



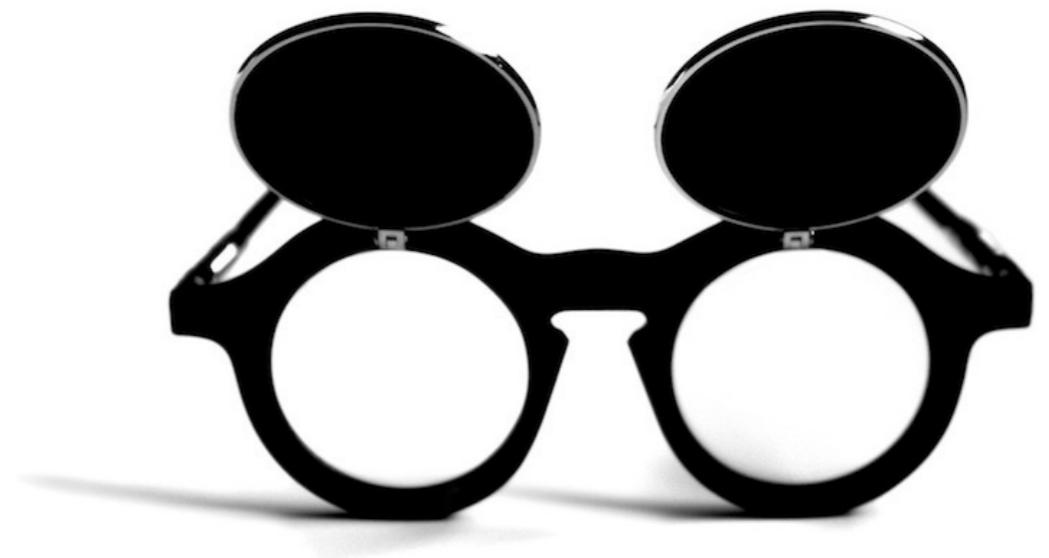
10 Things Every Molecular Biologist Should Know

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Edited By Nick Oswald

a **BitesizeBio.com eBook**

Preface



Molecular Biology is a funny old business...

Molecular biology is a funny old business. The ton of theoretical knowledge that we cram in during our undergrad years is scant preparation for life at the bench and leaves a lot to be said.

When we first hit the lab there are so many things to learn before we even get started that many things go unlearned.

How specific techniques work, what actually *is* in those kits we use and why we use certain approaches rather than others.

Often these nuggets of knowledge can mean the difference between understanding where an experiment is going wrong and not understanding, between getting a result and not getting one.

But normally, these nuggets can't be found in lectures or in text books. Instead they must be gleaned through hard on-the-job experience or passed down by word-of-mouth from more experienced mentors.

One of the core aims of Bitesize Bio (www.bitesizebio.com) is to make it easier to learn on the job as a molecular biologist by providing a place where these nuggets of vital, often over-looked knowledge can be found.

This short eBook provides 10 such nuggets, neatly contained in one place. From How SDS-PAGE works to the chemical reason why enzymes have optimal temperatures we hope that there will be something in here that will enlighten and entertain even the most experienced scientist.

If you like the information you find in this eBook - which is the first of many we'll be creating on a variety of topics - we would love you pass the file onto your friends to help spread the word about what we are doing at Bitesize Bio.

And if you are hungry for more please be sure to visit us at BitesizeBio.com to get the latest articles and eBooks we have on offer.

Finally if you have any suggestions for articles you'd like to see on Bitesize Bio, if you'd like to write something for the

website yourself, or if you'd just like to let us know what you think (good or bad!) of our efforts, please feel free to get in touch via the contact page at BitesizeBio.com.

Thanks for reading.

The Bitesize Bio team.

How SDS-PAGE Works



“If you are still wondering why the stacking gel is needed, think of what would happen if you didn’t use one.”

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) is commonly used in the lab for the separation of proteins based on their molecular weight. It's one of those techniques that is commonly used but not frequently fully understood. So let's try and fix that.

SDS-PAGE separates proteins according to their molecular weight, based on their differential rates of migration through a sieving matrix (a gel) under the influence of an applied electrical field.

Making the rate of protein migration proportional to molecular weight.

The movement of any charged species through an electric field is determined by it's net charge, it's molecular radius and the magnitude of the applied field. But the problem with natively folded proteins is that neither their net charge nor their molecular radius are molecular weight dependent. Instead, their net

charge is determined by amino acid composition i.e. the sum of the positive and negative amino acids in the protein and molecular radius by the protein's tertiary structure.

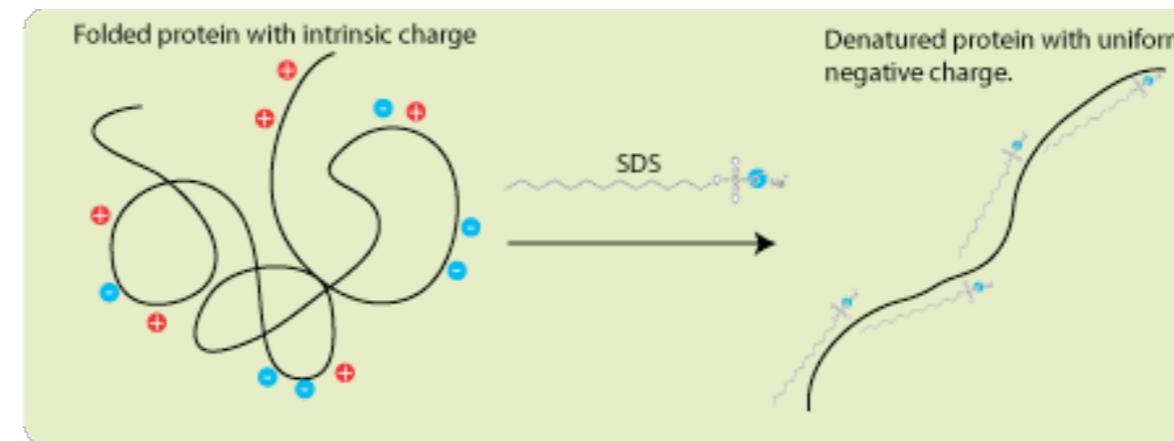
So in their native state, different proteins with the same molecular weight would migrate at different speeds in an electrical field depending on their charge and 3D shape.

To separate proteins in an electrical field based on their molecular weight, we need to destroy the tertiary structure by reducing the protein to a linear molecule, and somehow mask the intrinsic net

charge of the protein. That's where SDS comes in.

The Role of SDS (et al)

SDS is a detergent that is present in the SDS-PAGE sample buffer where, along with a bit of boiling, and a reducing agent (normally DTT or B-ME to break down protein-protein disulphide bonds), it disrupts the tertiary structure of proteins. This brings the folded proteins down to linear molecules.



SDS helps to disrupt the tertiary protein structure

SDS also coats the protein with a uniform negative charge, which masks the intrinsic charges on the R-groups. SDS binds fairly uniformly to the linear proteins (around 1.4g SDS/ 1g protein), meaning that the charge of the protein is now approximately proportional to its molecular weight.

SDS is also present in the gel to make sure that once the proteins are linearized and their charges masked, they stay that way throughout the run.

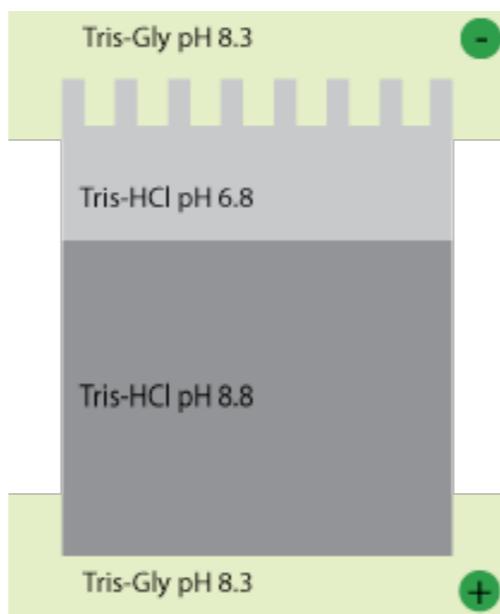
The dominant factor in determining the migration rate of an SDS-coated protein in a gel is its molecular radius.

SDS-coated proteins have been shown to be linear molecules, 18 Angstroms wide and with length proportional to their molecular weight, so the molecular radius (and hence their mobility in the gel) is determined by the molecular weight of the protein. Since the SDS-coated proteins have the same charge to mass ratio there will be no differential migration based on charge.

