0.0 LECTURE NOTES SECTION DAY OF COVERAGE

MOLECULAR TECHNIQUES LECTURE NOTES 2013

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SUBMIT TO OUR SCIENCE WRITING PROGRAMS.

0.1 CONTENTS:

1.1 DNA: General Info. DAY 1

First off, some basic things to consider that ultimately affect how you envision the behaviour of DNA in your experiments:

1. **DNA is a simple molecule** No really! it's got your A, T, C and G, which from the eyes of a chemist, are pretty similar anyway. What does this mean? It means "predictability" which is a very handy thing in research.
2. **DNA is negatively charged** Very much so – lots of electrons whizzing around those oxygen groups.
3. **DNA has lots of ring structures** You remember things like pi orbitals, and the difference between polar and non-polar bonds? Anyway, the point is that ring structures are generally quite hydrophobic.
4. **DNA is a very robust molecule.** Spat in your sample? Probably o.k. Left out at room temperature over the weekend? Not a problem. Generally, the easiest of the biological macromolecules to work with).
5. **DNA work has lots of toys.** (Talk about accessories! You simply have a lot of options as far as what you can do with this molecule, whether it is a procedure, or a specific enzyme, etc)

Some general common sense rules that are worth applying to all DNA experiments, and frankly, while we're at it, to all molecular experiments.

1. **Know the experiment's level of forgiveness.** Another way of saying that it pays to know the chemistry of your procedure. Inevitably, each experiment has a degree of forgiveness, which is a really useful thing to know. This allows you to gauge your level of care, which in turn will reflect on your efficiency as well as your ability to troubleshoot.
2. **Get your sample as pure as you can.** Although this is related to point one, consider the following thought: any impurity is an outright invitation for caveats. i) If you have contamination, your subsequent steps may be hindered, or worse, unduly affected. ii) If you have a contamination, you might just lose your sample outright. i.e. DNA doesn't like nucleases. And nucleases are "everywhere."
3. **If you had to choose, be gentle rather that be rough.** Doesn't hurt to be careful when handling material. i.e. keep everything cold, since these enzymes are much more active at physiological temperatures. (i.e. use of "ice cold" this and that") Wear gloves, etc.
4. **Know the idiosyncrasies of your molecule:** At times, you need to be aware of specific nuances that apply to your particular "brand" of molecule. For instances, genomic DNA is different from plasmid DNA is different from a PCR product is different from an EST fragment.
5. **Think carefully about how much stuff you actually need.** Small amounts generally easier to work with, machines are smaller, take less time to operate, etc, etc, etc… So, if you know, you don't need much, then only use that amount.

1.2 DNA: General Workflow. DAY 1

In general, when doing molecular work with DNA sequences, there are a number of basic things you're trying to do. Often, instructional manuals will attempt to classify this ilst as a "general workflow" which is akin to a sort of flowchart of events.

In a nutshell, this tends to involve the following:

1. Figure out what it is you're interested in: This step tends to be based on literature search, or via an observation that one has deemed intriguing. Basically, this narrows the boundaries of the DNA code you're keen to explore. A defining feature of this step is also figuring out where this sequence might come from, as well as its likely size. Both of these parameters will greatly influence subsequent steps.

2. Physically retrieve the sequence that you are interested in: Once you know what you're interested in, next comes the actual work to get it. This might involve working with tissue/cells which tends to incorporate various cell lysis and nucleic acid purification procedures. Or it may entail working with material that is provided in purified form as a pre-determine lab sample. As well, there are often steps to modify this DNA sample so that it can be used for subsequent steps,: this might include finessing its size, or simply amplifying it to appropriate amounts. Note that the most common way of performing all of these steps often involves the Polymerase Chain Reaction method.

3. Insert your sequence of interest into an appropriate vector. Although this will be described more fully, harbouring your DNA of interest in a specific vector will define how you intend to "characterize" your sequence of interest. i.e. Do you want to sequence it? Do you want to transcribe/translate into a protein? Do you want to insert your sequence into a host cell for observation? etc.

In all, this workshop will cover a wide variety of different techniques that provides a myriad of different options for you to cover this "general workflow."

Example Workflow

> GET TISSUE
> LYSE CELLS
> PURIFY GENOMIC DNA
> PRECIPITATE TO CONCENTRATE
> PCR DESIRED FRAGMENT
> CHECK ON AGAROSE GEL
> PURIFY FRAGMENT / QUANTITATE
> LIGATE INTO VECTOR
> SEQUENCE VECTOR (TO CHECK FRAGMENT)
> TRANSFORM/TRANSFECT INTO HOST

2.1 DNA: Genomic/Plasmid DAY 2

We'll talk later about plasmids, so will focus primarily on genomic DNA.

Genomic DNA? What is it? Well, it's actually trying to retrieve "all" of the sequence found within an organism. Wiki also says it succinctly:

"In modern molecular biology and genetics, the **genome** is the entirety of an organism's hereditary information. It is encoded either in DNA or, for many types of virus, in RNA. The genome includes both the genes and the non-coding sequences of the DNA/RNA."

From a practical point of view, if you're working with bacterial or eukaryotic samples, you are trying to isolate all DNA from your sample. By the way, many species of genomic DNA samples involve big, complicated pieces that often end up looking like snot in solution.

Why are you getting genomic DNA? (a couple of reasons)

1. Profiling/Fingerprint: This is the stuff you use to compare blood/semen samples. Essentially, your genomic DNA can lead to some form of reproducible data that provides a unique representation of the sample (i.e. band profile on a PCR DNA fingerprint for example)

2. First attempt at cloning a new gene. If you plan on looking for something completely new and novel. Therefore, you will need to go to the original source of that code which is the genome. And although, you may be able to get protein or mRNA information before this step, you will ultimately need to define it within a genomic context (i.e. what chromosome is it on, promoter regions, intron/exon organization, etc etc.)

3. Characterization/Diagnotics. Lots of examples in basic research. I.e. you've made a transgenic/knockout organism, and you need to check if indeed, the modification has occurred – this starts with a genomic prep. You have an interesting phenotype and you want to look deeper. i.e. a disease state. This hunt may often start from a genomic prep.

