ml |  | |  | | | | **To Prepare** | 1. Add broth base to 850 mL of ddH2O in an Erlenmeyer flask. 2. While stirring, add the glycerol. Stir until the broth base powder is completely dissolved. 3. Bring up to 900 mL with ddH2O in a graduated cylinder. 4. Aliquot into 90-mL, 450-mL, or 900-mL portions as needed. 5. Sterilize | | |  | | | | **To Sterilize** | Autoclave. | | |  | | | | **To Store** | At room temperature, indefinitely if not contaminated or discolored. | | |  | | | | **Usage** | In Recipe:   * Growing *Mycobacterium smegmatis* mc2155 * 1X 7H9 top agar | | |  | | | | **Notes** | 1. This medium will be diluted by the addition of 10 % (v/v) AD supplement (see Middlebrook 7H9 Liquid Medium: Complete). This is why medium is dispensed in 9/10 volumes (e.g., 450 mL). 2. 5 L of 7H9 liquid media should be sufficient per semester for a class of 20 students. | | |

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# **7H9 Liquid Medium(Complete)**

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| **content** | |  |  |  | | --- | --- | --- | | **Middlebrook 7H9 Liquid Medium: Complete (100 mL)**  **(For bacterial growth without Tween 80)** | | | | **For bacterial host** | *M. smegmatis* mc2155 only | | | **Ingredients** | **Amount** | **Final Concentration** | | 7H9 liquid medium: neat  AD supplement  CB stock  CHX stock  100 mM CaCl2 stock | 89 ml  10 ml  100 µl  100 µl  1 ml | 1X  10 %  50 µg/ml  10 µg/ml  1 mM | | **To Prepare** | All ingredients must be added to a sterile bottle or flask using aseptic technique. The final medium cannot be autoclaved or filter-sterilized. | | | **To Sterilize** | All ingredients are already sterile | | | **To Store** | At 4 ⁰C, for up to 30 days. Discard if media is discolored or contaminated. | | | **Usage** | In Recipe:   * Growing *Mycobacterium smegmatis* mc2155 | | | **Notes** | 1. Make complete 7H9 medium as needed; refrigeration may cause the CaCl2 to precipitate. If antimicrobials and AD supplement have been added, the medium must be stored at 4 °C. 2. A good rule of thumb is that CaCl2 should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because CaCl2 will precipitate out when cooling after autoclaving or when refrigerated. | | |

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# **7H9 Liquid Medium(Complete with Tween80)**

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| **name** | **7H9 Liquid Medium(Complete with Tween80)** |
| **imgtext** | **Recipe Cards** |
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| **content** | |  |  |  | | --- | --- | --- | | **Middlebrook 7H9 Liquid Medium: Complete with Tween80**  **(For bacterial growth with Tween 80)** | | | | **For bacterial host** | *M. smegmatis* mc2155 only | | | **Ingredients** | **Amount** | **Final Concentration** | | 7H9 liquid medium  AD supplement  CB stock  CHX stock  100 mM CaCl2 stock  20 % Tween80 stock | 90 ml  10 ml  100 µL  100 µL  1 ml  250 µL | 1X  10 %  50 µg/ml  10 µg/ml  1 mM  0.05 % | | **To Prepare** | All ingredients must be added to a sterile bottle or flask using aseptic technique. The final medium cannot be autoclaved or filter-sterilized. | | | **To Sterilize** | All ingredients are already sterile | | | **To Store** | At 4 ⁰C, for up to 30 days. Discard if media is discolored or contaminated. | | | **Usage** | In Recipe:   * Growing *Mycobacterium smegmatis* mc2155 | | | **Notes** | 1. In liquid culture, *Mycobacterium smegmatis* tends to clump. When liquid cultures are grown from bacterial colonies, Tween80 should be added to 0.05% to prevent clumping. 2. This culture is then sub-cultured into medium without Tween80 for use in phage infections. 3. Make complete 7H9 medium as needed; refrigeration will cause the calcium to precipitate. If antimicrobials and AD supplement have been added, the medium must be stored at 4 °C. 4. A good rule of thumb is that CaCl2 should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because CaCl2 will precipitate out when cooling after autoclaving or when refrigerated. | | |