The gel matrix

In an applied electrical field, the SDS-treated proteins will now move toward the cathode at different rates depending on their molecular weight. These different mobilities will be exaggerated by the high-friction environment of a gel matrix.

As the name suggests, the gel matrix used for SDS-PAGE is polyacrylamide, which is a good choice because it is chemically inert and, crucially, can easily



The discontinuous buffer system

be made up at a variety of concentrations to produce different pore sizes which, in turn, can produce varying separating conditions that can be adjusted to suit your needs.

The discontinuous buffer system and the stacking gel

To conduct the current through from the anode to the cathode through the gel, a buffer is obviously needed. Mostly we use the discontinuous Laemmli buffer system.

“Discontinuous” simply means that the buffer in the gel and the tank are different.

Typically, the system is set up with a stacking gel at pH 6.8, buffered by Tris-HCl, a running gel buffered to pH 8.8 by Tris-HCl and an electrode buffer at pH 8.3. The stacking gel has a low concentration of acrylamide and the running gel has a higher concentration capable of retarding the movement of the proteins.

So what's with all of those different pH's? Well, glycine can exist in three different charge states, positive, neutral or negative

depending on the pH. This is shown in the diagram below. Control of the charge state of glycine by the different buffers is the key to the whole stacking gel thing.

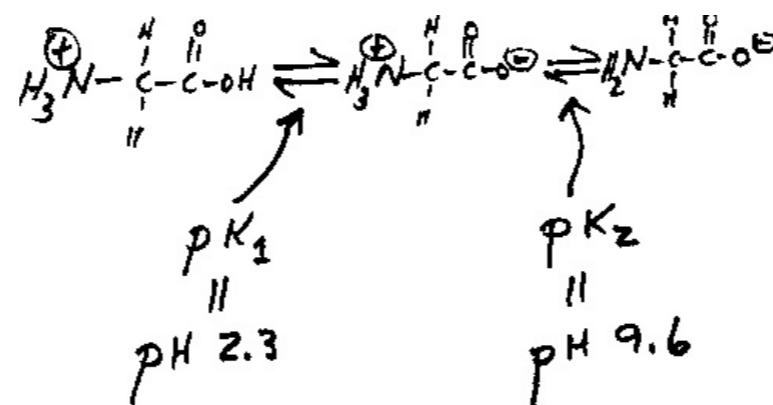
So here's how the stacking gel works. When the power is turned on, the negatively-charged glycine ions in the pH 8.3 electrode buffer are forced to enter the stacking gel, where the pH is 6.8. In this environment glycine switches predominantly to the zwitterionic (neutrally charged) state. This loss of charge causes them to move very slowly in the electric field.

The Cl⁻ ions (from Tris-HCl) on the other hand, move much more quickly in the electric field and they form an ion front that migrates ahead of the glycine. The separation of Cl⁻ from the Tris counter-ion (which is now moving towards the cathode) creates a narrow zone with a steep voltage gradient that pulls the glycine along behind it, resulting in two narrowly separated fronts of migrating ions; the highly mobile Cl⁻ front, followed by the slower, mostly neutral glycine front.

All of the proteins in the gel sample have an electrophoretic mobility that is intermediate between the extreme of the mobility of the glycine and Cl⁻ so when the two fronts sweep through the sample-well the proteins are concentrated into the narrow zone between the Cl⁻ and glycine fronts.

And they're off!

This procession carries on until it hits the running gel, where the pH switches to 8.8. At this pH the glycine molecules are mostly negatively charged and can migrate much faster than the proteins.



The effect of buffer pH on glycine charge

So the glycine front accelerates past the proteins, leaving them in the dust.

The result is that the proteins are dumped in a very narrow band at the interface of the stacking and running gels and since the running gel has an increased acrylamide concentration, which slows the movement of the proteins according to their size, the separation begins.

What was all of that about?

If you are still wondering why the stacking gel is needed, think of what would happen if you didn't use one.

Gel wells are around 1cm deep and you generally need to substantially fill them to get enough protein onto the gel. So in the absence of a stacking gel, your sample would sit on top of the running gel, as a band of up to 1cm deep.

Rather than being lined up together and hitting the running gel together, this would mean that the proteins in your sample would all enter the running gel at different times, resulting in very smeared bands.

So the stacking gel ensures that all of the proteins arrive at the running gel at the same time so proteins of the same molecular weight will migrate as tight bands.

Separation

Once the proteins are in the running gel, they are separated because higher molecular weight proteins move more slowly through the porous acrylamide gel than lower molecular weight proteins. The size of the pores in the gel can be altered depending on the size of the proteins you want to separate by changing the acrylamide concentration.

For a broader separation range, or for proteins that are hard to separate, a gradient gel, which has layers of increasing acrylamide concentration, can be used.

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How Proteases and Protease Inhibitors Work



“To defeat the protease, you must become the protease”

Proteases: wild, mysterious, destructive. What are these untamed elements ravaging your precious lysate?

How can a drop of EDTA or a smidge of “cocktail” protect that sample, which is gently cradling your hopes, your dreams, and your desire to survive the next lab meeting?

Brace yourself for a biochem flashback: in this section, I’ll explain the what and how of proteases and protease inhibitors, and we’ll do it with phrases like “reversible competitive inhibition” and “zymogen.”

Proteases: the good, the bad, the hydrolytic

All organisms have proteolytic and phosphorylating enzymes. You. Me. *Helicoverpa armigera*. While involved in everything from host defense to wound healing to viral replication, proteases can be broadly lumped into two camps based on what’s in their

active site and how it interacts with the target peptide bond.

Camp One hydrolyzes the bond through nucleophilic activation of a serine, threonine, or cysteine at its active site.

Camp Two, which includes the aspartic, glutamic, and metalloproteases, uses water itself for the hydrolysis.

These tidy camps break down under questioning about preferred targets and substrate affinity. Although Camp One’s serine proteases all agree that an active site serine is superior to the plebeian cysteine, they still have tense family dinners because of each of the proteases has a different target preference and structure.

These structural differences drive a protease’s specificity for where it will attack: terminal hydrolysis for the exopeptidases, internal hydrolysis for the endopeptidases.

Although the exopeptidases only choose between the amino or carboxyl terminus, the endopeptidases are gloriously picky.

To go back to the serine example, our friend trypsin hydrolyzes on the carboxyl side of positively charged arginine or lysine, unless these are followed by a proline.

And while its buddy chymotrypsin is also a serine protease, chymotrypsin simply doesn’t touch arginine or lysine and really just prefers aromatics like tyrosine, tryptophan, or phenylalanine.

The last thing a cell wants is these enzymes running amok, rampaging willy-nilly through the cytoplasm, chewing off-target, and generally behaving like – I say this with great familiarity – Ohio State football fans.

Regulation and segregation are the cell’s prime approaches for dealing with these enzymes: regulation by, for example, holding them in an inactive precursor state (a “zymogen”) and segregation to the lysosome or secretory pathways, among other locations.

Inhibitors: You can't stop with just one

All that chemical lysis, scraping, sonication, and freeze-thawing you inflict throws the whole system into chaos. No peptide bond is safe!

Exopeptidases attack the ends of amino acid chains while endopeptidases hydrolyze their internal links. Kinases phosphorylate and dephosphorylate at will. Good luck quantifying NOS induction or making a case for p53 activation by Western. Without inhibitors, your results will be inconsistent, your lab meetings agonizing, and your graduation date a pipe dream.

Tragically, there is no Wunderkind inhibitor that takes out all proteases, halts every phosphorylation event, and does some light tidying-up about the centrifuges. It takes a “cocktail” of approaches to stave off proteolysis. Protease inhibitors can act irreversibly, reversibly, and reversibly-under-duress,

and can be anything from small molecules like sodium fluoride to big honkin’ polypeptide analogues of evolution’s own microbial inhibitors. They can compete with substrates for an active site – sometimes destroying the site in the process – or selfishly hoard the precious, precious cations needed for protease function.

Competitive inhibitors act by binding in a substrate-like manner to the active site to block it or by binding and destroying it through modifications such as esterification. Many large competitive inhibitors boost their affinity and specificity by binding to the overlooked wallflower regions neighboring the hogs-all-the-glory active site. You have likely already come across competitive inhibitors. They include all sorts, from aprotinin, a 6 kDa polypeptide serine protease that is reversible if you ask politely at pH extremes, to sodium orthovanadate, a small molecule which inhibits the ever-gullible protein phosphotyrosyl phosphatases.