4. Studying genome for clues into gene expression and regulation: Although, usually the cDNA for a gene gets a lot of attention (this is a DNA copy of the messenger RNA directly responsible for coding the protein), this edited form of the gene misses out a lot of stuff that may be interesting or relevant to your research. I.E. Study gene expression regulation, euchromatin nuances, tissue/developmental specificity, spliced isoforms, etc.....

WORDS FOR SNOT

Buse	Norweigian
Morve	French
Muci	Spanish
Soplia	Russian
Moco	Spanish
Bishi	Mandarin
BayTay	Cantonese
Takony	Hungarian
Schleim	German
Sline	Serbian
Mochat	Arabic
Chewae	Punjabi
Snot	Dutch/Flemish
Seda	Gujarati
Smarkotch	Ukrainian
Glut	Polish
Myxa	Greek

2.2 DNA: Cell Lysis. DAY 2

IT'S IMPORTANT TO REALIZE that there are many variations of the lysis procedure. Some are quicker, some are more efficient, some are more expensive, some only work in certain situations. In our PCR experiment, we performed a simple boiling step to get the job done.

If there's time in class, we'll also mention the SDS/proteinase K method. It being a very standard procedure (often embedded in kits).

NOTE: **Large pieces of Genomic DNA can shear.** (not actually a big deal for most procedures but why take the risk). In this case, dealing with a piece of DNA that is ~100kb + in size. No vortexing, no vigorous pipetting/snip the end of your tip before use.

EXAMPLE OF COMMON LYSIS PROCEDURE: (SDS/Proteinase K):

1. Place the cells in a "physiological" like buffer. There are many varants of this, but the below is a good example.
   Tris (buffering agent -> good @pH6-8) Often at the 10 – 20mM range.
   EDTA chelates divalent cations which are necessary cofactors for DNase activity (way of shutting down nucleases) Usually at the <10mM range.
   NaCl at physiological concentration (generally considered to be 100 – 150mM) Keeps all molecules happy (particularily proteinase k)/prevents unwanted aggregation.

   NOTE that TE buffer is a commonly used buffer which contains the aforementioned Tris and EDTA.

2. Then add the reagents which are responsible for the lysis.

   SDS, nasty ionic detergent/ good at breaking membrane, general denaturant (inhibit enzyme activity). Since DNA is so robust, not really adversely affected by SDS treatment. Note: When dealing with plant material, a very common detergent in place of SDS is **Sarkosyl**. This essentially behaves in a similar manner to SDS.
   Proteinase K->serine protease (works well at 55C), used because it is very effective and not particularily susceptible to SDS, and other denaturants such as urea. Proteinase K will chew up protein, which helps lysis in general and frees up the DNA from any protein gunk associated with it (euchromatin structures/histones, etc). Best used FRESH (*quite an important step).

   Incubation step generally a minimum of an hour. Although most will allow the procedure to go longer (this may be largely dependant on material being used – i.e. many procedures outline an overnight or >16hrs incubation time). I.E. mouse tails -> may want to go overnight).

2.3A DNA: Nucleic Acid Purification. DAY 3

There are a number of different ways to purify nucleic acids (indeed, with our PCR experiment we are just using special CHELEX beads which are designed to pull out positively charged ions). For the remainder of the course, we'll be utilizing three other methodologies described below.

A. DIY METHODS

PHENOL/CHLOROFORM

NOTE that ***Phenol*** is pretty nasty! It will burn. (Apparently, someone once told me that if you cover 5% of your body, you will die). Since we are using reasonable amounts, do PHENOL addition in fumehood, but all manipulations can occur at the nech. If you get it on you -> not panic mode. Quickly rinse off with cold water.

Phenol, by the way, is usually buffer saturated. (i.e. when you buy it or make it, the solution comes in two layers -> top layer is excess buffer, and bottom layer is buffer saturated phenol. This is because the pH is important - In order for our purification to work, we need the PHENOL at a neutral pH.(acidic pH is not good as DNA becomes soluble in phenol). The fact that it is saturated is also important because this means that any additional aqueous (i.e. water) solution you add will create it's own fluid layer.
This purification procedure works on the principle of **"differential solubility".**

1. To your lysate, you will add an equivalent volume **Phenol/Chloroform/Isoamyl** alcohol. (usually at a volume ratio of 24:23:1). Phenol – organic solvent/ nucleic acids not soluble at all. Therefore, DNA/RNA will stay dissolved in aq phase. Lipids and polysaccharides preferentially go into the phenol phase. Proteins will also selectively go into phenol solution.
2. FURTHERMORE phenol also acts as a denaturant, proteins denature form aggregates and will collect at the interface. You will see GUNK @ the interface.
3. **Choloroform**, also has same general attributes as phenol (as far as solvent properties) but also stabilizes the rather unstable boundary between aq and organic layers. Isoamyl alcohol also contributes to interface stability and also helps prevent frothing.

Generally you do this step ~2 or 3 times. The more times you do it, the cleaner your sample (you may even note that the interphase gets cleaner and cleaner with each step). Note this procedure is very reliable and does not lose much DNA yield. This is probably why a lot of labs still like to use it. (BACK EXTRACT: adding extra aqueous to your organic samples).

Sometimes do a final **Choloform** step. Here, the interface is a little trickier to handle. But this step is a good safeguard to prevent any organic carrying over to your final aqueous solution. Likely finish with a chloroform step because it evaporates easily (?). In other words, this give you the option of leaving the lid open to really make sure ALL your organics are gone from your prep.

2.3B DNA: Nucleic Acid Purification. DAY 2/DAY 4

B. KIT BASED METHODS

pI BASED KITS

We'll first be checking out **Invitrogen's ChargeSwitch** kits, which rely on **magnetic beads** that contain polymers with **neutral pI**. Basically, if the environment is such that the net charge of the beads can bind to the DNA, you can release the DNA by altering the pH environment of the solution in the other direction (i.e. change the charge of the polymer). In short:

If pH < pI (more acidic), then the net charge of polymer linker becomes more positive (higher affinity for DNA)

If pH > pI (more basic), then the net charge of polymer linker becomes more negative (lower affinity for DNA)

Fast! But you would need to invest in special magnet racks.