# **7H9 Top Agar (1X)**

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| **name** | **7H9 Top Agar (1X)** |
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| **content** | |  |  |  |  | | --- | --- | --- | --- | | **1X Middlebrook Top Agar (TA; 100mL)** | | | | |  | | | | | **For bacterial host** | | *M. smegmatis* mc2155 only | | |  | | | | | **Ingredients** | **Amount** | | **Final Concentration** | | 100 mM CaCl2 stock  7H9 liquid media neat  2X TA | 1 mL  50 mL  50 mL | | 1 mM    1 X | |  | | | | | **To Prepare** | 1. Using aseptic technique, add 1 mL of CaCl2 stock to the 50 mL of 7H9 medium. 2. Place into a 55 °C water bath. 3. Melt the 2X TA in a microwave. Make sure the cap is loose! The agar must be completely melted, so carefully swirl the bottle and check to make sure there are no clumps. The 2X TA should come to a boil but not boil over. 4. Once the agar is completely melted, place the 2X TA into a 55 °C water bath. When both solutions are at 55 °C, aseptically add the medium-calcium mixture to the 2X TA and swirl. | | | |  | | | | | **To Sterilize** | All ingredients must be added using aseptic technique. The final medium cannot be autoclaved or filter-sterilized. | | | |  | | | | | **To Store** | In the 55 °C water bath ≤ 7days. Cooling to room temperature will cause the CaCl2 to precipitate out of solution.  Discard if media is discolored or contaminated.  Repeated melting and solidifying of top agar will cause the agar to deteriorate and not solidify properly on plates. | | | |  | | | | | **Usage** | In Protocol:  5.3, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.1,12.2 | | | |  | | | | | **Notes** | 1. The final agar concentration will be 0.4% 2. A good rule of thumb is that CaCl2 should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because CaCl2 will precipitate out when cooling after autoclaving or when refrigerated.   3. 3 – 5 mL of top agar is used per plating. | | | |

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# **7H9 Top Agar (2X)**

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| **content** | |  |  |  |  | | --- | --- | --- | --- | | **2X Middlebrook Top Agar (2X TA; 1 L)** | | | | |  | | | | | **For bacterial host** | | *M. smegmatis* mc2155 only | | |  | | | | | **Ingredients** | | **Amount** | **Final Concentration** | | **7H9 broth base**  **Agar**  **ddH2O** | | 4.7 g  8 g  1 L | 0.8 % | |  | | | | | **To Prepare** | 1. Place ddH2O and a stir bar, then broth base and agar, into an Erlenmeyer flask; put onto a stir plate. 2. Stir until the broth base powder is completely dissolved (the agar will not dissolve until it is heated). 3. Autoclave the flask, and cool to 55 °C in a 55 °C water bath. 4. Gently swirl the solution to evenly distribute the agar. 5. In a hood, using aseptic technique, dispense into sterile bottles using only half the available volume (e.g., for a 100 mL bottle, add 50 mL of 2X TA; this will allow you to add 50 mL of media directly to the molten 2X TA to prepare 1X TA). Keep any lids loose until the agar has solidified. 6. Tighten lids for storage. 7. To melt stored top agar for use, heat in a microwave using 30 second intervals until agar has completely melted. 8. Repeated melting and solidifying of top agar will cause the agar to deteriorate and not solidify properly on plates. | | | |  | | | | | **To Sterilize** | | Autoclave | | |  | | | | | **To Store** | | As a solid, store at room temperature, indefinitely. Discard if media is discolored or contaminated. | | |  | | | | | **Usage** | | In Recipe:   * 1X Middlebrook Top Agar   In Protocol:   * 12.1 | | |  | | | | | **Notes** | | 2.5 L of 2X top agar is sufficient for per semester for ~ 20 students | | |

# **Luria Agar (L-Agar) Plates**

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| **imgtext** | **Recipe Cards** |
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| **content** | |  |  |  |  | | --- | --- | --- | --- | | **Luria Agar (L-Agar) Plates (1 L)** | | | | |  | | | | | **For bacterial host** | | *M. smegmatis* mc2155 only | | |  | | | | | **Ingredients** | **Amount** | | **Final Concentration** | | Luria broth base  Agar  ddH2O  CB stock (if using)  CHX stock | 15.5 g  15 g  To 1 L  1.0 mL  1.0 mL | | 50 µg/mL  10 µg/mL | |  | | | | | **To Prepare** | 1. To 1 L of ddH2O in a large flask, add 15.5 g of Luria broth base and 15 g agar. Mix well with a magnetic stir bar. 2. Autoclave the flask. 3. Cool to 55 °C in a 55 °C water bath. 4. Once the agar has cooled to 50-55 °C, aseptically add antimicrobials as necessary. 5. Mix well by swirling, and avoid bubbles. 6. Using aseptic technique, pour agar into Petri dishes. 7. Allow the agar to set overnight.\* | | | |  | | | | | **To Sterilize** | Autoclave (see above) | | | |  | | | | | **To Store** | At 4 °C, for up to 3 months. Plates can still be used beyond 3 months, although the antimicrobials may have been partially degraded. Discard if plate is discolored or contaminated. | | | |  | | | | | **Usage** | In Protocol:  5.3, 5.4, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.2 | | | |  | | | | | **Notes** | 1. Prior to adding antibiotics or antimicrobials, the cooled medium can be aliquoted into sterile bottles, allowed to solidify, and stored at room temperature. The solidified agar can be melted by autoclaving for 5 mins, or in a microwave. Antimicrobials should only be added once the freshly melted agar has cooled to 55 ˚C. 2. Prior to use, agar plates stored at 4 ˚C should be allowed to warm to room temperature. 3. 1 L of agar is sufficient to prepare 50 agar plates. 4. \* Though the agar will set within 1 – 2 hours, allowing the plates to sit at room temperature overnight helps remove some water content within the agar. Drying plates in this manner helps prevent top agar, when used, from slipping on the surface of the agar. 5. Approximately 1,500 agar plates are used per semester per class of 20 students. Consider preparing plates in batches of 500 plates. | | | |