By contrast, the toddler-like chelators (motto: MINE!) take advantage of their

enhanced affinity for metal ions to complex with them so that nobody else can react with them. This matters because metalloproteases need zinc, magnesium, manganese, calcium, or even cobalt in their active site to activate water to hydrolyze peptide bonds to meet their quarterly hydrolysis goals. Take away that zinc with EDTA, complex up that calcium with EGTA, and it's bye-bye metalloprotease activity.

Conclusion: To defeat the protease, you must become the protease

Proteases are just some of the destructive forces nature aims at your beloved cell lysates, and protease inhibitors are one of the ways that the clever scientist can, judo-like, turn the universe against itself. For more information on beating the universe into submission, check out Emily Crow’s [“How To Preserve Your Samples In Western Blotting”](#), Jode Plank’s [“Streamline Your Western Blots”](#), on Bitesize Bio.

Agarose Gels Do Not Polymerize



“Polyacrylamide gel polymerization - Try saying that three times quickly!”

I was browsing a certain website the other day when I came across a protocol that advised: “When agarose starts to cool, it undergoes what is known as polymerization”

Polymerizing agarose? Someone is getting their gel matrices mixed up, and it’s not the first time I have heard this said, so it looks like some myth-busting is required.

So hunker down for a quick run down on the difference between polymerizing and non-polymerizing gel matrices.

Of the common gel matrices used in molecular biology, polyacrylamide, agar and agarose, polyacrylamide is the one that polymerizes. I’ll deal with agarose and agar later.

Polyacrylamide gel polymerization

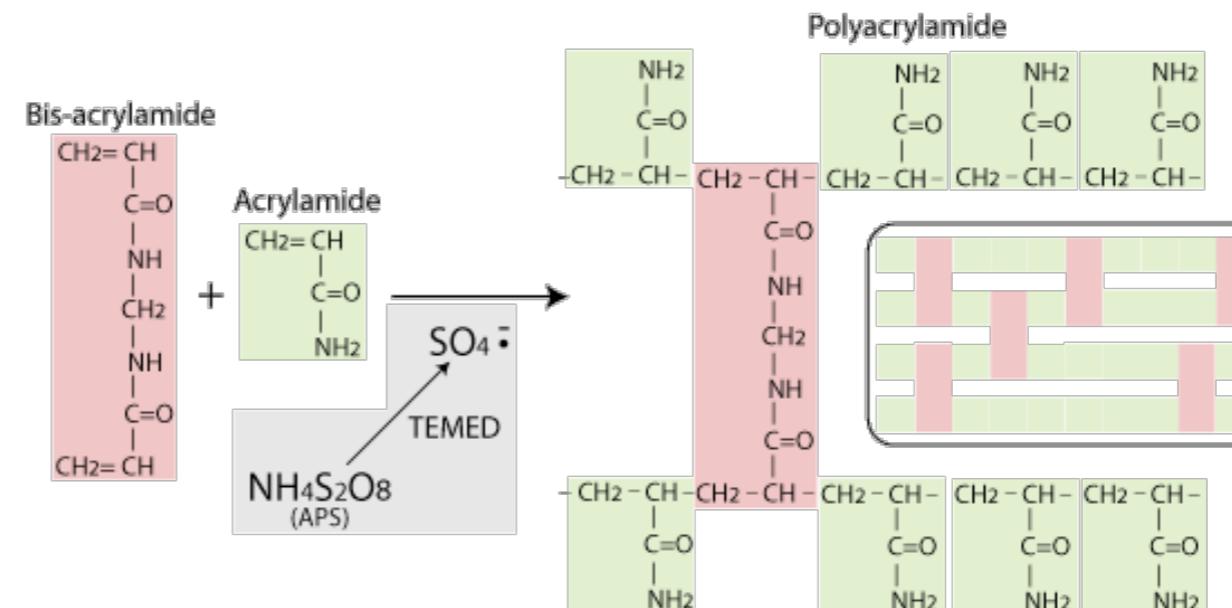
Polyacrylamide, used mainly for SDS-PAGE, is a matrix formed from monomers of acrylamide and bis-acrylamide. Its strengths are that is it

chemically inert – so won’t interact with proteins as they pass through – and that it can be easily and reproducibly made with different pore sizes to produce gels with different separation properties.

The polymerization reaction, shown in the diagram below, is a vinyl addition catalyzed by free radicals. The reaction is initiated by TEMED, which induces free radical formation from ammonium persulphate (APS). The free radicals transfer electrons to the acrylamide/bisacrylamide monomers, radicalizing

them and causing them to react with each other to form the polyacrylamide chain.

In the absence of bis-acrylamide, the acrylamide would polymerize into long strands, not a porous gel. But as the diagram shows, bis-acrylamide cross-links the acrylamide chains and this is what gives rise to the formation of the porous gel matrix. The amount of cross-linking, and therefore the pore size and consequent separation properties of the gel can be controlled by varying the ratio of acrylamide to bis-acrylamide.



Polyacrylamide gel polymerization - Try saying that three times quickly!

For more information on polyacrylamide gel polymerization see **Biorad Bulletin 1156**

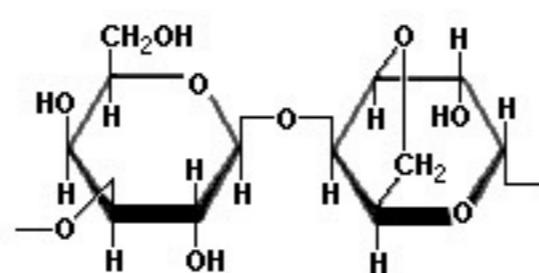
Agarose gel formation

So what about agarose? Well, agarose – the main component of the gelatinous agar that can be isolated from certain species of seaweed – is itself a polymer. But, polymerization is not the mechanism for agarose gel formation.

Chemically, agarose is a polysaccharide, whose monomeric unit is a disaccharide of D-galactose and 3,6-anhydro-L-galactopyranose which is shown in the diagram below.

In aqueous solutions below 35°C these polymer strands are held together in a porous gel structure by non-covalent interactions like hydrogen bonds and electrostatic interactions. Heating the solution breaks these non-covalent interactions and separates the strands. Then as the solution cools, these non-covalent interactions are re-established and the gel forms.

So agarose (and agar) gels form by gellation through hydrogen bonding and electrostatic interactions, not through polymerization.



Chemically, agarose is a polysaccharide, whose monomeric unit is a disaccharide of D-galactose and 3,6-anhydro-L-galactopyranose

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How Ethanol Precipitation of DNA and RNA Works



“First we need to know why nucleic acids are soluble in water...”

Ethanol precipitation is a commonly used technique for concentrating and de-salting nucleic acid (DNA or RNA) preparations in aqueous solution. The basic procedure is that salt and ethanol are added to the aqueous solution, which forces the nucleic acid to precipitate out of solution.

The precipitated nucleic acid can then be separated from the rest of the solution by centrifugation. The pellet is washed in cold 70% ethanol then after a further centrifugation step the ethanol is removed, and the nucleic acid pellet is allowed to dry before being resuspended in clean aqueous buffer.

So how does this work?

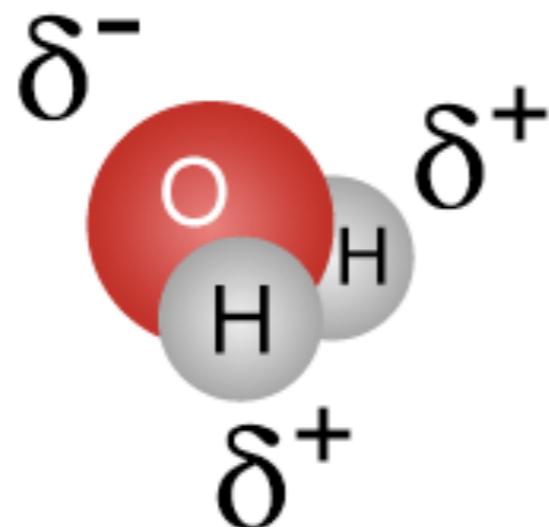
A bit about solubility...

First we need to know why nucleic acids are soluble in water. Water is a polar molecule – it has a partial negative charge near the oxygen atom due the unshared pairs of electrons, and partial positive charges near the

hydrogen atoms (see the diagram below).

