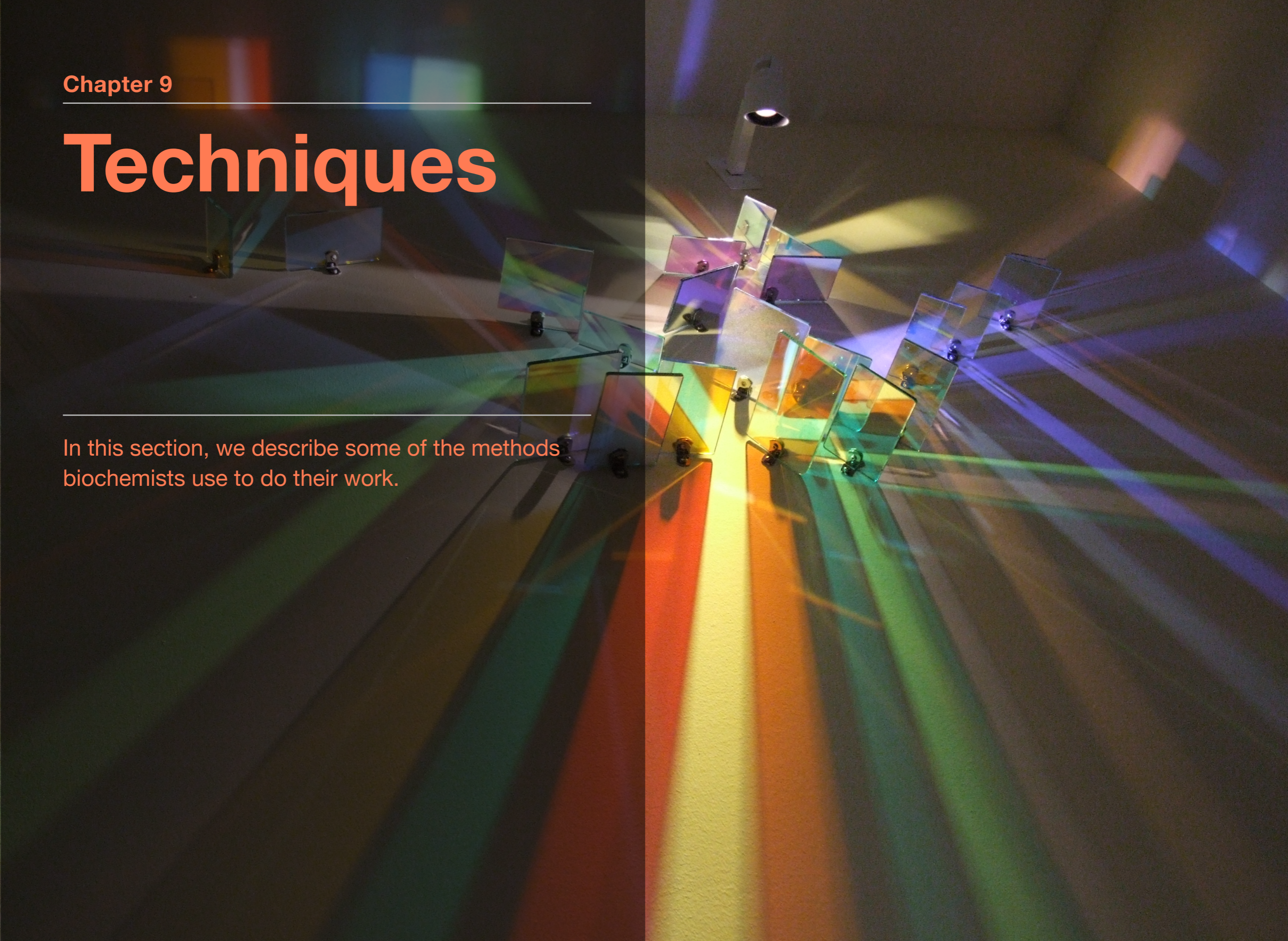


Techniques

In this section, we describe some of the methods biochemists use to do their work.



Techniques



introduction

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Introduction

The environment of a cell is very complex, making it very difficult, if not impossible, to study individual reactions, enzymes, or pathways within it. For this reason, biochemists prefer to isolate molecules (enzymes, DNAs, RNAs, and other molecules of interest) so they can be analyzed without interference from the millions of other processes occurring simultaneously in the cell. Many of the methods used in isolating molecules from cells involve some form of chromatography. To separate compounds from their cellular environments, one must first break open (lyse) the cells.

Cell Disruption

There are several ways to break open cells. Lysis methods include lowering the ionic strength of the medium cells are kept in. This can cause cells to swell and burst. Mild surfactants may be used to enhance the efficiency of lysis. Most bacteria, yeast, and plant tissues, which have cell walls, are resistant to such osmotic shocks, however, and stronger disruption techniques are often required.

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Enzymes may be useful in helping to degrade the cell walls. Lysozyme, for example, is very useful for breaking down bacterial walls. Other enzymes commonly employed include cellulase (plants), glycanases, proteases, mannases, and others.

Mechanical agitation may be employed in the form of tiny beads that are shaken with a suspension of cells. As the beads bombard the cells at high speed, they break them open. Sonication (20-50 kHz sound waves) provides an alternative method for lysing cells. The method is noisy, however, and generates heat that can be problematic for heat-sensitive compounds.

Another means of disrupting cells involves using a “cell bomb”. In this method, cells are placed under very high pressure (up to 25,000 psi). When the pressure is released, the rapid pressure change causes dissolved gases in cells to be released as bubbles which, in turn, break open the cells.

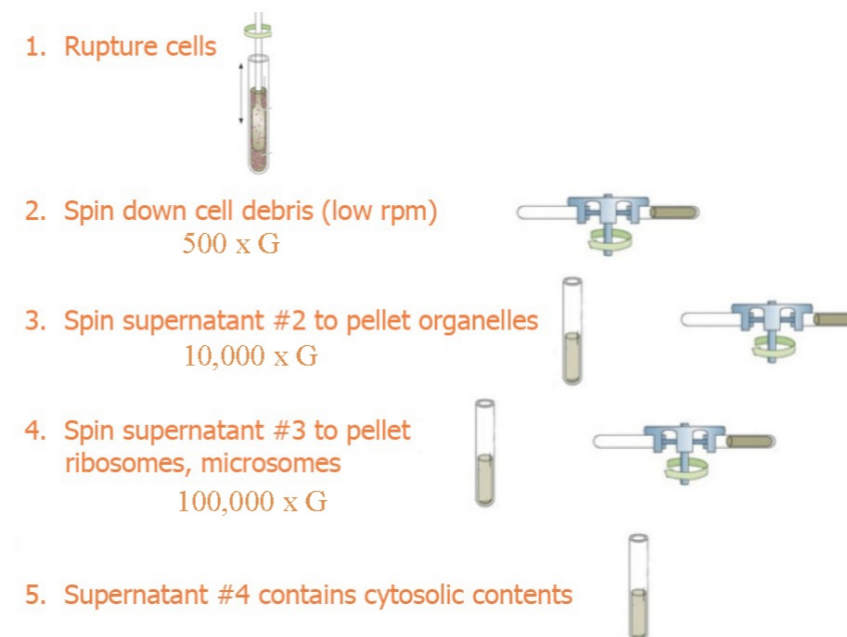
Cryopulverization is often employed for samples having a tough extracellular matrix, such as connective tissue or seeds. In this technique, tissues are flash-frozen using liquid nitrogen and then ground to a fine powder before extraction of cell contents with a buffer.

Whatever method is employed, the crude lysates obtained contain all of the molecules in the cell, and thus, must be further

processed to separate the molecules into smaller subsets, or fractions.