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# **Media Supplement for Streptomyces phage**

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| **name** | **Media Supplement for Streptomyces phage** |
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| **content** | |  |  |  | | --- | --- | --- | | **Media Supplement for *Streptomyces* phage (32.5 mL)** | | | | **For bacterial host** | *Streptomyces* spp. only | | | **Ingredients** | **Amount** | **Final Concentration** | | 1 M MgCl2  0.8 M Ca(NO3)2  40% Dextrose | 10 mL  10 mL  12.5 mL | 10 mM (after addition to culture media)  8 mM (after addition to culture media)  0.5% (after addition to culture media) | | **To Prepare** | 1. Aseptically add each component to a sterile container to mix or add each directly to cooled, sterile culture media (per 1 L). 2. Mix by gently swirling. | | | **To Sterilize** | Autoclave each component separately. | | | **To Store** | At room temperature, for up to 1 year. Discard if solution is discolored, contaminated or has precipitation. | | | **Usage** | Nutrient Broth with Media Supplement for *Streptomyces* phage, Nutrient Agar with Media Supplement for *Streptomyces* phage | | | **Notes** | If using a different species of *Streptomyces* for phage isolation, a different base media may be used with the same supplement. | | |

# **Nutrient Broth**

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| **name** | **Nutrient Broth** |
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| **content** | |  |  |  | | --- | --- | --- | | **Nutrient Broth (1 L)** | | | |  | | | | **For bacterial host** | *Streptomyces* spp. only | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | **Nutrient broth powder**  **ddH2O** | 8 g    To 1 L | 1X | |  | | | | **To Prepare** | 1. Mix 8 g of Nutrient broth powder with 1 L of ddH2O in a large flask and mix well with a magnetic stir bar. 2. Aliquot as needed. 3. Sterilize | | |  | | | | **To Sterilize** | Autoclave | | |  | | | | **To Store** | At room temperature or 4 °C. | | |  | | | | **Usage** | *Streptomyces* liquid culture growth for starter cultures. | | |  | | | | **Notes** | 1. Because *Streptomyces* cultures are prone to clumping when grown in liquid, it is highly recommended that all cultures contain 5% w/v PEG 8000, which serves as a physical dispersant, and that cultures be shaken vigorously in a baffled flask. PEG can be added to mixture prior to autoclaving. 2. 5 L of 7H9 liquid media should be sufficient per semester for a class of 20 students. | | |

# **Nutrient Broth(with Supplements for S.griseus)**

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| **templateId** | **chapterContent** |
| **name** | **Nutrient Broth(with Supplements for S.griseus)** |
| **imgtext** | **Recipe Cards** |
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| **content** | |  |  |  | | --- | --- | --- | | **Nutrient Broth with Media Supplement for *Streptomyces* phage (1 L)** | | | |  | | | | **For bacterial host** | *Streptomyces* spp. only | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | **Nutrient broth powder**  **ddH2O**  **Media Supplement for *Streptomyces* phage** | 8 g    To 1 L      32.5 mL | 1X | |  | | | | **To Prepare** | 1. Mix 8 g of Nutrient broth powder with 1 L of ddH2O in a large flask and mix well with a magnetic stir bar. 2. Aliquot as needed. 3. Autoclave. 4. Add Media Supplement for *Streptomyces* phage (32.5 mL/1L) after media has cooled. | | |  | | | | **To Sterilize** | Autoclave | | |  | | | | **To Store** | At room temperature or 4 °C. | | |  | | | | **Usage** | *Streptomyces* liquid culture growth in which phage infection will occur (including enrichment cultures) | | |  | | | | **Notes** | Because *Streptomyces* cultures are prone to clumping when grown in liquid, it is highly recommended that all cultures contain 5% w/v PEG 8000, which serves as a physical dispersant, and that cultures be shaken vigorously in a baffled flask. PEG can be added to mixture prior to autoclaving. | | |