Because of these charges, polar molecules, like DNA or RNA, can interact electrostatically with the water molecules, allowing them to easily dissolve in water.

Polar molecules can therefore be described as hydrophilic and non-polar



Polarity of water molecules. Oxygen atoms are slightly negatively charged, hydrogens are slightly positive

molecules, which can't easily interact with water molecules, are hydrophobic.

Nucleic acids are hydrophilic due to the negatively charged phosphate (PO_3^-) groups along the sugar phosphate backbone.

The role of the salt...

Ok, so back to the protocol. The role of the salt in the protocol is to neutralize the charges on the sugar phosphate backbone. A commonly used salt is sodium acetate. In solution, sodium acetate breaks up into Na^+ and $[\text{CH}_3\text{COO}]^-$.

The positively charged sodium ions neutralize the negative charge on the PO_3^- groups on the nucleic acids, making the molecule far less hydrophilic, and therefore much less soluble in water.

The role of the ethanol...

The electrostatic attraction between the Na^+ ions in solution and the PO_3^- ions are dictated by **Coulomb's Law**, which is

affected by the dielectric constant of the solution.

Water has a high dielectric constant, which makes it fairly difficult for the Na⁺ and PO₃⁻ to come together.

Ethanol on the other hand has a much lower dielectric constant, making it much easier for Na⁺ to interact with the PO₃⁻, shield its charge and make the nucleic acid less hydrophilic, causing it to drop out of solution.

The role of temperature...

Incubation of the nucleic acid/salt/ethanol mixture at low temperatures (e.g. -20 or -80C) is commonly cited in protocols as necessary in protocols.

However, according to Maniatis et al (Molecular Cloning, A Laboratory Manual 2nd Edition... 2nd edition?? – I need to get a newer version!), this is not required, as nucleic acids at concentrations as low as 20ng/mL will precipitate at 0-4C so incubation for 15-30 minutes on ice is sufficient.

The wash step with 70% ethanol...

This step is to wash any residual salt away from the pelleted DNA.

A few tips on nucleic acid precipitation...

Choice of salt

- Use Sodium acetate (0.3M final conc, pH 5.2) for routine DNA precipitations
- Use Sodium chloride (0.2M final conc) for DNA samples containing SDS since NaCl keeps SDS soluble in 70% ethanol so it won't precipitate with the DNA.
- Use Lithium Chloride (0.8M final conc) for RNA. This is because 2.5-3 volumes of ethanol should be used for RNA precipitation and LiCl is more soluble in ethanol than NaAc so will not precipitate, but beware – chloride ions will inhibit protein synthesis and DNA polymerase so LiCl is no good for RNA preps for *in vitro* translation or reverse transcription. In these cases, use NaAc.

- Use Ammonium acetate (2M final conc) for the removal of dNTPs, but do not use for preparation of DNA for T4 polynucleotide kinase reactions as ammonium ions inhibit the enzyme.

To increase the yield in precipitations of low concentration or small nucleic acid pieces (< 100 nucleotides)

- Add MgCl₂ to a final concentration of 0.01M
- Increase the time of incubation on ice before centrifugation to 1 hour.

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How Alkaline Lysis Works



“The easiest way to describe how alkaline lysis works is to go through the procedure and explain each step, so here goes....”

Alkaline lysis was first described by Birnboim and Doly in 1979 (*Nucleic Acids Res.* 7, 1513-1523) and has, with a few modifications, been the preferred method for plasmid DNA extraction from bacteria ever since. The easiest way to describe how alkaline lysis works is to go through the procedure and explain each step, so here goes.

1. Cell Growth and Harvesting

The procedure starts with the growth of the bacterial cell culture harboring your plasmid. When sufficient growth has been achieved, the cells are pelleted by centrifugation to remove them from the growth medium.

2. Re-suspension

The pellet is then re-suspended in a solution (normally called solution 1, or similar in the kits) containing Tris, EDTA, glucose and RNase A. Divalent cations (Mg^{2+} , Ca^{2+}) are essential for DNase activity and the integrity of the

bacterial cell wall. EDTA chelates divalent cations in the solution preventing DNases from damaging the plasmid and also helps by destabilizing the cell wall. Glucose maintains the osmotic pressure so the cells don't burst and RNase A is included to degrade cellular RNA when the cells are lysed.

3. Lysis

The lysis buffer (aka solution 2) contains sodium hydroxide (NaOH) and the detergent Sodium Dodecyl (lauryl) Sulfate (SDS).

SDS is there to solubilize the cell membrane. NaOH helps to break down the cell wall, but more importantly it disrupts the hydrogen bonding between the DNA bases, converting the double-stranded DNA (dsDNA) in the cell, including the genomic DNA (gDNA) and your plasmid, to single stranded DNA (ssDNA).

This process is called denaturation and is central part of the procedure (which is why it's called alkaline lysis!). SDS also

denatures most of the proteins in the cells, which helps with the separation of the proteins from the plasmid later in the process.

It is important during this step to make sure that the re-suspension and lysis buffers are well mixed, although not too vigorously.

Also remember that SDS and NaOH are pretty nasty so it's advisable to wear gloves and eye protection when performing alkaline lysis.

4. Neutralization

Addition of potassium acetate (solution 3) increases the pH of the mixture. Under these conditions the hydrogen bonding between the bases of the single stranded DNA can be re-established, so the ssDNA can re-nature to dsDNA.

This is the selective part. While it is easy for the small circular plasmid DNA to re-nature it is impossible to properly anneal those huge gDNA stretches.

This is why it's important to be gentle during the lysis step because vigorous

mixing or vortexing will shear the gDNA producing shorter stretches that can re-anneal and contaminate your plasmid prep.

While the double-stranded plasmid can dissolve easily in solution, the single stranded genomic DNA, the SDS and the denatured cellular proteins stick together through hydrophobic interactions to form a white precipitate. The precipitate can easily be separated from the plasmid DNA solution by centrifugation.

5. Cleaning and concentration

Now your plasmid DNA has been separated from the majority of the cell debris but is in a solution containing lots of salt, EDTA, RNase and residual cellular proteins and debris, so it's not much use for downstream applications. The next step is to clean up the solution and concentrate the plasmid DNA.

There are several ways to do this including phenol/chloroform extraction

followed by ethanol precipitation and affinity chromatography-based methods using a support that preferentially binds to the plasmid DNA under certain conditions of salt or pH, but releases it under other conditions. The most common methods are detailed in the Bitesize Bio article on [5 ways to clean up a DNA sample](#).

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How Silica Spin Column DNA and RNA Preps Work



“...the downside [of using kits] is that we don't actually know what is in the mysterious... set of solutions which... makes troubleshooting more difficult.”

Section 06

By Suzanne Kennedy

These days, most labs use commercial kits, which employ spin columns, for the isolation of nucleic acids. The spin columns contain a silica resin that selectively binds DNA/RNA, depending on the salt conditions and other factors influenced by the extraction method.

When things are going well, these kits make the whole process much easier and faster than the 'Methods of Old'.

However, the downside is that we don't actually know what is in the mysterious and proprietary set of solutions which, of course, makes troubleshooting more difficult.

In this section I'll explain how silica spin filter kits work and what is going on at each step.

I'll also go over some common problems specific to silica columns that can be overcome or avoided with just a little extra understanding.

Lysis

The lysis approach may vary based on whether you want DNA or RNA, but the common denominator is a lysis buffer containing a high concentration of chaotropic salt.

Chaotropes destabilize hydrogen bonds, van der Waals forces and hydrophobic interactions. Proteins are destabilized, including nucleases, and the association of nucleic acids with water is disrupted setting up the conditions for their transfer to silica.

Chaotropic salts include guanidine HCL, guanidine thiocyanate, urea, and lithium perchlorate.

Besides the chaotropes, lysis buffer usually contains detergents to help with protein solubilization and lysis.

There can also be enzymes used for lysis depending on the sample type. Proteinase K is one of these, and actually works very well in these denaturing buffers; the more denatured

the protein, the better Proteinase K works. Lysozyme, however, does not work in the denaturing conditions of the lysis buffer, so lysozyme treatment is usually done before adding the denaturing salts.

For plasmid preps, the lysis is very different from extraction for RNA or genomic DNA because the plasmid has to be separated from the genomic DNA first.