SILICA BEAD BASED KITS

(i.e. QIAprep) Nucleic acid purification kits more commonly involves the use of silica based beads which are specifically designed to interact with the very electrostatically charged nucleic acid molecules.
Our **QIAGEN** preps are a good example of how kits work in general:

1. Our DNA sample was first treated with a **salt solution** (something like NaI). This stuff is usually classed as a **chaotropic salt**, which is really a fancy way of calling a salt that is capable of altering structures by interacting with and thereby sequestering many molecules of water. In other words, salts like NaI can bind to several molecules of **water at high stoichiometry**, depriving water from the DNA structure in particular. This will alter its shape/charge/etc which makes it specifically bind to the silica beads (often pretreated themselves with ions to make them more amenable to specific binding).
2. We next do a wash, which in our case is called **NEW WASH** buffer. Not entirely sure what this is, but you can bet it doesn't affect the electrostatic state of your nucleic acid (likely an alcohol/water mixture). You don't want 100% water during the wash steps, or else your DNA will get the water back and fall off the beads!
3. Which is why the **final elution steps are water alone**.

THERE are lots of variations of these kits. Popular these days are versions that are set up in a **column format**. Arguably, most popular are those sold by **Qiagen**.

2.4 DNA: Precipitation.

DAY 3

PRECIPITATION OF DNA (alcohol and salt procedure)

To precipitate nucelic acids, you can add a high concentration of salt **(i.e. Sodium Chloride or Ammonium Acetate).** Why? Helps in the precipitation of the DNA in **EtOH**. NOTE: sometimes, you can also omit salt entirely (dependant on concentration of DNA). Salt will help neutralize negative charge of DNA (will also sequester the solvent molecules - in this case water) Salt will also interact with water, thereby weakening it's solvating prowess. This is commonly known as "**salting out**"

Use 100% EtOH. Time frame (show graph) EtOH generally helps because it is a much crappier solvent than something like water (which is very polar). DNA will tend to stay precipitated in **>65% Ethanol**. NOTE that efficiency of EtOH precipitation is dependant on a number of things. Temp, time, amount of DNA.

Can also use isopropanol for precipitation steps. RNA tends to stay soluble in this solvent (selective precipitation). which is why some people use it for this purpose. DNA will tend to stay precipitated in **>50% isopropanol**.

Once precipitated, DNA will tend to stay insoluble if kept in >**70% ethanol**. Hence, a lot of washing steps tend involve Ethanol based solutions, which is important as it removes any excess salt present in your sample. Final resuspension of sample in TE.

Graph below highlights how, generally speaking, unless working with very small amounts, DNA precipitates pretty quickly.

Ethanol Precipitation, *continued*

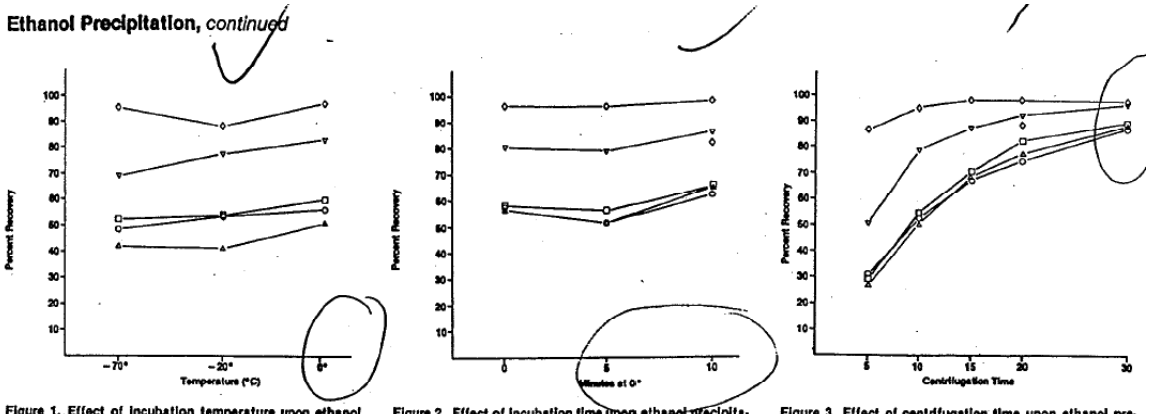


Figure 1. Effect of incubation temperature upon ethanol precipitation of DNA. All points are averages of two determinations. (○ = 0.6 ng, △ = 10 ng, □ = 100 ng, ▽ = 1 μg, and ◇ = 10 μg.)

Figure 2. Effect of incubation time upon ethanol precipitation of DNA. Note that two values are plotted for 10 μg, 10 minutes incubation. The lower value is believed to be due to experimental error. All other points are averages of two determinations. (○ = 0.6 ng, △ = 10 ng, □ = 100 ng, ▽ = 1 μg, and ◇ = 10 μg.)

Figure 3. Effect of centrifugation time upon ethanol precipitation of DNA. Note that two values are plotted for 10 μg, 20 minutes centrifugation. The lower value is believed to be due to experimental error. All other points are averages of two determinations. (○ = 0.6 ng, △ = 10 ng, □ = 100 ng, ▽ = 1 μg, and ◇ = 10 μg.)

2.5 DNA: Quantitation DAY 2

Spectrophotometry readings: Using **UV absorbance** to ascertain DNA/RNA amounts and purity. Ring structures can absorb UV wavelengths. BUT Lots of things have ring-structures (including nucleic acids, proteins, organics, detergents, lipids, the list goes on and on...)

i.e. Both DNA and RNA -> ring structure absorbs strongly at ~ 260nm and ~280nm.
Protein => some amino acids like W and P and Y also flouresce strongly at ~ 260nm and ~280nm.

Bottom line is that you have to take numbers with a grain of salt. Very rough estimate, since lots of things absorb at UV wavelengths. Also quite **sensitive to pH** which is why dilutions are often done with TE buffer at a particular pH, that was also used to calibrate the spectrophotometer.