# **Nutrient Agar Plates(with Supplements for S.griseus)**

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| **name** | **Nutrient Agar Plates(with Supplements for S.griseus)** |
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| **content** | |  |  |  | | --- | --- | --- | | **Nutrient Agar with *Streptomyces* phage supplement (NA+) Plates (1 L)** | | | | **For bacterial host** | *Streptomyces* spp. only | | | **Ingredients** | **Amount** | **Final Concentration** | | **Nutrient agar powder**  **ddH2O**  **Media Supplement for *Streptomyces* phage** | 23 g  To 967.5 L      32.5 mL | 1X | | **To Prepare** | 1. Mix 23 g of Nutrient agar powder with ddH2O to 967.5 mL total volume in a large flask and mix well with a magnetic stir bar. 2. Autoclave the flask. 3. Cool to 55 °C in a 55 °C water bath. 4. Once the agar has cooled to 50-55 °C, aseptically add 32.5 mL of sterile Media Supplement for *Streptomyces* phage. 5. Mix well by swirling, and avoid bubbles. 6. Using aseptic technique, pour agar into Petri dishes. 7. Allow the agar to set overnight.\* | | | **To Sterilize** | Autoclave (see above) | | | **To Store** | At room temperature or 4 °C, for up to 3 months. Discard if plate is discolored or contaminated. | | | **Usage** | In *Streptomyces* plate growth protocols. | | | **Notes** | 1. Prior to adding media supplements, the cooled medium can be aliquoted into sterile bottles, allowed to solidify, and stored at room temperature. The solidified agar can be melted by autoclaving for 5 mins, or in a microwave. Supplements should only be added once the freshly melted agar has cooled to 55 ˚C. 2. Prior to use, agar plates stored at 4 ˚C should be allowed to warm to room temperature. 3. \* Though the agar will set within 1 – 2 hours, allowing the plates to sit at room temperature overnight helps remove some water content within the agar. Drying plates in this manner helps prevent top agar, when used, from slipping on the surface of the agar. 4. 1 L of agar is sufficient to prepare 50 agar plates. 5. Approximately 1,500 agar plates are used per semester per class of 20 students. Consider preparing plates in batches of 500 plates. | | |

# **PYCa Agar Plates**

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| **content** | |  |  |  | | --- | --- | --- | | **PYCa Agar Plates (1 L)** | | | |  | | | | **For bacterial host** | General (excluding *M. smegmatis* and *Streptomyces* spp.) | | |  | | | | **Ingredients** | **Amount** | **Final Concentration** | | Agar  Yeast extract  Peptone  1 M Cacl2  Dextrose (40 %)  ddH2O  CHX stock | 15.0 g  1 g  15 g  4.5 mL  2.5 mL  990 mL  1.0 mL | 1.5 %  1 g/L  15 g/L  4.5 mM  0.1 %    10 µg/mL | |  | | | |  | | | | **To Prepare** | **Note**: A video protocol for this recipe card is available [**here**](https://seaphages.org/video/55).     1. Place water and a stir bar, followed agar, peptone and yeast extract, into an Erlenmeyer flask. Put onto a stir plate. 2. Stir until the yeast extract and peptone are completely dissolved (the agar will not dissolve until it is heated). 3. Autoclave the flask, and cool to 60 °C in a 60 °C water bath. 4. In a hood, aseptically add dextrose and CaCl2. 5. Add antibiotics/antimicrobials stock solutions, if using. 6. Mix well by swirling, and avoid bubbles. 7. Using aseptic technique, pour agar into Petri dishes. 8. Allow the agar to set overnight.\* | | |  | | | | **To Sterilize** | Autoclave | | |  | | | | **To Store** | At 4 °C, for up to 3 months. Plates can still be used beyond 3 months, although the antimicrobials may have been partially degraded. Discard if plate is discolored or contaminated. | | |  | | | | **Usage** | In Protocol: 5.3, 5.4, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.2 | | |  | | | | **Notes** | 1. Prior to adding antibiotics or antimicrobials, the cooled medium can be aliquoted into sterile bottles, allowed to solidify, and stored at room temperature. To melt many batches of solidified agar, autoclave for ~ 10 -15 mins. Alternatively, melt the agar in small bathes using a microwave. Antimicrobials should only be added once the freshly melted agar has cooled to 55 ˚C. 2. Prior to use, agar plates stored at 4 ˚C should be allowed to warm to room temperature. 3. \* Though the agar will set within 1 – 2 hours, allowing the plates to sit at room temperature overnight helps remove some water content within the agar. Drying plates in this manner helps prevent top agar, when used, from slipping on the surface of the agar. 4. 1 L of agar is sufficient to prepare 50 agar plates. 5. Approximately 1,500 agar plates are used per semester per class of 20 students. - Consider preparing plates in batches of 500 plates. - Often, a limiting factor to preparing plates in large quantities is adequate space at 4 ˚C to store those plates. | | |