If you throw in chaotropes, you'll release everything at once and won't be able to differentially separate the small circular DNA from the high molecular weight chromosome.

So in plasmid preps the chaotropes are not added until after lysis and the salts are used for binding.

A more in-depth explanation of alkaline lysis for plasmid preps can be found in the previous section "How Alkaline Lysis Works"

Binding

The chaotropic salts are critical for lysis, but also for binding of the nucleic acids to the silica resin.

To enhance the binding of nucleic acids to silica, alcohol is also added at the binding stage. Most of the time this is ethanol but sometimes it may be isopropanol.

The concentration of ethanol, and the volume used during binding, both have big effects. Too much (concentration or volume) and you'll bring in a lot of degraded nucleic acids and small species that will influence UV260 readings and throw off some of your yields. Too little, and it may become difficult to wash away all of the salt from the membrane.

The important point here is that the ethanol influences binding and the amount added is optimized for whatever kit you are using. Modifying that step can help change what you recover so if you are having problems

and want to troubleshoot recovery, this can be a step to evaluate further.

Another way to diagnose problems is to save the flow-through after binding and precipitate it to see if you can find the nucleic acids you are searching for. If you used an SDS-containing detergent in lysis, try using NaCl as a precipitant to avoid contamination of the DNA or RNA with detergent.

Washing Steps

Your lysate was centrifuged through the silica membrane and now your DNA or RNA should be bound to the column and the impurities, protein and polysaccharides, should have passed through.

But, the membrane is still dirty with residual proteins and salt. If the sample was from plants, there will still be polysaccharides, maybe some pigments too, left on the membrane, or if the sample was blood, the membrane might be tinted brown or yellow.

The wash steps serve to remove these impurities. There are typically two washes, although this can vary depending on the sample type. The first wash will often have a low amount of chaotropic salt to remove the protein and colored contaminants. This is always followed with an ethanol wash to remove the salts. If the prep is something that didn't have a lot of protein to begin with, such as plasmid preps or PCR clean up, then only an ethanol wash is needed.

Removal of the chaotropic salts is crucial to getting high yields and purity of DNA or RNA. Some kits will even recommend washing the column twice with ethanol. If salt remains behind, the elution of nucleic acid is going to be poor, and the A230 reading will be high, resulting in low 260/230 ratios.

Dry Spin

After the ethanol wash, most protocols have a centrifugation step to dry the column. This is to remove the ethanol and is essential for a clean eluant. When 10 mM Tris buffer or water is applied to the

membrane for elution, the nucleic acids can become hydrated and will release from the membrane. If the column still has ethanol on it, then the nucleic acids cannot be fully rehydrated.

Skipping the drying step results in ethanol contamination and low yields.

The main indication of this problem comes when you try to load the sample onto an agarose gel; the DNA will not sink, but floats in the buffer, even in the presence of loading dye. Another indicator of ethanol contamination is that if you put the sample in the -20C, it doesn't freeze.

Elution

The final step is the release of pure DNA or RNA from the silica. For DNA preps, 10 mM Tris at a pH between 8-9 is typically used. DNA is more stable at a slightly basic pH and will dissolve faster in a buffer. This is true even for DNA pellets. Water tends to have a low pH, as low as 4-5 and high molecular weight DNA may not completely

rehydrate in the short time used for elution. Elution of DNA can be maximized by allowing the buffer to sit in the membrane for a few minutes before centrifugation.

RNA, on the other hand, is fine at a slightly acidic pH and so water is the preferred diluent. RNA dissolves readily in water.

What other things can go wrong with silica spin columns?

Low yields: If you experience yields lower than you expected for a sample, there are many factors to think about.

Usually it is a lysis problem, and incomplete lysis is a very common cause of low yields.

Alternatively, the problem could be incorrect binding conditions.

Make sure to use fresh high quality ethanol (100% 200 proof) to dilute buffers or for adding to the binding step.

Low quality ethanol or old stocks may have taken on water and not be the correct concentration. If the wash buffer is not made correctly, you may be washing off your DNA or RNA.

Low Purity: If the sample is contaminated with protein (low 260/280) then maybe you started with too much sample and the protein was not completely removed or dissolved.

If the sample has poor 260/230 ratio the issue is usually salt from the bind or the wash buffer. Make sure that the highest quality ethanol was used to prepare wash buffers and if the problem continues, give the column an additional wash.

Some samples have a lot more inhibitors compared to others. Environmental samples are especially prone to purity issues because humic substances are solubilized during extraction. Humics behave similarly to DNA and are difficult to remove from the silica column. For this type of sample, specialized techniques

exist to remove the protein and humics prior to the column step.

Degradation: This is more of a concern for RNA preps and an article that gives specific advice can be found in the Bitesize Bio article “[Troubleshooting RNA Isolation](#)”.

Mainly with RNA, degradation occurs from improper storage of the sample or an inefficient lysis, assuming of course that you eluted with RNase-free water. For DNA preps, degradation is not a huge problem because for PCR, the DNA can be sheared and it works fine. But if you were hoping not to have so much sheared DNA, then you may have used too strong a lysis method.

PCR Clean-up Special

Considerations: PCR clean-up is the easiest of all silica spin column techniques, because it simply involves adding a high concentration of binding salts (typically between 3-5 volumes of salt per volumes of PCR reaction) and centrifuging the column.

So when PCR Clean-up kits fail, it can be particularly frustrating. The first

question I ask people is “did you check the results of the PCR on a gel?” because you cannot UV check a PCR reaction and get an accurate reading.

There is way too much in a PCR reaction absorbing UV at 260: nucleotides, detergents, salts, and primers. In my experience, apparent failure of a PCR clean-up kit to work frequently is normally caused by a PCR reaction that has failed and so there was nothing to clean up in the first place.

But if you know you had a strong PCR product, the best approach is to just save your flow-through fraction after binding. If the DNA doesn't bind, that's where it is. You can always rescue it and then clean it up again. And then call tech support and ask for a replacement kit.

If you liked this, you'll also like:

[Recycle Those DNA Extraction Columns](#)

by Liam Thompson at [BitesizeBio.com](#)

You know those ridiculously priced and throw-away DNA mini, midi and maxi-prep columns? Well the good news is that you can actually re-use them if you are reasonably careful at regenerating them, with this simple and cheap method..

[...\[click to read more\]](#)

How DNA Ligation Works



“Don’t be scared off by the chemistry, this is worth taking some time to understand!”

DNA ligase ([EC 6.5.1.1](#)) covalently joins the phosphate backbone of DNA with blunt or compatible cohesive ends (see figure below) and it's natural role is in repairing double strand breaks in DNA molecules.

In molecular biology it is commonly used for the insertion of restriction enzyme-generated DNA fragments into vector backbones. Commercial ligases are supplied with a reaction buffer containing ATP and Mg²⁺, which are both essential for ligase activity.

Since ATP can be damaged by repeated freeze-thaw cycles, it is advisable to make aliquots of the buffer (see the Bitesize Bio article "[5 DNA ligation tips](#)" for more information).

The ligation reaction itself has two basic steps. Firstly the DNA ends have to collide by chance and stay together long enough for the ligase to join them.

This is the most inefficient part of the reaction, but is easier at low temperatures.

Why? Well, as you will probably know, all molecules move faster at higher temperatures so you can imagine that it is going to be easier for two DNA ends to collide and stay together if they are gently floating through the solution at low temperature, rather than whizzing about as they would be at higher temperatures. For cohesive ends, there is an additional reason; lower temperatures stabilize the hydrogen bonding between the complementary nucleotides, which really helps to keep things in place.

The second step is the enzymatic reaction, which is shown schematically on the next page.

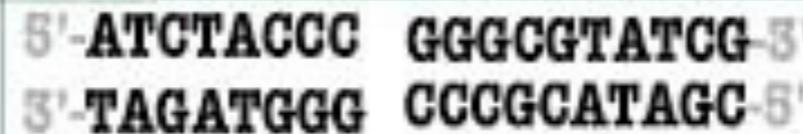
DNA ligase catalyzes the joining of the 3'-OH to the 5'-phosphate via a two step mechanism. First, the AMP nucleotide, which is attached to a lysine residue in the enzyme's active site, is transferred to the 5'-phosphate. Then the AMP-phosphate bond is attacked by the 3'-OH, forming the covalent bond and releasing AMP.