You can also assess purity by looking at ratios of 260 over 280.
DNA A260/A280 ~1.8
RNA A260/A280 ~2.0

These are good ratios for purified product. If you get good numbers here, then maybe you can also get good quantitation numbers (NOTE: that the conversion for O.D. numbers to DNA amounts is the constant **50ug/ml per O.D260nm** value, LIKEWISE, the constant for RNA is **40ug/ml per O.D.260nm** value.)

Other wavelengths of interest include: A230 (good especially when phenol is in the mix, since phenol absorbs most strongly at 270nm).

Quantitation Assays: Usually rely on stoichiometry binding between reagents like Ethidium Bromide, SYBR dyes to correlate with a DNA amount. Generally a more robust, more expensive manner of determining amounts. ALONG the same lines, DNA fragment quantitation can be done by comparing band intensities in an ethidium bromide stained gel – i.e. use a marker as a gauge.

2.6 DNA: Agarose Gel Electrophoresis. DAY 1/2

Using Agarose. Polysaccharide polymer. Used because of its ability to form pore sizes capable of resolving ~ 0.2kb to ~60kb, (200bp to 60,000bp).
Essentially, creates a big mesh of fibers that your DNA has to pass through. Electrical charge is the driving force and things will separate according to size.

DNA works well in this set-up because for things to separate in correlative manner, all DNA species generally have about the same charge density. ALSO, in our case, the DNA we are looking at has been cut with restriction enzymes - therefore all DNA fragments are predominantly linear in shape.

Loading buffer:
Glycerol: thickens sample up so that it doesn't float away after you load it into the well
0.1 M EDTA stop reagent for the assay
1% SDS help denature the RE stop the reaction
0.1% bromophenol blue. Dye. Helps you visualize sample when loading. Will run towards +electrode. Can use it as a rough idea of where your DNA may be running (dependant on gel%)

Running buffer:
Tris Borate EDTA (**TBE**) In this case, borate is your ion, which allows the generation of an electric field in the gel set-up.
Your common alternative is **TAE**: Here, acetate acts as an alternative ion - is often used because it works and is much cheaper. Need 50c for 10L of TAE, (need $10 for 10L of TBE)

But tris/borate has a significantly better buffering capacity, which means gel running is more reliable especially at **high voltages** (for speed), or **long running times** (i.e. overnight**)**. BUT borate (when preparing the gel in microwave or oven) also forms complexes with the agarose sugar monomers/polymers. Can be a problem if using procedures to isolate band from a gel (i.e. melting of gel is required), although most band extraction kits come with chemistry to deal with this.

Quick mention of **Sodium Borate** systems (cheaper, *very* fast, but resolution can be affected – Kern and Brody, *BioTechniques* Feb 2004)

Visualizing the DNA:
Most people now use SYBR stains Examples include **SYBR SAFE,/SYBR Green I/ SYBR GOLD** which are exceptionally sensitive nucleic acid gel stains with bright fluorescence when bound to dsDNA and low background in gels, making it ideal for detecting dsDNA in gels using laser scanners or standard UV transilluminators. Compared to Ethidium Bromide, these stains are generally about a hundred fold more sensitive, and less carcinogenic to boot! (a bit more expensive mind you, and you will need a particular filter in your light box to see it – although this isn't that expensive). Stain can occur during run, or after run.

Alternatively, you also have **Ethidium Bromide** (carcinogen), also sensitive stain that interchelates DNA (which has the added ability of slightly uncoiling it). Need to use a UV lightbox to see it – take care to use appropriate eye shielding.
Some labs add EtBr into gel. Some add it after gel has run (i.e. stain with solution containing EtBr) -> adding it into the gel is much easier, but if the apparent molecular weight of closed circular DNA is particularily important to you, it may be worth adding after so that it doesn't affect its molecular weight.
FOR EtBr, you should inquire at your health, safety department, as many research facilities are phasing out EtBr use.

3.1 CLONING: What is a Vector? DAY 2

Vectors are essentially shuttles that carry your DNA. At the most basic level, almost all vectors have the following traits (sorry for the long long titles):

UPKEEP: Naked DNA (no matter how important it is to your research) does not upkeep itself. Therefore, to do this, you need your "**DNA of interest**" to replicate independently of integration into host genome. i.e. the vector allows the use existing replication machinery of host organism. OR another way to get around this, is for the vector to contain information that can drive integration into the host genome.

PLACE TO PUT DNA IN: all vectors generally have defined places where you can insert your DNA of interest. This is usually at a place called the **Multiple Cloning Site (MCS**), and is governed by (i) convenience (i.e. easy to put something in this area), or (ii) geography (you put your DNA "here" because it is next to some element – say a promoter – that you want to use to act on your DNA of interest." Vector nomenclature, while hardly poetic, is usually a reflection of what the **MCS** and it's surrounding geography is all about.

SOMETHING THAT LETS YOU KNOW IF THE VECTOR IS IN THE ORGANISM:
All vectors will generally contain a **selectable marker** (notable exception is where GM crops are involved). This is usually a gene that confers resistance to some sort of drug. Commons ones used in research field include antibiotics such as **ampicillin** (common for e.coli), and **neomycin** (common in mammalian cell cultures). On the other hand, some organisms will rely on **auxotrophic** traits. This turns up a lot in yeast work, whereby host cells are mutant and unable to make a nutrient like **tryptophan**. Sooo... In order for these strains to grow, you either need to supply it in media, or you insert a vector into the yeast that reconstitutes the ability to make **trp**..

SOMETHING THAT LETS YOU KNOW IF THE DNA OF INTEREST YOU PUT IN IS ACTUALLY IN THE VECTOR, WHICH IN TURN IS IN THE ORGANISM:
There are a number of strategies that let you determine whether your DNA of interest actually made it into the vector. These strategies often entail some form of **insertional inactivation**, or ligation trick (i.e. CIP assay, T overhang systems).