# **PYCa Liquid Medium**

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| **name** | **PYCa Liquid Medium** |
| **imgtext** | **Recipe Cards** |
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| **content** | |  |  |  | | --- | --- | --- | | **PYCa Liquid Medium (1 L)** | | | | **For bacterial host** | General (excluding *M. smegmatis* and *Streptomyces* spp.) | | | **Ingredients** | **Amount** | **Final Concentration** | | Yeast extract  Peptone  ddH2O  1 M CaCl2 stock  Dextrose (40 %)  CHX stock | 1.0 g  15.0 g  990 ml  4.5 ml  2.5 ml  1 ml | 1 g/L  15 g/L    4.5 mM  0.1 %  10 µg/ml | | **To Prepare** | 1. Add 990 mL of ddH2O into a large flask. Then weigh out and add peptone and yeast extract to the flask. 2. Stir until completely dissolved. 3. Autoclave. 4. Once cool, aseptically add dextrose and CaCl2. 5. Aliquot as 100-mL, 500-mL, or 1000-mL portions, in sterile vials, as needed.\* 6. If using CHX, add prior to use.   \* It may be more convenient to aliquot the media (lacking dextrose and calcium chloride) into vials prior to sterilization by autoclaving. In this case, the vials do not need to be sterile, but dextrose and calcium chloride stock solutions should still only be added after sterilization. You will also need to adjust the volume of dextrose and calcium chloride stock solutions added to each vial. | | | **To Sterilize** | Autoclave. | | | **To Store** | At room temperature, indefinitely, or at 4 °C for up to 30 days if antimicrobials have been added.  Discard if media is discolored or contaminated. | | | **Usage** | In Protocol: 5.2, 5.5, & 12.1 | | | **Notes** | 1. If antimicrobials have been added, the medium must be stored at 4 °C. 2. 5 L of PYCa liquid media should be sufficient per semester for a class of 20 students. | | |

# **PYCa Top Agar**

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| **name** | **PYCa Top Agar** |
| **imgtext** | **Recipe Cards** |
| **img** | **recipe-cards.svg** |
| **content** | |  |  |  | | --- | --- | --- | | **PYCa Top Agar (PYCa TA at 1X concentration; 1 L)** | | | | **For bacterial host** | General (excluding *M. smegmatis* and *Streptomyces* spp.) | | |  |  |  | | **Ingredients** | **Amount** | **Final Concentration** | | Agar  Yeast extract  Peptone  1 M Cacl2  Dextrose (40 %)  ddH2O | 4.0 g  1 g  15 g  4.5 mL  2.5 mL  990 mL | 0.4 %  1 g/L  15 g/L  4.5 mM  0.1 % | | **To Prepare** | **Note**: You may choose to prepare a large volume of media and then transferring aliquots into smaller vials after sterilization (as described below), or preparing the media in smaller vials before sterilization. If choosing the latter, adjust the weight and volumes of each media component accordingly.     1. Add water and a stir bar, followed by agar, peptone and yeast extract, into an Erlenmeyer flask; put onto a stir plate. 2. Stir until the yeast extract and peptone is completely dissolved (the agar will not dissolve until it is heated). 3. Autoclave the flask. 4. Once autoclaved and cooled slightly (not below 60 ˚C), add dextrose and CaCl2, using aseptic technique. Gently swirl the flask to mix the solution. 5. In a hood, using aseptic technique, dispense into 50 mL or 100 mL sterile bottles. 6. If using immediately, maintain top agar in the molten form by placing it at 55 ˚C. If not, keep bottle lids slightly loose until the agar has solidified. Once solidified, tighten lids for storage. 7. To melt stored top agar for use, make sure the cap is loose, and heat in microwave using 30 second intervals. The top agar should come to a boil but not boil over. As the agar must be completely melted, **carefully** and gently swirl the bottle with the loose cap facing away from you and others. Check to make sure there are no clumps. 8. Once the agar is completely melted, place the top agar into a 55 °C water bath to cool before use. 9. Repeated melting and solidifying of top agar will cause the agar to deteriorate and not solidify properly on plates. | | | **To Sterilize** | Autoclave | | | **To Store** | * As a solid, store at room temperature, indefinitely. Discard if media is discolored or contaminated. * As molten agar, store for up to 10 days. Agar stored in its molten form deteriorates over time and may not solidify properly on plates. | | | **Usage** | In Protocol:  5.3, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.1, 12.2 | | | **Notes** | 5 L of PYCa top agar is sufficient for per semester for ~ 20 students . | | |

# **Propagating Bacteria and Phage**

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| **name** | **Propagating Bacteria and Phage** |
| **child** | **true** |
| **appendix** | **false** |
| **templateId** | **chapterContent** |
| **name** | **Growing bacteria from a frozen stock** |
| **templateId** | **chapterContent** |
| **name** | **Growing liquid cultures of bacteria from a single colony** |
| **templateId** | **chapterContent** |
| **name** | **Preparing a culture without Tween80 from one with Tween80** |
| **templateId** | **chapterContent** |
| **name** | **Preparing frozen stocks of bacteria** |
| **templateId** | **chapterContent** |
| **name** | **Preparing Additional Phage Lysate & Long-term Storage** |
| **templateId** | **chapterContent** |
| **name** | **Received Biologicals from the Program? Follow these Next Steps.** |