Fractionation

Fractionation of samples typically starts with centrifugation. Using a centrifuge, one can remove cell debris, and fractionate organelles, and cytoplasm. For example, nuclei, being relatively large, can be spun down at fairly low speeds. Once nuclei have been sedimented, the remaining solution, or supernatant, can be centrifuged at higher speeds to obtain the smaller organelles, like mitochondria. Each of these fractions will contain a subset of the



Fractionation by Centrifugation

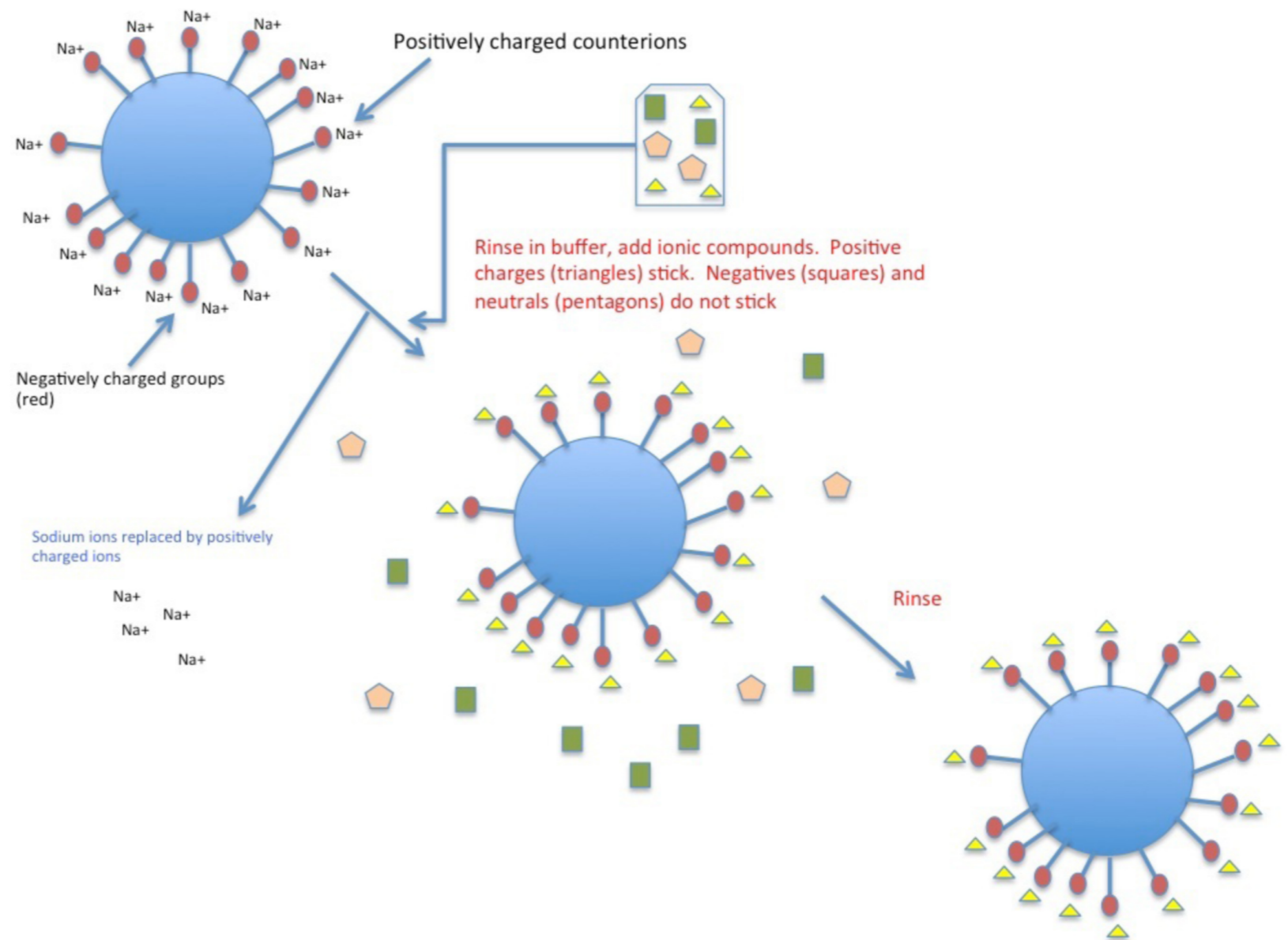
molecules in the cell. Although every subset contains fewer molecules than does the crude lysate, there are still many hundreds of molecules in each. Separating the molecule of interest from the others is where chromatography comes into play. We will consider several separation techniques.

Many chromatographic techniques are performed in “columns.” These are tubes containing the material (called the “support”) used to perform the separation. Supports are designed to exploit the chemical, or size, differences of the many molecules in a mixture. Columns are “packed” (filled) with the support and a buffer or solvent carries the mixture of compounds to be separated through the support. Molecules in the sample interact differentially with the support and consequently, will travel through it with different speeds.

Ion Exchange Chromatography

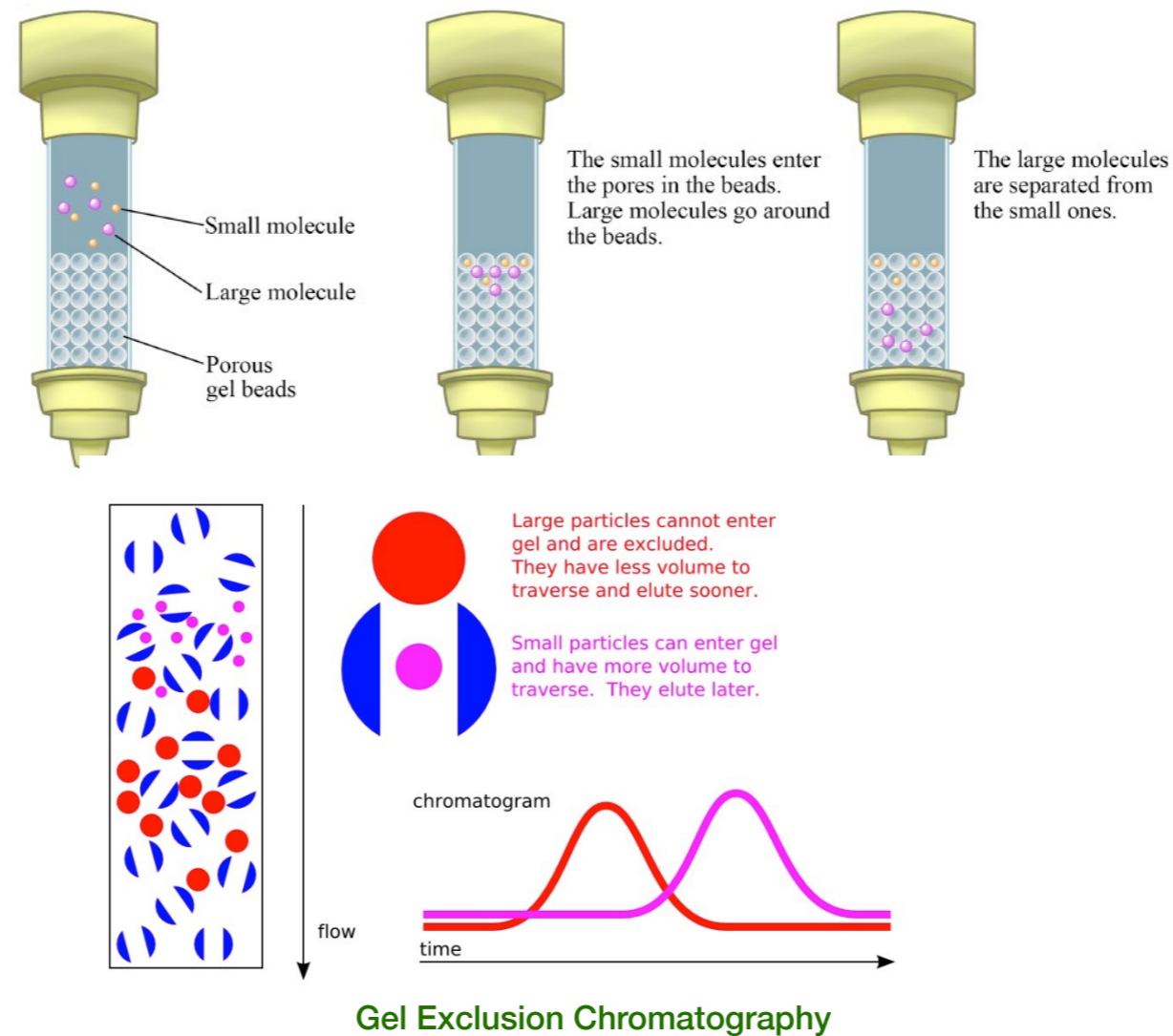
In ion exchange chromatography,

the support consists of tiny beads to which are attached chemicals possessing a charge. Each charged molecule has a counter-ion. The figure shows the beads (blue) with negatively charged groups (red) attached. In this example, the counter-ion is sodium, which is positively charged. The negatively charged