To allow the enzyme to carry out further reactions the AMP in the enzyme's active

Cohesive ends



Blunt ends

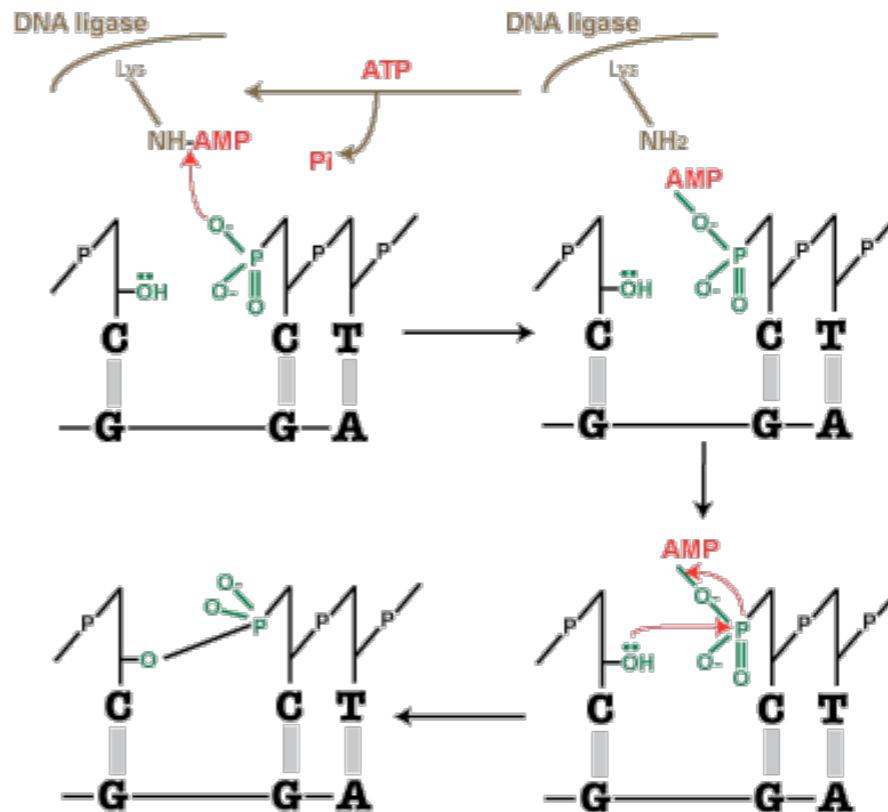


The DNA ligase can covalently join blunt ends, but cohesive ends are a bit easier because the hydrogen bonds hold the cohesive ends in the right place for the enzyme to do the job.

site must be replenished by ATP.

The DNA ligase enzyme has optimal activity at 25°C so the ligation reaction is carried out at a temperature that is a trade-off between the optimal temperatures for bringing the DNA ends together (1°C) and the enzymatic reaction (25°C). Normally 1hr at 16°C is fine but since bringing the DNA ends together is the least efficient part of the reaction favoring this by lowering the temperature to 4°C can give even more efficiency.

However, the enzyme will work very slowly at this temperature so a long (e.g. overnight) incubation time is required.



The DNA ligation mechanism. Don't be scared off by the chemistry, this is worth taking some time to understand!

If you liked this, you'll also like:

Pinpointing DNA Ligation Problems

by Nick Oswald at BitesizeBio.com

How Colorimetric Assays Work



“...even the most traditional of molecular biologists perform chemical reactions nearly every day”

One of my colleagues, a very good molecular biologist, told me that the only time she uses chemistry is when she needs to calculate molarities.

I, of course, scoffed at this statement, and tried to remind her of all the chemistry she uses daily.

I may be a bit biased since I am a chemist, but surely, all the chromophores, fluorophores, and imaging probes, etc., involve some knowledge of chemistry and chemical reactions. She shrugged, and replied that they're all supplied in kits.

So, in defense of all chemistry nerds out there, here is my rebuttal: even the most traditional of molecular biologists perform chemical reactions nearly every day.

From purification by ion exchange resins and affinity columns, to calculating protein concentrations and coupling bioconjugates, chemistry is behind it all. For this article I will focus on a couple of colorimetric assays and hopefully convince you that knowledge of these chemical reactions is helpful in

performing these assays and understanding the results, as well as in troubleshooting when things go awry.

By taking advantage of the electronic properties and specific wavelengths of chromophores, we can deduce a great deal of information. Colorimetric assays help us to distinguish a particular enzyme from another, to quantitate catalytic activity, as well as inhibition of this activity, to generate proliferative and toxicity profiles, and even to determine the concentration of proteins in solution. They are a simple and convenient way to visualize biological processes. As a former PI of mine used to say, in his thick German accent, while pointing to his eyes, "Nature has provided us with our own spectrophotometers, no?"

Even more appealing, many colorimetric assays are commercially available as kits, usually with a detailed protocol. In fact, colorimetric assays are used so regularly in chemistry and biology, that to cover them all would take an entire book. So, for simplicity's sake, this article will focus on two phosphatase assays.

Kinases and phosphatases mediate a variety of cellular processes like metabolism, gene transcription and translation, protein-protein interactions, and apoptosis, through protein phosphorylation and dephosphorylation. For this reason, many different technologies, including various colorimetric assays, exist to detect these enzymes and their activity.

para-Nitrophenylphosphate Assay Mechanism

para-Nitrophenylphosphate (pNPP) is widely used as a synthetic substrate to measure the catalytic activity of various phosphatases.

The phosphate group of pNPP is cleaved by the enzyme to yield p-nitrophenol, which is also colorless since the wavelength maximum for the electronic excitation of p-nitrophenol in water is 318 nm. However, under alkaline conditions, the p-nitrophenol is converted to the p-nitrophenolate anion, resulting in a bathochromic shift (a change in absorbance of a compound to a longer wavelength based on solution conditions) to around 400 nm. This is at the blue edge of the electromagnetic spectrum, but since

we see the color of the reflected light (as opposed to the absorbed light), the solution looks yellow.

Things to Keep in Mind...

One of the great selling points of the pNPP assay is its simplicity, so it's easy to see why one might forget that this **bathochromic** (a great trivia word!) shift is a crucial element to the assay.

What if, for example, the enzymatic activity requires an optimum pH in the acidic range, as so happens to be the case with acid phosphatase?

In that instance, the end point addition of a strong base (such as sodium hydroxide or potassium hydroxide) is necessary in order to obtain the desired yellow color.

Malachite Green Assay Mechanism

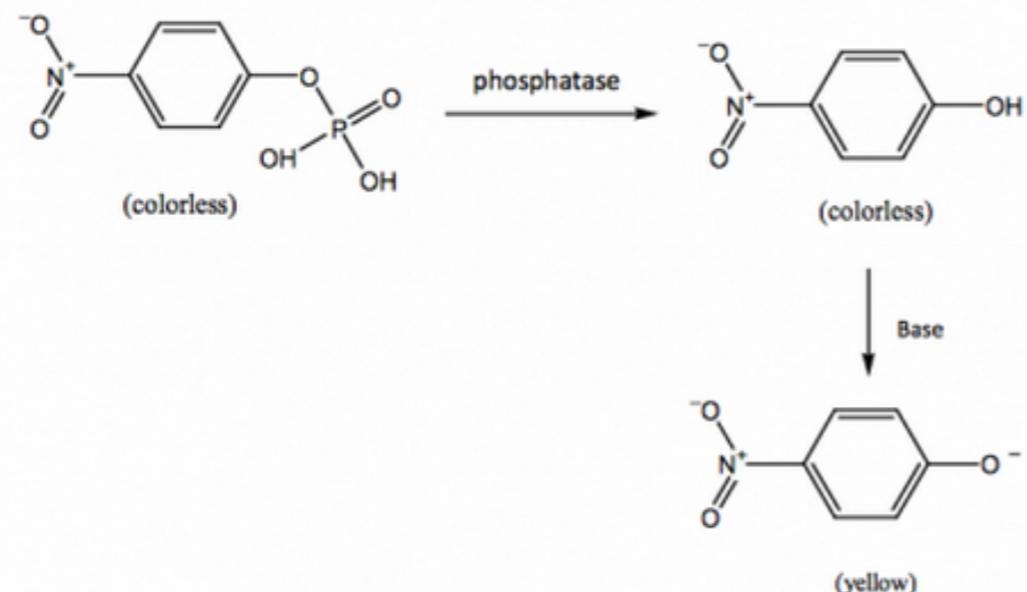
The para-nitrophenylphosphate assay is great, but what if your favorite phosphatase doesn't efficiently metabolize pNPP? Another commercially available phosphatase assay is the malachite green assay. This simple assay method is based on the complex

formed between malachite green, ammonium molybdate, and free orthophosphate (aka inorganic phosphate, Pi) under acidic conditions.