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# **Growing bacteria from a frozen stock**

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| **content** | |  |  | | --- | --- | | **Growing bacteria from a frozen stock (Streak Plate)** | | |  | | | **To Prepare** | Note: A video demonstrating this protocol is available at [**here**](https://seaphages.org/video/54/).     1. Refer to Host Basics in the Phage Discvery Guide for specific guidelines when working with a particular bacterial host. In particular, take note of the appropriate media (agar) and incubation temperature. 2. Label an appropriate agar plate. 3. Once you are ready to streak a plate, obtain your frozen stock from the freezer and complete Step 4 quickly to minimize the amount of thawing of your frozen stock. 4. Using an inoculation loop or sterile wooden stick, scrape the surface of a frozen stock, and immediately return the stock to the freezer. 5. Aseptically spread the bacteria at the end of the loop or wooden stick onto the agar plate by streaking for single colonies. 6. Incubate for 2 – 3 days at the appropriate temperature. 7. Monitor growth over time to ensure bacterial growth parameters are as expected (e.g. time for colony formation and color/texture of colonies. 8. Once single colonies are visible and large enough for picking (see image of plate in “Host Basics” of the Phage Discovery Guide), plates should be stored either at room temperature or at 4 ˚C. To prevent over drying, the plates should be wrapped in parafilm. | |  | | | **To Store** | At room temperature or at 4°C. | |  | | | **Usage** | In Protocol: Streak plate | |  | | | **Notes** | 1. This is your P0FF (passage 0 from frozen) stock. Depending on the host bacteria, this stock culture can be stored at room temperature or at 4°C for several weeks. We recommend storage for no more than a couple of weeks. 2. We suggest that you start P0FF stock cultures every week or every other week. | |

# **Growing liquid cultures of bacteria from a single colony**

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| **content** | |  |  | | --- | --- | | **Growing liquid cultures of bacteria from a single colony** | | |  | | | **To Prepare** | Note: A video demonstrating this protocol is available at [**here**](https://seaphages.org/video/56/).     1. Refer to Host Basics in the Phage Discovery Guide for specific guidelines when working with a particular bacterial host. In particular, take note of the appropriate media (liquid) and incubation temperature. 2. Add 50 mL of the appropriate liquid medium to a 250-mL baffled flask. If using, add antibiotics or antimicrobials. 3. Using an inoculation loop or sterile wooden stick, pick a small piece from the middle of a single colony of bacteria on an agar plate. Avoid using colonies from plates older than 2 weeks. 4. Aseptically transfer the bacteria from the loop or wooden stick into the medium in the flask. 5. **Tween80 should be added to 0.05% when culturing bacteria that tend to clump (e.g. *M. smegmatis* mc2155).** Cultures prepared with Tween80 must be subcultured to remove Tween80. See [instructions for subculturing from cultures prepared with Tween80.](#_hhsh3yxflam7) 6. Incubate, with shaking (250 rpm), at the recommended temperature. 7. Monitor growth over time to ensure culture growth parameters are as expected (e.g. duration before culture is expected to become saturated) 8. Once the culture is saturated, store at room temperature.   Important: Whenever a liquid culture of bacteria is prepared, the culture should be tested as follows:   * Prepare a streak plate from the culture, and monitor for the expected growth rate and colony morphology of the bacterial strain. If the growth rate or colony morphology is not as expected, the culture may be contaminated. * Prepare a top agar lawn using an aliquot of the bacterial culture, and test the ability of a host-specific phage to infect the top agar lawn using a spot test Protocol 5.6. If the host-specific phage is unable to infect the bacterial lawn, the bacterial culture may be contaminated. | |  | | | **To Store** | At room temperature or at 4°C, for 1 – 2 weeks. | |  | | | **Usage** | In Protocol:   * Growing liquid cultures for use in phage experiments | |  | | | **Notes** | 1. This is your P1FF (passage 1 from frozen) stock. This stock culture can be stored at room temperature, the duration for which will depend on the bacterium. Check Host Basics in this Guide for information about a specific bacterium. 2. It can be aseptically aliquoted into smaller volumes for storage (this minimizes the potential for contamination). 3. We recommend that you start P1FF stock cultures weekly, depending on use and the size of a given class. Regardless, because of the possibility of contamination, a backup P1FF culture should be available at all times. 4. Single colonies from an agar plate can be used to inoculate multiple P1FF cultures for as long as the plate remains uncontaminated. 5. If your PIFF does **NOT** contain Tween80, this culture can be used for experiments with phage. 6. Tween80 is added to cultures for some bacteria to minimize clumping during bacterial growth. **The presence of Tween80, however, will likely inhibit phage infection. For this reason:** To grow liquid cultures for use in phage experiments, sub-inoculate from a P1FF culture into liquid media without Tween80. For instructions, see the recipe card “Growing liquid cultures of bacteria for use in phage experiments.” | |