Cation Exchange Chromatography

groups are unable to leave the beads, due to their covalent attachment, but the counter-ions can be “exchanged” for molecules of the same charge. Thus, a cation exchange column will have positively charged counter-ions and positively charged compounds present in a mixture passed through the column will exchange with the counter-ions and “stick” to the negatively charged groups on the beads. Molecules in the sample that are neutral or negatively charged will pass quickly through the column. On the other hand, in anion exchange chromatography, the chemical groups attached to the beads are positively charged and the counter-ions are negatively charged. Molecules in the sample that are negatively charged will “stick” and other molecules will pass through quickly. To remove the molecules “stuck” to a column, one simply needs to add a high concentration of the appropriate counter-ions to displace and release them. This method allows the recovery of all components of the mixture that share the same charge.



Gel Exclusion Chromatography

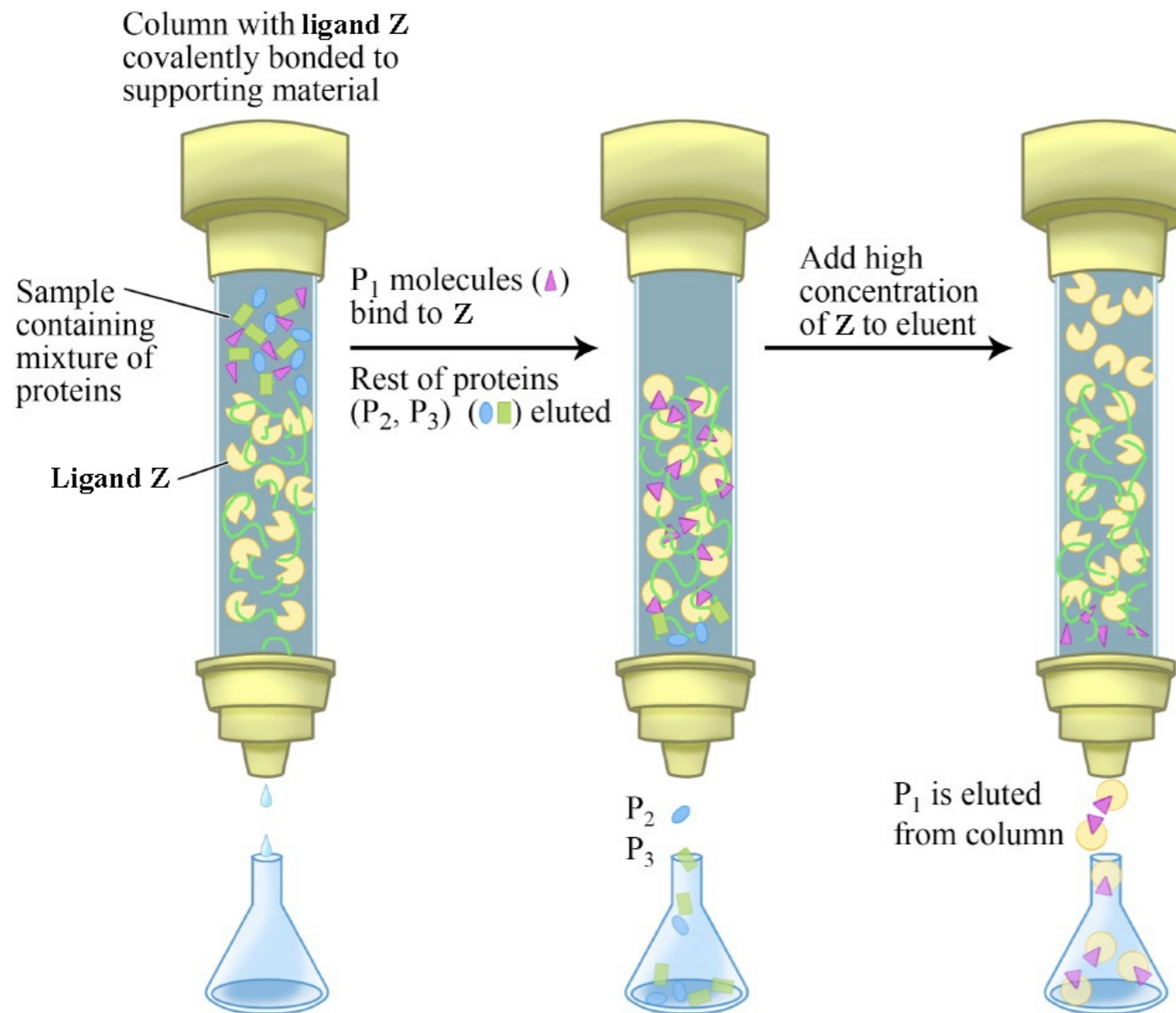
<https://en.wikipedia.org/wiki/File:SizeExChrom.png>

weight will not fit into the tunnels. Molecules with sizes larger than the exclusion limit do not enter the tunnels and pass through the column relatively quickly by making their way between the beads. Smaller molecules, which can enter the tunnels, do so, and thus, have a longer path

Gel Exclusion Chromatography

Gel exclusion chromatography (also called molecular exclusion chromatography, size exclusion chromatography, or gel filtration chromatography) is a low resolution isolation method that employs a cool “trick.” This involves the use of beads that have tiny “tunnels” in them that each have a precise size. The size is referred to as an “exclusion limit,” which means that molecules above a certain molecular

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Affinity Chromatography

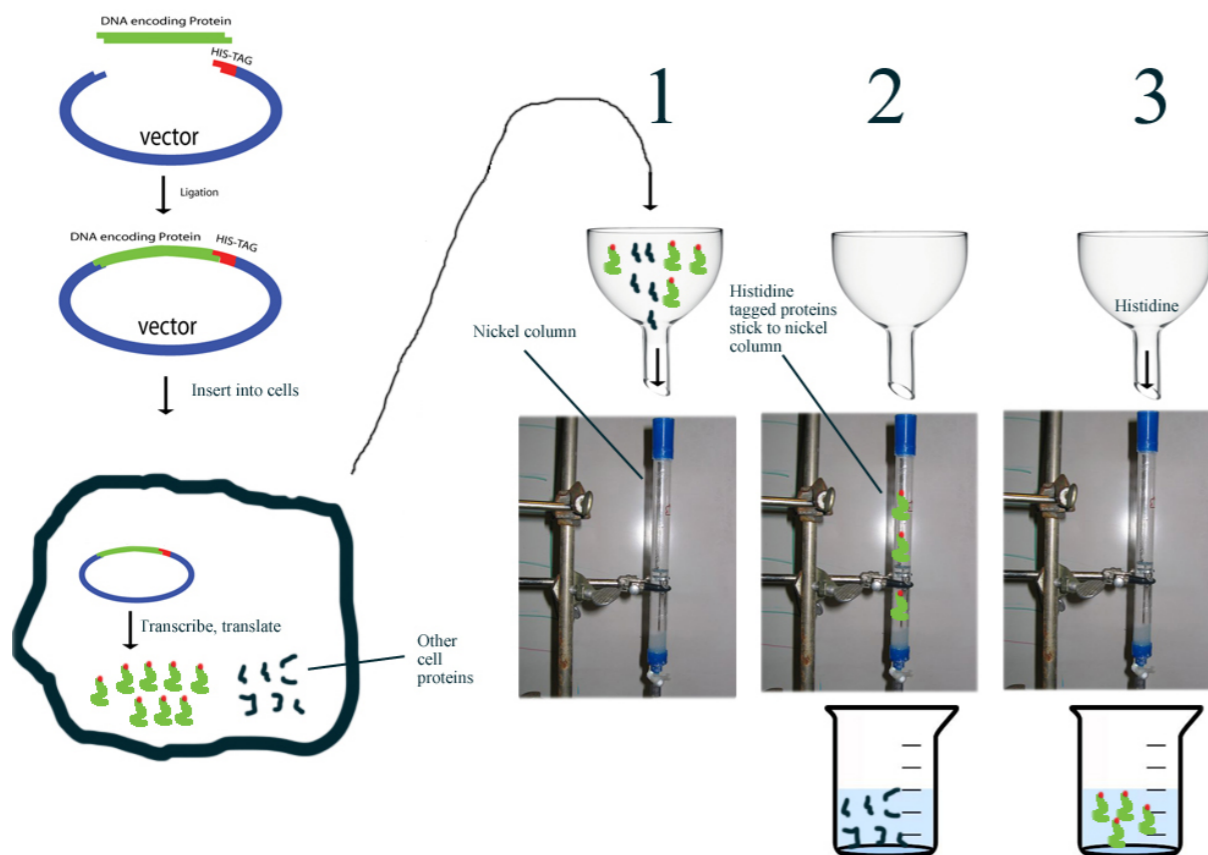
that they take in passing through the column. Because of this, molecules larger than the exclusion limit will leave the column earlier, while those that pass through the beads will elute from the column later. This method allows separation of molecules by their size.