Orthophosphate, liberated from a phosphorylated substrate upon cleavage by the phosphatase, forms a complex with ammonium molybdate in a solution of sulfuric acid. The formation of the malachite green phosphomolybdate complex, measured at 620-650 nm, is accordingly directly related to the free orthophosphate concentration. Therefore, it is possible to quantify phosphorylation and phosphate release from protein phosphatase substrates.

Things to Keep in Mind...

This assay measures only inorganic free phosphate; organophosphates (lipid-bound or protein-bound phosphates) must first be hydrolyzed and neutralized prior to measurement. Knowledge of the different classes of organophosphates and their respective different free energies of hydrolysis aids in the optimization of assay conditions. Higher-energy organophosphates (acetal phosphates,



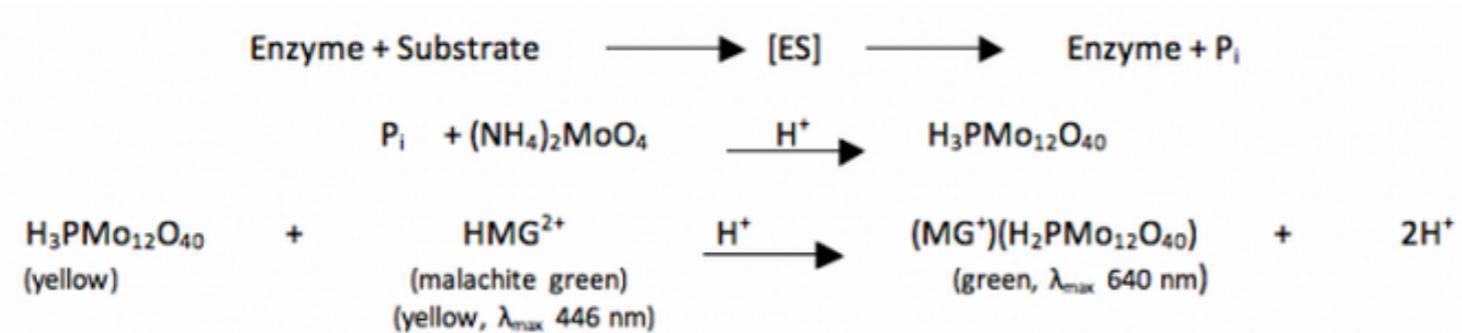
para-Nitrophenylphosphate: From Colorless to Yellow

phosphoanhydrides, etc) possess acid-labile phosphate groups that can be released into solution by incubation at low pH. On the other hand, lower-energy organophosphates (phosphoesters) are stable in acidic solution and require much harsher conditions (such as thermal decomposition) to be detected by this method.

In the presence of a large excess of malachite green, the 3:1 ion associate ($(\text{MG}^+)_3(\text{PMo}_{12}\text{O}_{40} \text{ 3-})$) can easily form and precipitate in the acidic aqueous solution. To stabilize the 1:1

ion associate in the aqueous solution, polyvinylalcohol is added to the solution.

Another thing to take into consideration during the assay is the possibility of any redox reactions that could interfere with the assay. Molybdenum is a transition metal and so exists in many oxidation states. In the molybdate anion, it has an oxidation state of +6. Reduction of the acidified Mo(VI) solution by organic compounds, like ascorbic acid and reducing sugars (i.e. glucose), or by inorganic compounds, like SnCl_2 , creates the Mo(IV) species, which is blue in color.



"No sleep 'til 640nm", By Malachite Green

There's much more where those came from

These two examples barely skim the surface of the colorimetric assays. Just think of all the catalase assays for the detection of hydrogen peroxide (like Sigma's quioneimine derived assay), and of course, the very popular MTT-based assays for measuring cytotoxicity. A better understanding of the chemistry involved in these assays helps to provide a deeper understanding of the reactions, as well as any required troubleshooting procedures.

Colorimetric assays are just one of the many ways in which chemistry and biology work hand-in-hand in scientific research. I think it's fair to say that the multi-disciplinary approach is now recognized as a vital way to move forward in the drug discovery process.

My chemical biology professor commented that in the 'olden days' it was enough to study medicinal chemistry and that the pharmaceutical companies would teach you the rest. Now, medicinal chemists are

expected to understand pharmacology, cell biology, and molecular biology techniques. It takes an entire team of people, involving many specialities, in order to be successful in developing safe and effective drugs. But it is not enough simply to involve many collaborators; I would argue that researchers on all sides need to learn to occasionally think alike. So hopefully the next time my aforementioned colleague performs a colorimetric assay she can don her ‘chemistry hat’ in addition to her ‘molecular biology hat’, and remember that drug research is an interdisciplinary art.

Want More Chemistry for Biologists?

Take a look at this free webinar series from Peter Kirsop at BitesizeBio.com

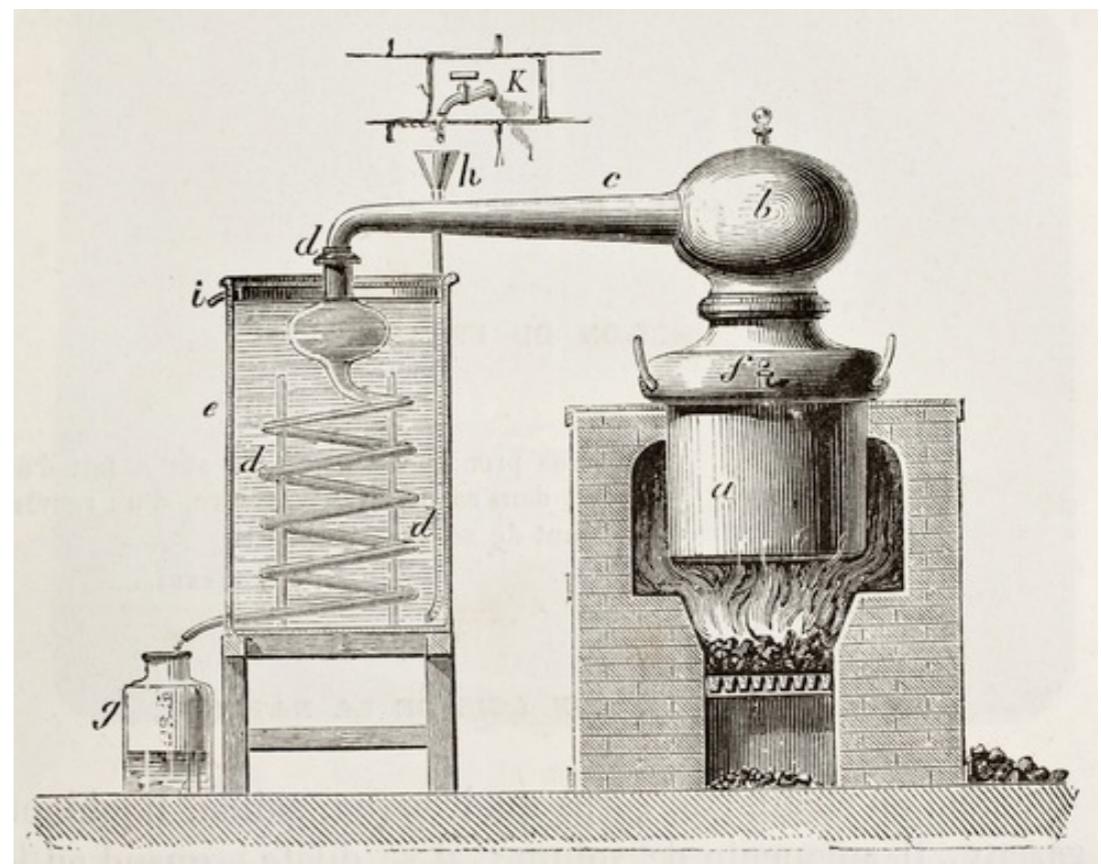
Chemistry for Biologists

Covered in this series:
Biological Organic Molecules

The Importance of Stereochemistry in Biological Molecules

Features of Organic Molecules

How Lab Grade Water is Purified



“...in practice a number of techniques are used, each of which can remove a different set of impurities.”