# **Preparing a culture without Tween80 from one with Tween80**

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| **content** | |  |  | | --- | --- | | **Preparing a culture without Tween80 from one with Tween80** | | |  | | | **To Prepare** | Note: This protocol assumes a saturated liquid culture has been prepared, and contains Tween80.     1. Add 50 mL of the appropriate liquid media (i.e., without Tween80) to a 250-mL baffled flask. For larger volumes, adjust accordingly (see notes). If using, add antibiotics or antimicrobials. 2. Aseptically, add a 1:1000 volume of the saturated culture containing Tween80. For example, for a 50-mL culture, add 50-µL of your culture that contains Tween80. 3. Incubate, with shaking (250 rpm), at the recommended temperature. | |  | | | **To Store** | At room temperature or at 4 °C. | |  | | | **Usage** | In Protocol: 5.2 - 7.2 | |  | | | **Notes** | Note: A culture prepared from a single colony is known as a P1FF. A new culture prepared from a PIFF is known as a P2FF.     1. The addition of small (≤1:1000) volumes of the PIFF culture (containing Tween80) when preparing a sub-culture without Tween80 reduces the likelihood of bacterial clumping in subculture. If clumping does occur, continue incubating the subculture up to an additional 48 hours. 2. This culture can be stored at room temperature, the duration for which will depend on the bacterium. Check Host Basics in this guide for information about a specific bacterium. 3. To prepare additional cultures without Tween80, it is best to go back to the initial P1FF stock culture, so long as the P1FF culture is not contaminated, is not clumpy, or has not been stored for beyond the recommended time (see Host Basics in this guide) 4. The volume of a culture should be approximately one-fifth the size of the flask. For example, for 200 mL of bacterial culture, use a 1-L baffled flask. This maximizes aeration when the bacteria are grown on a shaker. 5. Tween80 will inhibit phage infection. It is advisable to double-check that any medium that will be used in phage experiments does not include Tween80. | |

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# **Preparing frozen stocks of bacteria**

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| **content** | |  |  | | --- | --- | | **Preparing frozen stocks of bacteria** | | |  | | | **To Prepare** | Note: A video demonstrating this protocol is available at [**here**](https://seaphages.org/video/57/).     1. Label the appropriate number of cryo-tubes with the name of the bacteria, the date of preparation, and initials of the person preparing the stock. 2. Add 300 µL of 40 % glycerol to each tube. 3. Add 700 µL of bacterial culture to each tube. 4. Mix by inversion. 5. Store at -80 °C. | |  | | | **To Store** | at -80 °C, indefinitely. | |  | | | **Notes** | 1. To use frozen bacterial stocks, refer to the recipe card “Growing bacterial cultures from frozen a stock.” 2. It is important that only one person access any given glycerol stock. When a glycerol stock is first used, it should be labelled with the name of the person who is using it. | |

# **Preparing Additional Phage Lysate & Long-term Storage**

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| **content** | |  |  | | --- | --- | | **Preparing Additional Phage Lysate & Long-term Storage** | | |  | | | **To Prepare** | 1. First, determine the titer of your phage lysate by performing a full plate titer (Protocol 6.5). 2. Based on the calculated titer, prepare webbed plates (Protocol 7.1). *Note: You can flood those plates from Step.1 that are webbed or have very high densities of plaques. This lysate should also be titered as part of Step.4.* 3. Prepare additional lysate by flooding the webbed plates (Protocol 6.3). Proceed to Step 4, ideally as soon as Protocol 6.3 is complete. 4. Determine the titer of the freshly prepared phage lysate by performing a full plate titer (Protocol 6.5). Ideally, the titer of the lysate will be at 109 pfu/ml or greater. If the titer is below 109 pfu/ml, return to Step 2 and attempt the following modification: When webbed plates have been identified, incubate those webbed plates for an additional 24 - 48 hrs such that the bacteria remaining within the 'webbing' have also been lysed by phage before proceeding to Step 3. 5. Next: 6. **To prepare freezer stocks**, add 1 ml of the high-titer phage lysate to a labeled cryotube, then add 70 µL of DMSO. Mix by inversion and store at -80 °C. Ideally, 3 - 4 freezer stocks will be prepared. 7. **To maintain a working stock,** store the lysate at 4 °C. **Important**: The stability of phage particles in lysate stored at 4 °C will vary from phage to phage. Generally, phage stability parallels the titer, with phages more stable at higher titers. Regardless, the titer of phage lysates can drop from one day to another, or remain stable for weeks. It is therefore important to recalculate the titer of your lysate periodically. 8. **To archive phage lysates,** refer to the archiving protocol (Protocol 7.3). **Note:** You will receive bar-coded cryo-tubes and DMSO from the program in the fall of Year 1. All samples for archiving at the University of Pittsburgh should be prepared as described in the Phage Discovery Guide. | |  | | | **To Store** | Freezer stocks -80 °C, indefinitely. Working stocks at 4 °C, with the titer checked periodically (e.g. biweekly) | |  | | | **Notes** | 1. To use frozen phage lysates, scrape a few microliters from the surface of the frozen lysate using a sterile micropipette tip, and immediately return the stock to the freezer to prevent thawing of the frozen stock. Repeated freeze-thaw cycles can damage phage particles. 2. Transfer the scraped lysate to a microcentrifuge tube pre-filled with 100 µL of phage buffer. 3. The resuspension can then be used to prepare a working stock of phage lysate. 4. Avoid thawing the frozen stock. | |