Affinity Chromatography

Affinity chromatography is a very powerful technique that exploits the binding affinities of target molecules (typically proteins) for substances covalently linked to beads. For example, if one wanted to separate all of the proteins in a sample that bound to ATP from proteins that do not bind ATP, one could covalently link ATP to support beads and then pass the sample through column. All proteins that bind ATP will “stick” to the column, whereas those that do not bind ATP will pass quickly through it. The proteins adhering to the column may then released from the column by adding ATP.

High Performance Liquid Chromatography (HPLC)

HPLC (also sometimes called High Pressure Liquid Chromatography) is a powerful tool for separating smaller molecules based on their differential polarities. It employs columns with supports made of very tiny beads that are so tightly packed that flow of solvents/buffers through the columns requires the application of high pressures (hence the name). The supports used can be polar (normal phase separation) or non-polar (reverse phase separation). In normal phase separations, non-polar molecules elute first followed by the more polar compounds. This order is



Histidine Tagging

switched in reverse phase chromatography. Of the two, reverse phase is much more commonly employed to due more reproducible chromatographic profiles (separations) that it typically produces.

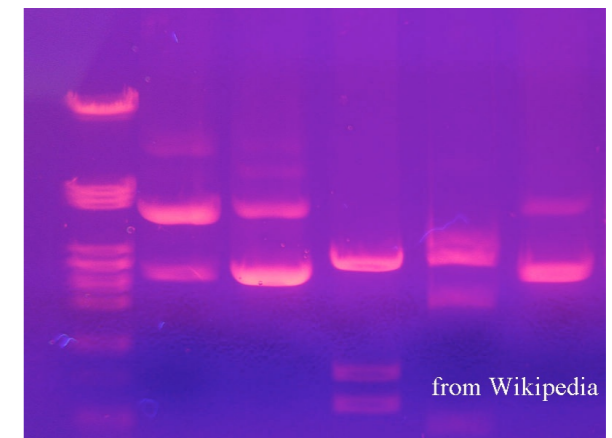
Histidine Tagging

Histidine tagging is a powerful tool for isolating a recombinant protein from a cell lysate. It relies on using recombinant DNA techniques to add codons specifying a series of histidines

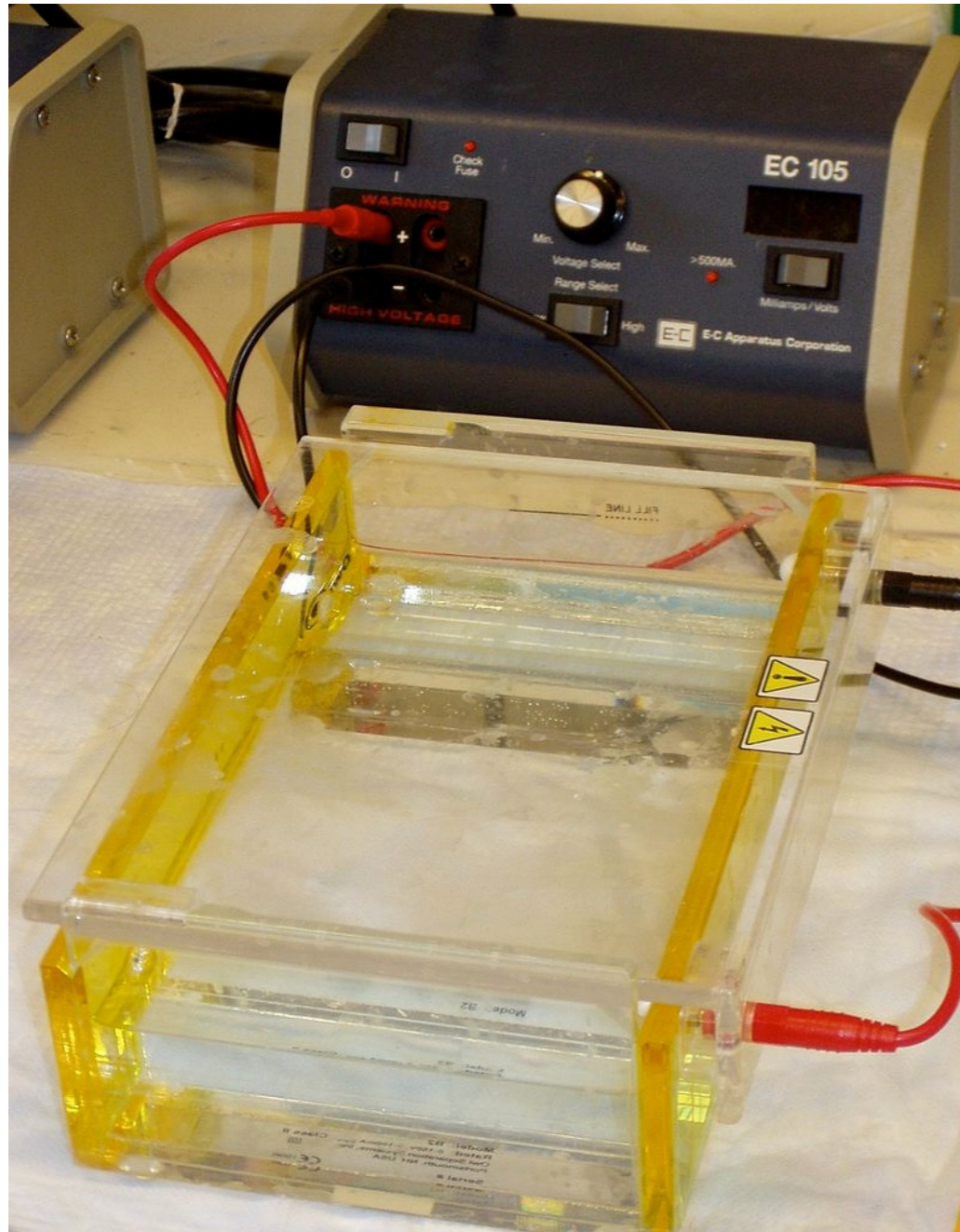
(usually six) to the coding sequence for a protein. The protein produced when this gene is expressed has a run of histidine residues fused at either the carboxyl or amino terminus to the amino acids in the remainder of the protein. The histidine side chains of this “tag” have an affinity for nickel or cobalt ions, making separation of histidine-tagged proteins from a cell lysate is relatively easy. Simply passing the sample through a column that has immobilized nickel or cobalt ions allows the histidine-tagged proteins to “stick,” while the remaining cell proteins all pass quickly through. The histidine-tagged proteins are then eluted by addition of imidazole (which is chemically identical to the histidine side chain) to the column. Histidine tags can be cleaved off using endopeptidases.