3.2 CLONING: Choosing a vector I DAY 2

There are two general things to think about. First of all is the principle that size matters. i.e. **How big is my DNA of interest?** Is it a relatively small <10,000bp cDNA, or is it a chunk of chromosome >300,000bp? In this respect, here is a run down of common vector types:

1 PLASMID VECTORS: such as **pUC18**. (used in our experiment):
a) selectable marker: **ampicillin resistance cassette**. Therefore if you want to ensure that your bug has gotten the plasmid, then you just test for the bug's ability to survive under drug treatment.
b) **Blue white screening**: which sort of works like this -> Things to point out ->*LacZ* gene (**b-galactosidase**) Bugs will constitutively express a lac repressor. Which interacts with *lacUV* promoter. Therefore, no transcription of beta-galactosidase. But if you add lactose -> lac repressors fall off promoter. What you usually use is IPTG (**isopropyl-b-D-thiogalactoside**) which is an analog of lactose.
Anyways, if there is NO insert -> *lacZ* gene will get transcribed. Codes for a b-galactosidase.
If there is an insert -> something else will get made. Frameshift mutations/insertional inactivation. Will not get *lacZ* transcribed.
c) MCS: Small area of the plasmid where the vector designers have decided to put lots of unique restriction sites in a small area. I.e. try to make it as easy as possible for you to have a convenient nice restriction site that works for your cloning strategy.

2 BACTERIOPHAGE VECTORS: (very old school – but will cover as background necessary): fancy word for virus that infects bacteria. Good for moving around ~20kb worth of DNA. Good example of bacteriophage vector is the "**lambda DNA**."
This thing is about 49kb in size, containing essential and non-essential regions as outlined in figure. Essentially, the key idea is to take advantage of the viral pathways to get your DNA inside. I.E. you can package your DNA into a bacteriophage structure, which can infect and shunt your DNA into the host cell (a bug). ONCE the DNA is inside, the bacteriophage will still go about making more DNA, and making all the necessary components to make more of itself. This is essentially how you upkeep your DNA.
The only technical difficulty is how you would "harvest material" . i.e. your DNA is not in the bacteria per se, but rather is inside the bacteriophage, and the logistics of getting bacteriophage cultures are more difficult (i.e. plaques on a plate)... I.E. WHY phage systems are now rarely used. The other reason is because of...

3. COSMID VECTORS: Good for packaging 30-45kb of material. Think of it as an unworthy bacteriophage. I.e. Even essential regions have been deleted (this is why it can carry more than normal bacteriophage vectors) but special **cohesive sites (COS SITES)** are still present. Therefore, still has enough genetic information to get packaged but not enough to "be a bacteriophage" i.e. doesn't have information to make more bacteriophage.

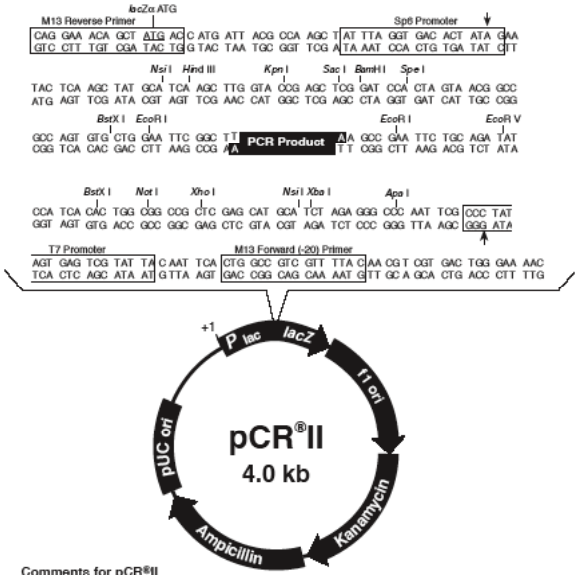
NOW you can use a KIT to supply materials (bacteriophage bits and packing machinary) needed for packaging of your DNA into a "pseudo" bacteriophage. Then your bacteriophage can infect a host organism, and effectively transform it (deliver your DNA). ONCE, DNA gets in, the COS sites will direct recircularization and you now essentially have a **BIG PLASMID** (therefore can use normal plasmid techniques for isolation).

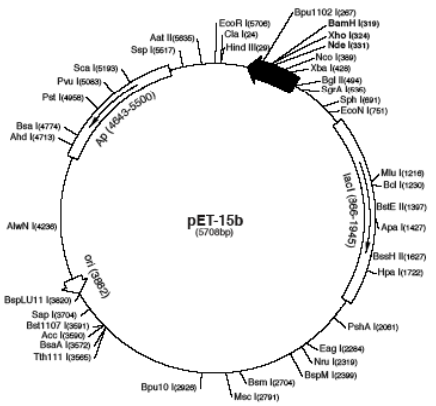
4. ARTIFICIAL CHROMOSOME SYSTEMS – BACs and YACs: "The realization that the components of a eukaryotic chromosome that are required for stable replication and replication in YEAST, are VERY SMALL very DEFINED sequences" CAN TAKE UP TO 1 Mb!!
NOTE that **BACs** or **Bacterial Articifical Chromosomes** (sometimes known as **PACs** or **plasmid artificial chromosome**) are more common nowadays. Similar to YACs in principle in that they allow incorporation of a huge amount of DNA **(up to 300kb).** The difference is that you can work in *e. coli* which is much easier than yeast manipulation

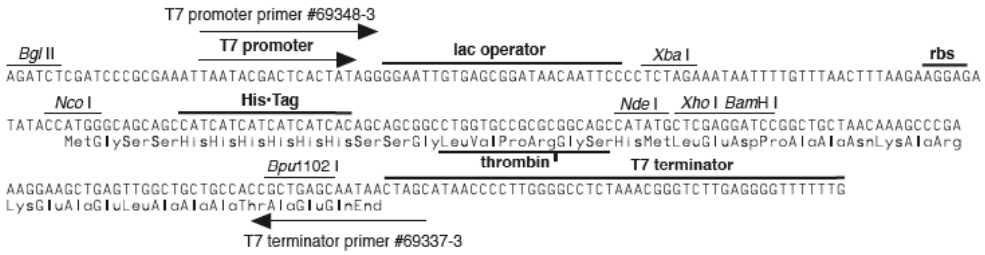
3.3 CLONING: Choosing a vector II

DAY 2

The other constraint, of course, is what you want to do with your DNA of interest, and quite literally, to say that the **world is your oyster** is very much a reality. Here are two examples of the sorts of things you need to think about.







pET-15b cloning/expression region

3.4 CLONING: Plasmid Preps. DAY 4

Isolating plasmid DNA from other types of DNA (i.e. genomic) is actually very simple. In short, it usually involves a denaturing step, followed by a quick renaturing step. The IDEA is that plasmid DNA being much smaller, can renature relatively easily - consequently, once back to normal it can go into solution easily. Something like genomic DNA will have an incredibly hard time renaturing because it is simply too big and too complicated. It doesn't renature effectively and instead tangles up and precipitates out.
IF YOU THINK ABOUT IT, you have now separated your plasmid (in solution) from your genomic (out of solution) prep. You simply have to centrifuge away the genomic pellet, and you are left with your plasmid DNA (+ all the other cellular crap like proteins, etc etc --> however, now you can use any standard DNA purification procedure).