There's something in the water, and it would love to go after your experiments.

Straight out of the tap, water contains microorganisms, endotoxins, DNase and RNase, salts and other impurities that could gobble up your experiment in one bite.

Of course we avoid this drama completely by using purified water from which these nasties have been removed. But how is this purification done?

Well in practice a number of techniques are used, each of which can remove a different set of impurities.

So here are the techniques:

1. Distillation. A technology as old as the hills (or at least as the stills that were hidden in the hills). Water is heated to its boiling point then condensed back to liquid. This will remove many impurities but impurities

with a boiling point equal to or less than that of water will also be carried over in to the distillate.

2. Microfiltration. In this technique, pressure is used to force the water through a filter with pore sizes of 1 to 0.1 micron in order to remove particulate matter. Filter diameters lower than 0.2 micron removes bacteria – so-called cold sterilization.

3. Ultrafiltration uses even smaller pore sizes (down to 0.003 micron). These are essentially molecular sieves, which remove molecules with a diameter larger than the pore size. It can be used to remove viruses, endotoxins, RNase and DNase

4. Reverse osmosis. If you thought that ultrafiltration used impressively small pore sizes, you'll be even more impressed by reverse osmosis. Reverse osmosis filters have pore sizes of less than 0.001 microns, which allows them to sieve ions depending on their diameter. This is used for desalting the water.

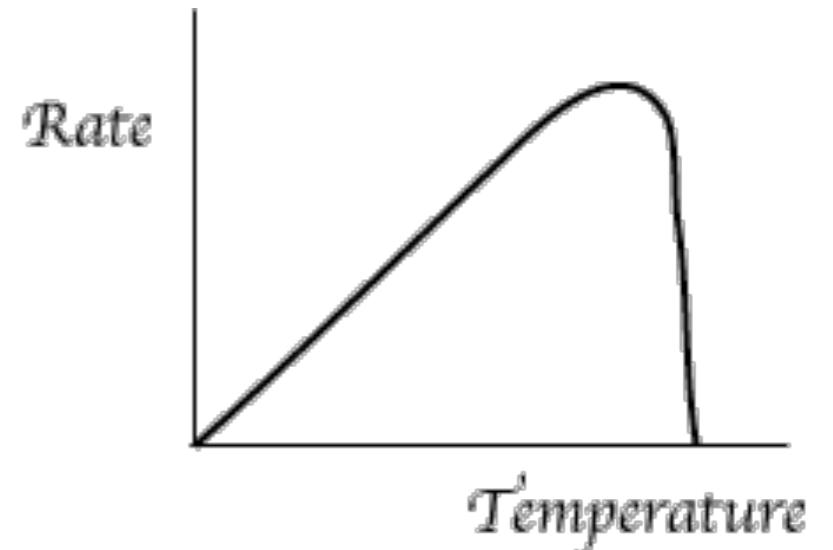
5. Filtration through a bed of activated carbon is useful for removing things like chloride ions and organic compounds, which are adsorbed onto the surface of the carbon.

6. UV radiation. We all know what UV radiation, at specific wavelengths, can do to DNA and microorganisms. So UV is an obvious way to remove microorganisms from the water. It can also clean up the water by breaking down certain organic compounds into less harmful products.

7. Deionization/ Ion exchange. This technique removes ions from the water by passing it through a resin bed containing a mixture of cationic and anionic resins. Positive ions in the water are attracted to the anionic resin particles and negative ions are (yes, you've guessed it) attracted to the cationic resins. The result is that nicely deionised water comes out of the other end of the resin bed.

Commercially available water, or water purification systems will typically use a combination of these. The higher the water purity grade, the more techniques used.

(Exactly) Why Enzymes Have Optimal Temperatures



“Actually it’s reassuringly simple...”

Every biologist is familiar with the profile of the rate of an enzymatic reaction versus temperature as shown in the figure.

We know that enzymes from *E.coli* or warm-blooded animals tend to have an optimum around 37°C while those from thermal vent bacteria have much higher optimal temperatures.

Surprisingly, I find that many biologists don't have a grasp of why enzymes have these temperature profiles. Actually it's reassuringly simple.

Chemists have a rule of thumb that a 10°C increase in temperature gives a doubling of the reaction rate. This rule is loosely derived from the [Arrhenius equation](#).

Basically, as the temperature increases so does the kinetic energy of the reactants. This increased kinetic energy means that the reactants are more likely to collide with enough energy to allow the reaction to occur, so the higher the temperature, the higher the reaction rate.

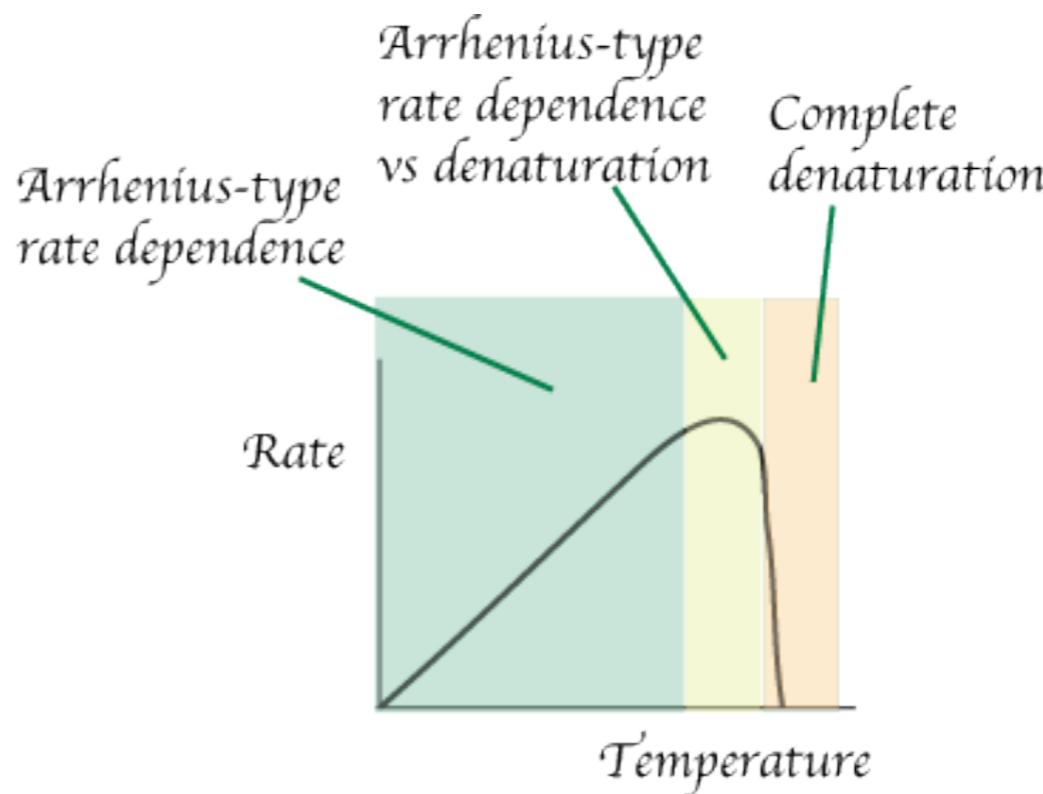
Going Up

The first part of the reaction rate profile (shown below in green), where the rate is increasing with the temperature, follows the Arrhenius equation. If the enzyme was completely stable even at high temperatures the reaction rate would continue to increase with temperature until something else

happened, like one of the reactants became limiting for example.

Plateauing...

The reaction rate begins to plateau then fall in the yellow highlighted section of the graph. This is due to the temperature approaching the point at which the enzyme begins to denature (and therefore lose activity).



A typical plot of enzymes optimal temperature (rate vs temperature). The shape is caused by competition between the rate increasing with temperature (in line with Arrhenius equation) vs the breakdown of the enzyme at higher temperatures.

Going down

At even higher temperatures (the red section) the enzyme is fully denatured and no activity remains.

The temperature at which the denaturation occurs is dependent on the structure of the enzyme, which in turn is related to its evolutionary origin. Thus, E.coli enzymes have evolved to cope with temperatures of around 37°C while enzymes from thermal vent bacteria have been forced to evolve in such a way that they can remain stable at far higher temperatures.

So an enzyme's optimal temperature is a trade-off between the Arrhenius-type dependence on temperature (the hotter the reaction, the faster the rate) and the instability of the enzyme as it approaches then reaches its denaturation temperature.

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