Electrophoresis

DNA molecules are long and loaded with negative charges, thanks to their phosphate backbones. Electrophoretic methods separate large molecules, such as DNA, RNA, and proteins based on their charge and size. For DNA and RNA, the charge of the nucleic acid is proportional to its size (length). For proteins, which do not have a



DNA Fragments Separated by Agarose Gel Electrophoresis



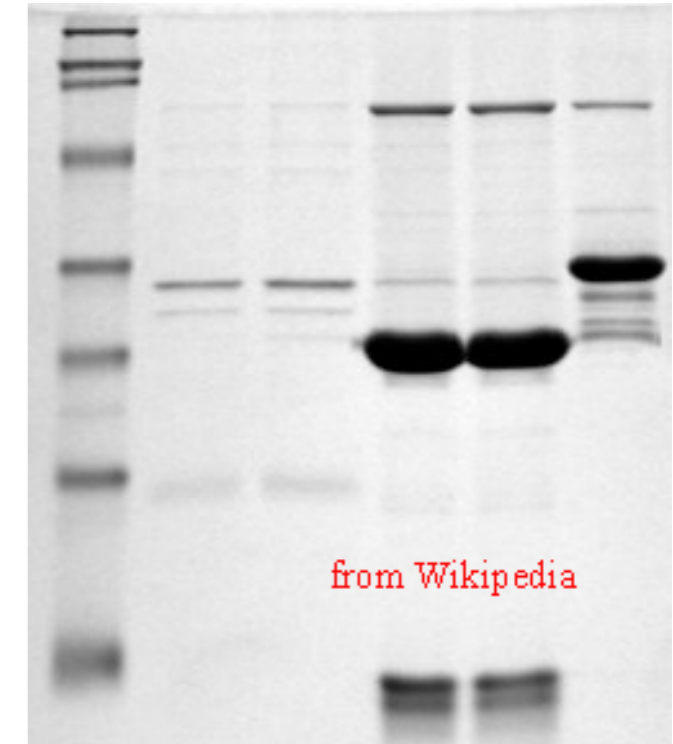
Agarose Gel Electrophoresis

uniform charge, a clever trick is employed to make them mimic nucleic acids.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method for separating nucleic acids. It is worth noting that nucleic acids are the largest molecules found in cells, in some cases by orders of magnitude. Agarose provides a matrix which encases a buffer. The matrix provides openings for macromolecules to move through and the largest macromolecules have the most difficult time navigating, whereas the smallest

macromolecules slip through it the easiest. Unlike column chromatography, electrophoresis uses an electric current as a force to drive the molecules through the matrix. Since the size to charge ratios for DNA and RNA are constant for all sizes of these nucleic acids, the size per force is also constant (since force is directly proportional to charge), so the molecules simply sort on the basis of their size - the smallest move fastest and the largest move slowest. Visualization of the DNA fragments in the gel is

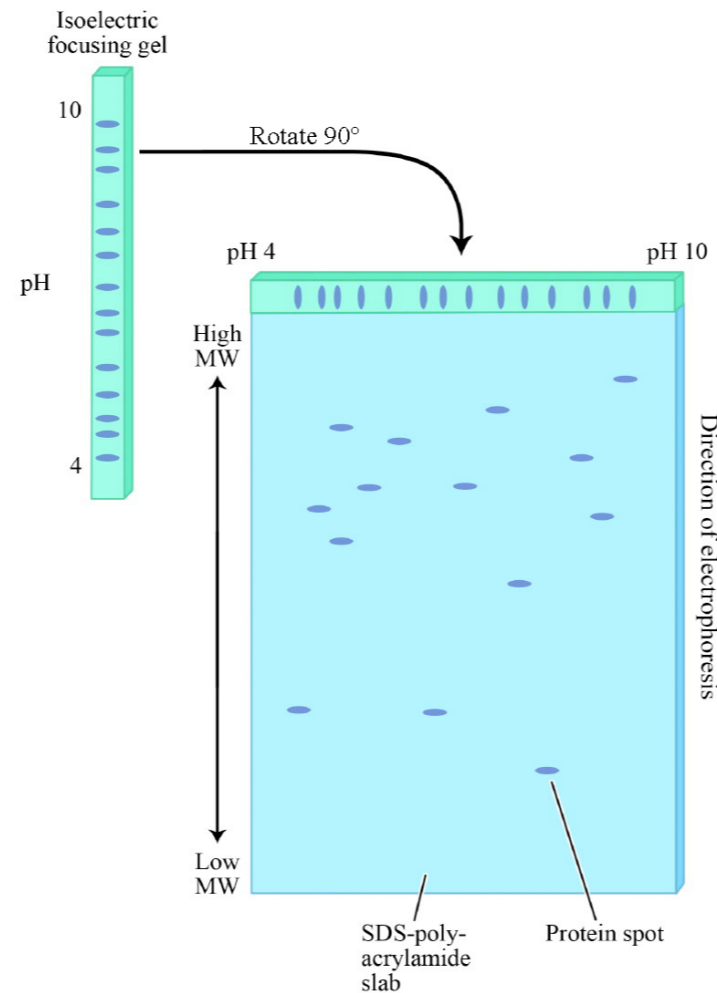


SDS-PAGE Separation of Proteins

made possible by addition of a dye, such as ethidium bromide that fluoresces under ultraviolet light.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Like DNA and RNA, proteins are large macromolecules. Proteins, however, vary tremendously in their charge. Whereas double-stranded DNA is rod-shaped, most proteins are globular (folded up). Further, proteins are considerably smaller than nucleic acids, so the openings of the matrix of the agarose gel are simply too large to effectively provide separation. Consequently, unlike nucleic acids, proteins cannot be effectively separated by electrophoresis on agarose gels. To separate proteins by electrophoresis, one must make several modifications. First, a matrix made by polymerizing and crosslinking acrylamide units is employed. One can adjust the pore size of the matrix readily by changing the percentage of acrylamide in the gel. Higher percentages of acrylamide create smaller pores and are more effective in separating smaller molecules,



2D Gel Electrophoresis

whereas lower percentages of acrylamide reverse that. Second, proteins must be physically altered to “present” themselves to the matrix like the negatively charged rods of DNA. This is accomplished by treating the proteins with the detergent called SDS (sodium dodecyl sulfate). SDS denatures the proteins so they assume a rod-like shape and the SDS molecules coat the proteins such that the exterior surface is loaded with negative charges proportional to the mass, just like the backbone of DNA. Third, a “stacking gel” may be employed at the top of the gel to provide a way of compressing the samples into a tight band before they enter the main polyacrylamide gel (called the resolving gel). Just as DNA fragments get sorted on the basis of size (largest move slowest and smallest move fastest), the proteins migrate through the gel matrix at rates inversely related to their size. Upon completion of the electrophoresis, there are several means of staining to visualize the proteins on the gel. They include

reagents, such as Coomassie Brilliant Blue or silver nitrate (the latter is much more sensitive than Coomassie Blue staining and can be used when there are very small quantities of protein).

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Isoelectric Focusing

Proteins vary considerably in their charges and, consequently, in their pI values (pH at which their charge is zero). Separating proteins by isoelectric focusing requires establishment of a pH gradient in an acrylamide gel matrix. The matrix's pores are adjusted to be large to reduce the effect of sieving based on size. Molecules to be focused are applied to the gel with the pH gradient and an electric current is passed through it. Positively charged molecules, for example, move towards the negative electrode, but since they are traveling through a pH gradient, as they pass through it, they reach a region where their charge is zero and, at that point, they stop moving. They are at that point attracted to neither the positive nor the negative electrode and are thus "focused" at their pI. By using isoelectric focusing, it is possible to separate proteins whose pI values differ by as little as 0.01 units.

2D Gel Electrophoresis

Both SDS-PAGE and isoelectric focusing are powerful techniques, but a clever combination of the two is a powerful tool of proteomics - the science of studying all of the proteins of a cell/tissue simultaneously. In 2D gel electrophoresis, an extract containing the proteins is first prepared. One might, for example, be studying the proteins of liver tissue. The liver cells are lysed and all of the proteins are collected into a sample. Next, the sample is subjected to isoelectric focusing as described earlier, to

separate the proteins by their pI values. Next, as shown on the previous page, the isoelectric gel containing the separated proteins is rotated through 90° and placed on top of a regular polyacrylamide gel for SDS-PAGE analysis (to separate them based on size). The proteins in the isoelectric gel matrix are electrophoresed into the polyacrylamide gel and separation on the basis of size is performed. The product of this analysis is a 2D gel, in which proteins are sorted by both mass and charge.