Most common way of doing this is known as the **ALKALINE LYSIS METHOD** (which also makes an appearance in practically all kit based plasmid prep methodologies)

Here the idea is to chemically denature and renature.
1. Need to open the cells up. NaOH and SDS. Ruptures cells, and denatures everything. Low pH specifically breaks H bonds in dsDNA.

O.K. so your test tube is now this messy mix of denatured stuff.
Genomic DNA (big) -> denatured.
Plasmid DNA (small) -> denatured.
Proteins -> denatured.

THROW IN salt that is acidic (KAc pH4.8).
Salt helps in the precipitation process. Acid -> causes things to go back to neutral. DNA can renature BUT HAPPENS VERY QUICKLY.

Large DNA renatures as a MESS. Small DNA renatures O.K.
So, genomic DNA will precipitate out (should see a white mess), but your plasmid DNA will now be in solution. TA DA!
Move onto purification/precipitation step.

ALTERNATIVE QUICK AND DIRTY PLASMID PREP METHOD:
Via causing the cells to lyse by using STET + lysozyme. and then the trick is to boil and then cool (this provides the denature and renature step)

4.1A ENZYMES: RE digests I

READ ON YOUR OWN

Generally speaking, molecular biology enzymes are like your high maintenance buddies. It really pays to know them well, and they will end up being very useful to you. We will focus on a lot of details with regards to **Restriction Endonucleases (REs),** so that you're aware of the sorts of nuances entailed.

NOTE: Enzymes are pretty expensive so you want to keep them cold and keep them clean. This means you always use a freezer box when keeping them at your bench. This means that if you don't have a freezer box, you do everything at the freezer! This means no double dipping.
NOTE: bug a vendor and get some free catalogs – these are very useful reference source. Especially useful is the New England Biolabs catalog.

1. WHAT IS A RE?: Think of it as an enzyme that cuts or degrades DNA but in a very specific manner. There are actually several classes of REs, but the most commonly used are affectionately categorized by the type of cut they leave. In shop talk, the two common types are referred to as "**sticky end**" and "**blunt end**" cuts.
In terms of physiological or historical background:
*All started in 1970. Hamilton Smith at John Hopkins University studying Hemophilus influenza. -> extracts from this bug could cleave DNA at very precise points. Turns out, bugs have sets of restriction enzymes which are believed to serve as their "immune system" of sorts protecting them from virus infection (viral DNA gets cleaved). To date well over 3000 diff REs to choose from, which were found from screening >10000 bacteria.

2. WHY ARE YOU USING A RE?: Generally speaking, you are trying to create a specific cut, often to obtain a particular fragment or chunk of DNA. However, RE can be used to simply "alter" the size of a DNA sample to fulfill a particular purpose – a good example is to use a RE to cut genomic DNA down to size for effective agarose gel viewing. In some respects, this ties into the idea of WHICH ONE TO USE?

3. HOW MUCH TO USE?: Amount? Very simple. 1 unit of enzyme is enough (theorectically) to do whatever it's suppose to do (in this case "cut") 1ug DNA in a specific amount of time at the defined temperature (in a reaction volume <50µl)
BUT it's interesting to point out that every enzyme can come with its own personality. (which, in turn, can be dependant on the batch or company they come from). Common restriction enzymes generally work quite well, and arguably, any enzyme that is not "fancy" is more reliable.
NOTE that generally speaking blunt cutters seem to be more finicky than sticky cutters.
ALSO NOTE that researchers often add more enzyme than is theorectically needed (likely to ensure that the reaction will go to completion, but probably it's simply impossible to pipette anything less than 1.0µl accurately anyways).

4.1B ENZYMES: RE digests II

READ ON YOUR OWN

4. CONDITIONS OF THE REACTIONS: enzymes will always come with "special" buffer. Generally, the buffer is all about making your enzyme happy - keeps it at a particular pH (usually around 7 to 8), a particular salt concentration, and has cofactors like Mg in it.

Salt is the most varied consideration in getting a reaction to work (In fact, in the not so recent past, buffers use to be separated simply as low, med and high salt.). This is particularily true if you wanted to cut your DNA with >2 different REs (double digest). In the past, you would cut first with RE that worked at lower [salt] / bring up the [salt] for second enzyme. (this would be an example of a tough dilution question)
Nowadays, there are handy dandy buffer activity charts – lists % activity for an enzyme under different buffers.

5. OTHER THINGS YOU CAN ADD. **Rnasel** – get rid of extra RNA in your prep. **BSA** – (albumen) generic protein. Proteins have an uncanny ability to destabilize when solvated in a VERY dilute concentration (why? Too much water solvating protein) Companies try to fix this by adding more (generic) protein in solution so that overall [protein] is higher. **Spermidine** (* not used as much anymore): Added b/c of its ability to interact with polymerases and other DNA binding proteins. **DTT:** reducing agent. Dithiothrietol -> during purification process by company -> May get unwanted disulfide bond formation, which would affect enzyme structure.

6. TIME AND TEMPERATURE: only guidelines. In terms of RE, the unit value is usually under time constraint of 1 hour. Therefore, 1 hour is the bare minimum. However, it is considered safe to leave digests going overnight, which is often suggested if it is crucial for you to get complete digest. Some enzymes sensitive to high temperatures: useful if you want to inactivate it (heat inactivation).