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# **Received Biologicals from the Program? Follow these Next Steps.**

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| **content** | |  |  | | --- | --- | | **Received Biologicals from the Program? Follow these Next Steps.** | | |  | | | **Bacteria** | Bacterial strains are typically shipped as glycerol/freezer stocks to program members, and are intended for use in preparing additional freezer stocks for long-term storage.  Upon receiving these glycerol/freezer stocks, store these stocks at -80 °C.    To prepare additional glycerol/freezer stocks:   1. Streak out bacteria to obtain single colonies. Follow the instructions provided in ''[Growing bacteria from a frozen stock](#_geztkxm6nfck)''. 2. Within days of obtaining single colonies, prepare a liquid culture of the bacterial strain. Follow the instructions provided in '['Growling liquid stock of cultures from a single colony](#_bobu0vfovern)''. 3. Once the liquid culture is saturated, prepare additional frozen stocks following the instructions provided in ''[Preparing frozen stocks of bacteria](#_jmyhhejerih)''.     Important: Whenever a liquid culture of bacteria is prepared, the culture should be tested as follows:   * Prepare a streak plate from the culture, and monitor for the expected growth rate and colony morphology of the bacterial strain. If the growth rate or colony morphology is not as expected, the culture may be contaminated. * Prepare a top agar lawn using an aliquot of the bacterial culture, and test the ability of a host-specific phage to infect the top agar lawn using a spot test Protocol 5.6. If the host-specific phage is unable to infect the bacterial lawn, the bacterial culture may be contaminated. | |  | | | **Phage** | Phage are typically shipped as working stocks (i.e. the stock lacks a cryo-protectant and should not be placed in a freezer). Upon receiving phage lysate from the program, store the lysate at 4 °C. Within weeks of receiving the lysate, prepare additional lysate for long-term storage and for use in research following instruction provided in ''[Preparing Additional Phage Lysate & Long-term Storage](#_uenprs70iuu6)''. | |

# **Notes**

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# **Notes About Recipes:**

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| **Content** | Notes About Recipes:About ddH2O Water should be double-distilled at a minimum. Filtered water (e.g., Millipore) is ideal. Regardless of water availability, consistency of preparation is essential (i.e., always use the same water).   About Labeling Clearly label all media and solutions, and date the additions of supplements. Sterile solutions should also be clearly labeled as such, and any autoclave tape (indicating sterility) should be left on the bottles.   About Adjusting the pH of Solutions Be sure to check each recipe for the proper acid or base solution that should be used to adjust the pH (if needed) of each solution or reagent. |

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# **Autoclave Setting**

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| **Content** | When using an autoclave, selecting the correct settings is crucial. Cycle Type: Most autoclaves are equipped with 3 settings: Liquid, Wrapped (or Vacuum), andUnwrapped (or Gravity).   * **Liquid Cycle**: When sterilizing liquids by autoclave, the LiquidCycle should be selected. The Liquid Cycle is programmed to release the chamber pressure slowly at the endof the cycle, which prevents your super-heated liquids from rapidly bubbling over. * **Wrapped (or Vacuum) Cycle**: Most consumables and glassware used,as well as biohazard waste generated, should be sterilized in an autoclave using the Wrapped (or Vacuum)Cycle. This cycle mechanically replaces the air in the chamber with steam, which allows the steam to accessareas where air may otherwise be trapped (e.g. pipette tip boxes). Note that this cycle is notpre-programmed to release chamber pressure slowly, and should not be used for liquids. * **Unwrapped (or Gravity) Cycle**:Unwrapped utensils can be sterilized using this cycle. Unlike the Wrapped Cycle, the Unwrapped cycleuses gravity to displace the air in the chamber. As such, air trapped in containers (e.g. pipette tipboxes) may not be replaced with steam, limiting sterilization.    Cycle temperature and duration:  * **Liquids:** Liquids should be autoclaved at 121 ˚C (250 ˚F). The cycle duration varies depending on the amount of liquid being autoclaved. As a rule, 500 ml of liquid should be autoclaved for 40 mins. An extra5 minutes should be added to the overall duration for each additional 500 ml of liquid. * **Non-liquids:** Most consumables and glassware used, as well as biohazard waste generated, should be sterilized in an autoclave set at 121 ˚C (250 ˚F) for 20 mins. |

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