The power of 2D gel electrophoresis is that virtually every protein in a cell can be separated and appear on the gel as a distinct spot. In the figure, spots in the upper left correspond to large positively charged proteins, whereas those in the lower right are small negatively charged ones. It is possible using high-throughput mass spectrometry analysis to identify every spot on a 2D gel. This is particularly powerful when one compares protein profiles between different tissues or between the samples of the same tissue treated or untreated with a particular drug.

Cleavage Reagent

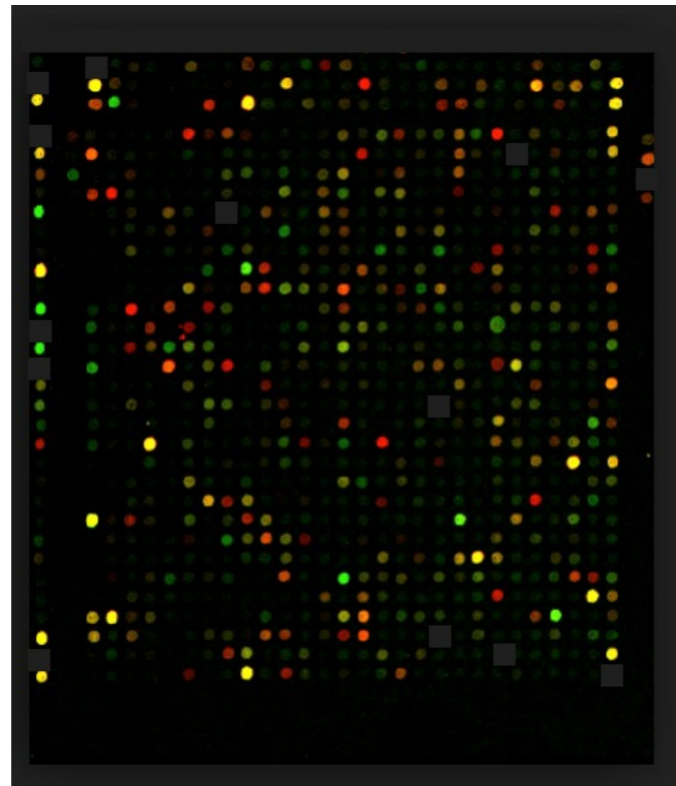
1. Cyanogen bromide
2. Hydroxylamine
3. 2-Nitro-5-thiocyanobenzoate
4. Trypsin
5. Chymotrypsin
6. Thrombin
7. Elastase

Specificity

COOH side of MET
ASP-GLY bonds
NH₂ side of CYS
COOH side of LYS or ARG
COOH side of TYR, TRP, PHE, LEU, MET
COOH side of arginine
ALA-ALA or ALA-GLY bonds

Protein Cleaving Agents

Comparison of a 2D separation of a non-cancerous tissue with a cancerous tissue of the same type provides a quick identification of proteins whose level of expression differs between them. Information such as this might be useful in designing treatments or in determining the mechanisms by which the cancer arose.



Microarray

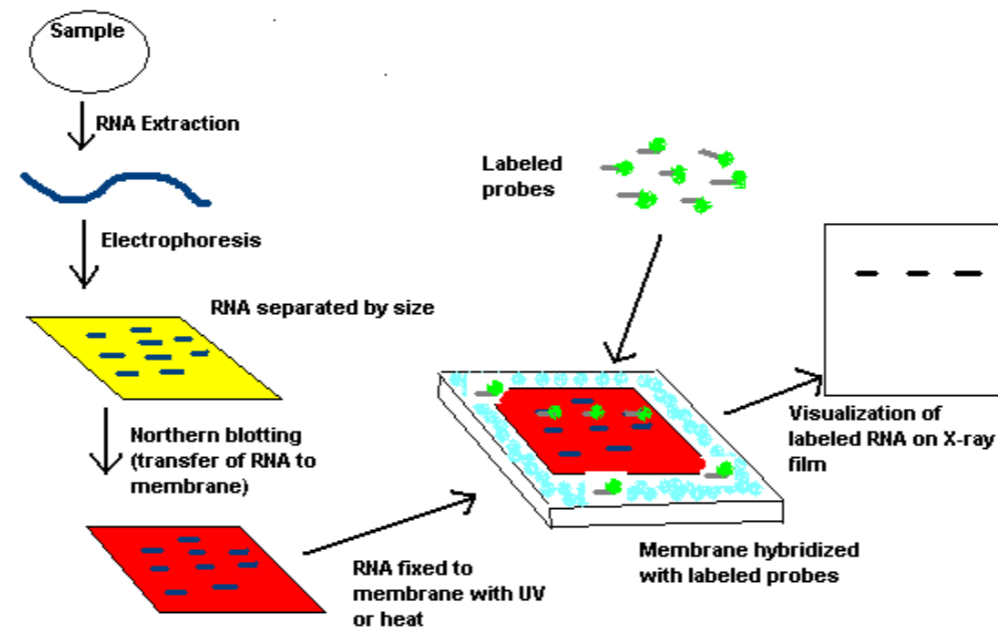
Protein Cleavage

Working with intact proteins in analytical techniques, such as mass spectrometry, can be problematic. Consequently, it is often desirable to break a large polypeptide down into smaller, more manageable pieces. There are two primary approaches to accomplishing this - use of chemical reagents or use of proteolytic enzymes. The table on the previous page shows the cutting specificities of various cleavage agents.

Microarrays

2D gels are one way of surveying a broad spectrum of molecules simultaneously. Other approaches to doing the same thing involve what are called microarrays. DNA microarrays, for example, can be used to determine all of the genes that are being expressed in a given tissue, simultaneously. Microarrays employ a grid (or array) made of rows and columns on a glass slide, with each box of the grid containing many copies of a specific molecule, say a single-stranded DNA molecule corresponding to the sequence of a single unique gene. As an example, consider scanning the human genome for all of the known mRNA sequences and then synthesizing single stranded DNAs complementary to each mRNA. Each complementary DNA sequence would have its own spot on the matrix. The position of each unique gene sequence on the grid is known and the entire grid would represent all possible genes that are expressed. Then for a simple gene expression analysis, one could take a tissue (say liver) and extract the mRNAs from it. These mRNAs represent all the genes that are being expressed in the liver at the time the extract was made. The mRNAs can easily be tagged with a colored dye (say blue). The mixture of tagged mRNAs is then added to the array and base-pairing conditions are created to allow complementary sequences to find each other. When the process is complete, each liver mRNA should have bound to its corresponding gene on the array, creating a blue spot in that box on the grid. Since it is known which genes are in which box, a

blue spot in a box indicates that the gene in that box was expressed in the liver. The presence and abundance of each mRNA may then readily determined by measuring the amount of blue dye at each box of the grid. A more powerful analysis could be performed with two sets of mRNAs, each with a different colored tag (say blue and yellow). One set of mRNAs could come from the liver of a vegetarian (tagged blue) and the other from a meat eater (tagged yellow), for example. The mRNAs are mixed and then added to the array and complementary sequences are once again allowed to form duplexes. After unhybridized mRNAs are washed away, the plate is analyzed. Blue spots in grid boxes correspond to mRNAs present in the vegetarian liver, but not in that of the meat eater. Green spots (blue plus yellow) would correspond to mRNAs present in equal abundance in the two livers. The intensity of each spot would also give information about the relative amounts of each mRNA in the tissues. Similar analyses could be done, using cDNAs instead of mRNA. Peptide microarrays have peptides bonded to the glass slide instead of DNA and can be



Northern Blotting Procedure

used to study the binding of proteins or other molecules to the peptides.