7. QUALITY OF YOUR DNA SAMPLE : As a whole, restriction digests are very forgiving. BUT no organics (i.e. phenol or chloroform), as they will affect RE viability.
METHYLATION: will also affect the ability to be cleaved. IN actual fact, this is what protects the bacteria from its own restriction enzyme defence system. I.e. it has a corresponding methylation enzyme that protects bacterial DNA.
This is why most e. coli strains that you work with have dam- and dcm- nomenclature. This means that these methylation enzymes have been knocked out so that they won't affect the ability to cut DNA isolated from these bacteria. -> important point since all organisms capable of methylation to some degree.

7. STAR ACTIVITY: This is when your RE gets sloppy, loses specificity. The point being to remember that these are enzymes, and to think of the sequence specificity as its substrate. Things that can cause non-specific cleaving include things that can affect the enzyme-substrate relationship:

(i) beyond saturating kinetics: too much of anything
(ii) change active site: buffer conditions, contaminants.

8. STOPPING REACTION: Easy. +EDTA or heat the sucker, as some enzymes are heat sensitive. (Stick in a 65C waterbath for 10min). When in doubt, purify the DNA out, by doing something like the phenol chloroform.

4.2 ENZYMES: Ligases/Phosphatases DAY 2

LIGATION REACTION: Ligase: enzyme that can anneal two pieces of compatible ends of DNA together.
The important nuance to consider is that the 5' end of your DNA ends need to be phosphorylated.
From a compatibility point of view, if you are dealing with sticky ends (i.e. they have the overhangs), the overhangs MUST be complementary to each other. Blunt ends can be useful in that all blunt ends are compatible with each other. NOTE that ligation works way better for sticky ends then for blunt ends.

T4 Ligase is considered the workhorse for this particular procedure. Conditions vary greatly depending on the one you use, and where you bought it from, but nowadays, the procedure can be done at room temperature in as little as 15 to 60 minutes.
** can't directly check if ligation worked except by virtue of getting bugs to grow after transformation.
->useful to try different insert:vector ratios. Molar ratios that often tried 1:1, 3:1, 5:1 etc. Generally more insert is better, but every ligation is different.

CALF INTESTINAL PHOSPHATASE (CIP) Can also use alkaline phosphatase: Used to dephosphorylate 5' phosphates. Useful to desphorylate a cut vector, which prevents recircularization of vector unless an insert can get in and provide the 5' phosphases. (i.e. it's a way to make sure that any colonies you see on the ligation plates MUST have an insert in the multiple cloning site)

5.1 TRANSFORMATION: DNA into your host. DAY 2

The story so far… You have just done a ligation reaction where you have added a cut vector and a fragment together. ->You want to know if the ligation worked…
FIRST: you are going to put the ligation mix into bacteria, and plate the bacteria onto ampicillin supplemented media.

Therefore, in order for bugs to grow -> MUST have ampR. Therefore must have plasmid. But plasmid must be circularized in order for it to get continuously and independantly replicated.
Therefore, LIGATION HAS to work to get all of this.

Is the insert in? Use the white blue system (your plates will also have **X-GAL**)

TRANSFORMATION:
TRANSDUCTION: all three about getting DNA into your organism.
TRANSFECTION:

Transformation is DNA into a **prokaryote** (except for yeast), Transduction is the use of **infection** (i.e. viruses) to get DNA inside, and Transfection is DNA into a **eukaryote** (except for yeast).
With bugs: Two main techniques: **CaCl2 and heat shock treatment** and **electroporation**.
In both cases need to make **COMPETENT** cells. Cells who are primed and ready for acceptance of DNA. Generally involves a series of growth steps so that bugs are at the just the right stage of growth.

In **CaCl method -> competent cells have been treated with CaCl2. Essentially the membranes are thrashed around. BOTTOM LINE: cells are **delicate**.
NOBODY REALLY KNOWS WHY or HOW CaCl2 + heat shock works, but it does. AND… it is easy to do!
Ice step is believed to allow the DNA to adhere to membrane. Heat shock may make the membrane move around more (fluid membrane). Membrane gets weaker, holes get bigger? DNA falls in?
37 incubation allows thrashed cells to recuperate. Also gives time for plasmid to replicate so that bugs are ampR

Electroporation: will give much higher transformation efficiencies. ZAP a current through the bugs.
Again nobody really knows whats going on. A bit more versatile (i.e. most organisms don't have a heat shock procedure). Microbiologists working on other bugs pseudomonads, bacilli, strept etc etc will use this piece of machinary.
Competent cells are much easier to prepare. Procedure very quick. MORE EXPENSIVE… those cuvettes are generally not reusable, machine cost several thousand dollars.
Different bugs use different setting, but the main idea is to vary amount of voltage applied. Dependant on the bug, the cuvette, etc etc. All of this will translate to some magic current value which is what causes this whole thing to work.
BECAUSE OF THIS, you need to be careful with salt content in your cells or ligation mix. Making competent cells usually involves successive water washes.

V=IR

If you are not careful because of salt, or air pockets, etc etc.. You get something called **ARCING**. This is a small explosion happening, which usually consists of a bang (to varying degrees), cracking of the cuvette, a flash of light. General scariness but not at all dangerous.

Transfection: getting DNA into **eukaryotic** cells: Most common method is still to use **electroporation**. ZAP the buggers!! Other alternatives include things like the **Gene GUN** (Ballistic approach), and **viral** mechanisms.

6.1 PROTEINS: General Info DAY 3

First off, some basic things to consider that ultimately affect how you envision the behaviour of protein in your experiments:

1. **Proteins are not so simple** Basic idea is that with DNA, you've got 4 different components (nucleotides), which are all essentially quite similar anyways. WITH proteins. **20 different amino acids**. All with **different** sorts of properties, **different** sorts of charges, biochemical attributes. Consequently, proteins as a population are tough to predict.

2. **Proteins can be high maintenance:** Add to that the fact that proteins can often be high maintenance/delicate themselves, and you have an invitation for frustration. I.e. they degrade, enzymatically go off, they aggregate, they complex, etc.