Blotting

Blotting provides a means of identifying specific molecules out of a mixture. It employs three main steps. First, the mixture of molecules is separated by gel electrophoresis. The mixture could

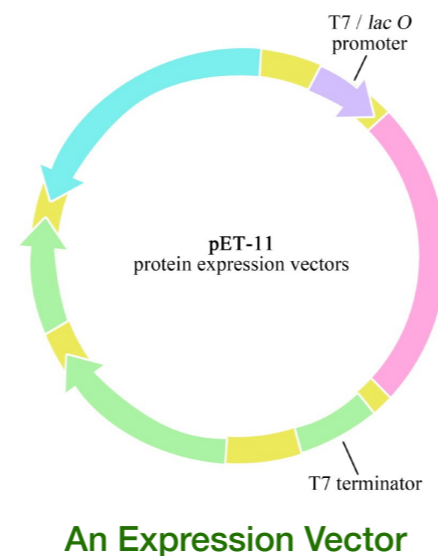
be DNA (Southern Blot), RNA (Northern Blot), or protein (Western Blot) and the gel could be agarose (for DNA/RNA) or polyacrylamide (for protein). Second, after the gel run is complete, the proteins or nucleic acids in the gel are transferred out of the gel onto a membrane/paper that physically binds to the molecules. This “blot”, as it is called, has an imprint of the bands of nucleic acid or protein that were in the gel (see figure at left).

The transfer can be accomplished by diffusion or by using an electrical current to move the molecules from the gel onto the membrane. The membrane may be treated to covalently link the bands to the surface of the blot. Last, a visualizing agent specific for the molecule of interest in the mixture is added to the membrane. For DNA/RNA, that might be

a complementary nucleic acid sequence that is labeled in some fashion (radioactivity or dye). For a protein, it would typically involve an antibody that specifically binds to the protein of interest. The bound antibody can then be targeted by another antibody specific for the first antibody. The secondary antibody is usually linked to an enzyme which, in the presence of the right reagent, catalyzes a reaction that produces a signal (color or light) indicating where the antibody is bound. If the molecule of interest is in the original mixture, it will “light” up and reveal itself.

Making Recombinant DNAs

Molecular biologists often create recombinant DNAs by joining together DNA fragments from different sources. One reason for making recombinant DNA molecules is to enable the production of a specific protein that is of interest. For example, it is possible to engineer a recombinant DNA molecule containing the gene for human growth hormone and introduce it into an organism like a bacterium or yeast, which could make massive quantities of the human growth hormone protein very cheaply. To do this, one needs to set up the proper conditions for the protein to be made in the target cells. For bacteria, this typically involves the use of plasmids. Plasmids are



circular, autonomously replicating DNAs found commonly in bacterial cells. Plasmids used in recombinant DNA methods 1) replicate in high numbers in the host cell; 2) carry markers that allow researchers to identify cells carrying them (antibiotic resistance, for example) and 3) contain sequences (such as a promoter and Shine Dalgarno sequence) necessary for expression of the desired protein in the target cell. A plasmid that has all of these features is referred to as an expression vector (see an example in the figure at left). Plasmids may be extracted from the host, and any gene of interest may be inserted into them, before returning them to the host cell. Making such recombinant plasmids is a relatively simple process. It involves 1) cutting the gene of interest with a restriction enzyme (endonucleases which cut at specific DNA sequences); 2) cutting the expression plasmid DNA with restriction enzyme, to generate ends that are compatible with the ends of the gene of interest; 3) joining the gene of interest to the plasmid DNA using DNA ligase; 4) introducing the recombinant plasmid into a bacterial cell; and 5) growing cells that contain the plasmid. The bacterial cells bearing the recombinant plasmid may then be induced to express the inserted gene and produce large quantities of the protein encoded by it.

Polymerase Chain Reaction (PCR)

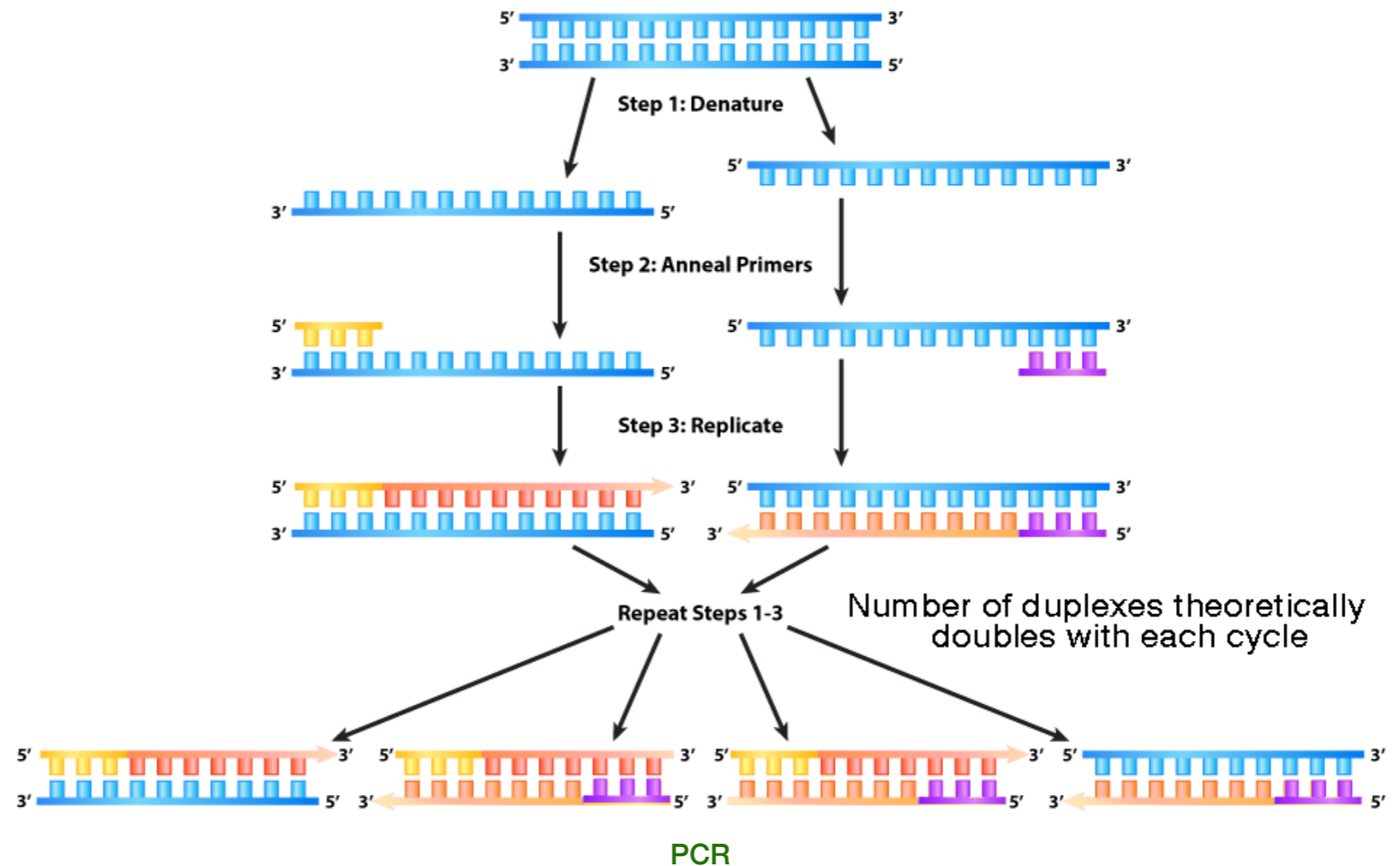
PCR allows one to use the power of DNA replication to obtain large amounts of a specific DNA in a short time. As everyone

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knows, cell division results in doubling the number of cells with each round of division. Each time cells divide, DNA must be replicated, as well, so

the amount of DNA is doubling as the cells are doubling. Kary Mullis recognized this fact and came up with the technique of PCR, which mimics DNA replication. In contrast to cellular DNA replication, which amplifies all of a cell's DNA during a replication cycle, PCR is used to replicate only a specific segment of DNA. This segment of DNA, known as the target sequence, is replicated repeatedly, to obtain millions of copies of the target. Just as in DNA replication, PCR requires a template DNA, 4 dNTPs, primers to initiate DNA synthesis on each strand, and a DNA polymerase to synthesize the new DNA copies. In PCR, the primers bind to sequences flanking the target region that is to be amplified, and are present in large excess over the template. The DNA polymerase used is chosen to be heat stable, for reasons that will be clear shortly. The first step of each PCR cycle involves separating the strands

of the template DNA so that it can be replicated. This is accomplished by heating the DNA to near boiling temperatures. In the next step of the cycle, the solution is cooled to a temperature that favors complementary DNA sequences finding each other. Since the primers are present in great excess over the template, they can readily find and base-pair with the complementary sequences in the template on either side of the target sequence. In the third step in the cycle, the DNA polymerase (which has not been denatured during the heat

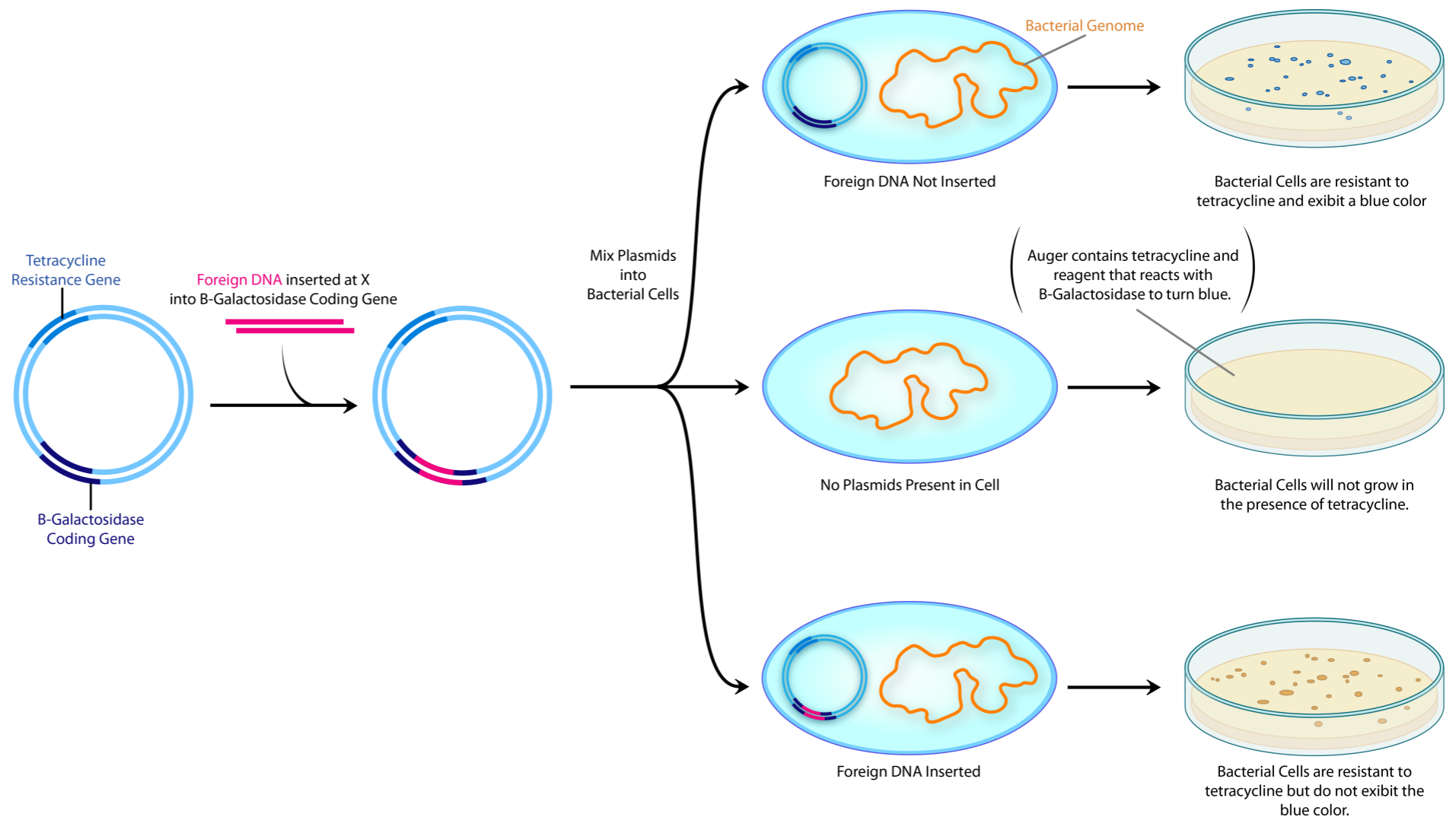


treatment because it is thermostable) extends the primer on each strand, making copies of both DNA strands and doubling the amount of the target sequence. The cycle is then repeated, usually about 30 times. At the end of the process, there is a theoretical yield of 2^{30} more of the target DNA than there was in the beginning.

Lac Z Blue-White Screening

A powerful tool for biotechnologists is the *lac Z* gene. You may recall from an earlier section on the control of gene expression, that *lac Z* is part of the *lac* operon of *E. coli* and encodes the enzyme β galactosidase. This enzyme catalyzes the hydrolysis of lactose into glucose and galactose, allowing the bacteria to use lactose as an energy source. β galactosidase can also break down an artificial substrate called X-gal to

produce a compound that is blue in color. X-gal can thus be used to test for the presence of active β galactosidase. With this background, we can now look at how the *lac Z* gene can be of help to molecular biologists when they create recombinant plasmids. In the example described earlier, the gene for human growth hormone (hGH) was inserted into a plasmid. As we noted, the plasmid, as well as the hGH gene are cut with restriction



Blue-White Screening Strategy

endonucleases to create compatible DNA ends that can be ligated. While the ends of the hGH gene are, indeed, capable of being ligated to the ends of the plasmid, the two ends of the plasmid could also readily rejoin. In fact, given that the two ends of the plasmid are on the same molecule, the chances of their finding each other are much higher than of a plasmid end finding an hGH gene. This would mean that many of the ligated molecules would not be recombinants, but simply recircularized plasmids. Five percent of the plasmids having inserts of the hGH gene would be very good. That would mean that 95% of the bacterial colonies arising from transformation would contain the original plasmid rather than the recombinant. To make the process of screening for the relatively rare recombinants simpler, plasmids have been engineered that carry the *lac Z* gene, modified to contain, with the coding sequence, restriction enzyme recognition sites. If one of these sites is used to cut open the plasmid and a gene of interest is inserted, this disrupts the *lac Z* gene. If the plasmid simply recircularizes, the *lac Z* gene will be intact. To find which bacterial colonies carry the recombinant plasmids, X-Gal is provided in the plates. Bacterial colonies containing plasmids with the *lac z* sequence disrupted by an inserted gene will not produce functional β galactosidase. The X-Gal will not be broken down and there will be no blue color. By contrast, bacterial cells with recircularized plasmids having no inserted hGH gene will make functional β galactosidase, so in the presence of X-Gal and IPTG these colonies will produce a blue color. This is summarized in the figure on the previous page.

Reverse Transcription

According to the central dogma, DNA codes for mRNA, which codes for protein. An exception to this rule is seen with the retroviruses, RNA-encoded viruses that have a phase in their replication cycle during which their genomic RNA is copied into DNA by a virally-encoded enzyme known as reverse transcriptase. The ability to convert RNA to DNA can be useful in the laboratory. For example, the power of PCR can be brought to RNA by converting RNAs of interest to DNA and then amplifying them by PCR. With reverse transcriptase, this is readily accomplished. First, one creates a DNA oligonucleotide to serve as a primer for reverse transcriptase to use on a target RNA. The primer must, of course, be complementary to a segment (near the 3' end) of the RNA to be amplified. The RNA template, reverse transcriptase, the primer, and four dNTPs are mixed. With one round of replication, the RNA is converted to a single strand of DNA, which can be separated from the RNA either by heating or by the use of an RNase to digest the RNA. The product of this process is called a complementary DNA (cDNA).

Jump to Chapter

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