3. **Protein work still has lots of toys.** (Although definitely not to the extent that DNA has. Also, one of the most powerful reagents you need – which may not be commercially available – is a specific antibody)

4. **Protein work is more fun(?):** interesting to note that when pressed for opinion, most will say that protein work tends to be more rewarding, interesting, and challenging.

We're going to focus on two techniques, the first of which (show Nature Table of Contents) will be the **Western Blot analysis**, and the second is the more fancy **2D gel electrophoresis analysis**.

BE CAREFUL! keep things cold, but be sensitive to the structure of the proteins you work with – even cold temperatures can be detrimental to your experiments.

QUANTITATION: (a few options)

- Run a gel. Compare band with preweighed amounts of standard protein (like albumin) -> works really well, kind of labour intensive.
- USE colorimetric tests. BCA/Coomassie Blue tests works best. Easy to do, but can be very susceptible to chemicals that are commonly used in the buffers you store your proteins in (i.e. presence of detergents, tris, etc etc)
- ABSORBANCE at 280nm. very rough! ~1.0 O.D. = 1mg/ml of protein (but as an example for IgG it's closer to 1.4mg/ml)

6.2 PROTEINS: SDS PAGE. DAY 3/4

GENERAL IDEA: You have a slurry of proteins, i.e. when you lyse a particular population of cells --> millions of proteins. YOU WANT to see if a specific protein is present in this slurry and you are particularily interested in observing its molecular weight at the same time.

HOW DOES IT WORK?
SAMPLE PART: As proteins are quite diverse (many sizes, many shapes, many variable charge properties – all things that will affect mobility), you need a reagent that can equalize these parameters.

This is what your sample buffer is all about.
* has dye (so that you can see stuff)
* has glycerol (makes sample heavy so that sample will flow into wells)
* has SDS (VERY IMPORTANT) coats proteins with negative charge (now all proteins have same charge density), and denatures proteins to uniform shape (rod-like shape). Now all proteins have equivalent shape as well.
* has beta-mercaptoethanol or DTT (dithiothreitol) these are reducing agents. will reduce and break disulfide bonds.

GEL PART: first you need to RUN A GEL. Often called **polyacrylamide gel electrophoresis (PAGE)**.
(NOTE that acrylamide is a nasty nasty neurotoxin). Acrylamide + bis-acrylamide form basis of mesh like structure. TEMED and ammonium persulfate actually catalyse the crosslinking. More specifically these two cause the production of free radicals resulting in covalent cross-linking of your acrylamide fibres.
- Amounts of TEMED+ammonium persulfate dictate rate of polymerization
- Amounts of acrylamide+bis-acrylamide dictate pore sizes in mesh.

Laemmli system. (also called a discontinuous gel system) – a system to ensure you get nice tight bands.

THE PLAYERS:
STACK (low %acryl, pH6.8, Chloride ions) Cl- (leading ion) moves faster than proteins.
RESOLVING: (set %acryl, pH8.8, Chloride ions)
RUNNING BUFFER: glycine ions. (trailing ion)

Start gel. Cl in stack and resolving gels moves quickly, glycine from buffer enters stacking gel and moves slowly. You can envision that the Cl ions move SO fast, that there is an area of low conductance between the Cl and glycine ions. Proteins are caught in the middle and consequently move slowly.
When proteins reach boundary between STACK and RESOLVE, they see the resolving gel which has smaller pore sizes making it tough for the protein to enter (this is also made worse by the fact that the Cl ions are so far away that proteins aren't very attracted towards the positive electrode). **BUT**, when the **slow** glycine ion reaches the interface it will become a much **better** glycine ion (because of the different pH).
NET EFFECT is that all your protein sample will tighten into a sharp band at the boundary, and then get separated according to size (proteins will be able to move into resolving because the glycine ion is pulling it towards the positive electrode)
NOTE that because of this set up, the slowest part of the gel run is usually the stack.

STAINING:
coomassie blue (0.3ug to 1ug per band) can probably see a band as faint as 100ng
silver staining 2 to 5ng (MUCH MORE SENSITIVE) very dirty procedure. If you want figure quality data -> equipment must be ultra clean!!
ponceau red: reversible stain.

6.3 PROTEINS: Western Blot. DAY 5

WESTERN PART: essentially a procedure that allows you to probe for a specific protein using an ANTIBODY. main idea. USE A MEMBRANE. (this is why it's called a blot).

TRANSFER: There are two main types of membrane. (i) Nitrocellulose and (ii) Nylon based mambranes. Essentially, both rely on being hydrophobic to adhere to molecules of interest (i.e. protein, but also DNA and RNA). Standard membranes generally have a net negative charge.

nitrocellulose is cheaper, lower binding capacity, can be brittle, not fancy.

nylon is more expensive, higher binding capacity, not brittle, lots of fancy variations out there.

O/H picture of transfer. MOVE your proteins from the gel to the membrane by electric current.

DEVELOPING THE WESTERN: classical blot procedure: BASIC STEPS are...

- **STEP 1:** incubate membrane with **BLOCK buffer** (~2hrs) solution that covers the rest of the membrane with generic protein (usually 5.0% milk powder or BSA)
- **STEP 2**: WASH membrane a bit (~5min) (usually **TTBS**, tween-20, tris buffered saline)
- **STEP 3**: incubate membrane with PRIMARY Ab (~2hrs): Ab that is specific for your protein of interest.
- **STEP 4**: WASH several times (~1/2 hr)
- **STEP 5**: incubate with SECONDARY Ab (~1hr): Ab that is specific for the Fc portion (constant region) of the primary Ab. Also has an enzyme attached to it that can convert some substrate into an observable product (=MEANS OF DETECTION). i.e. it glows, it changes colour, etc, etc)
- **STEP 6**: WASH SEVERAL TIMES (~1/2 hr)
- **STEP 7**: ADD substrate.

TOTAL TIME: ~6hr procedure.

QUICK METHOD (millipore) Uses **PVDF** membrane **(immobulon P)** -> is highly hydrophobic form of nylon based membrane that particularly "hates water"
THEREFORE, in theory, solutions can't actually touch membrane unless the membrane is prewet. **DRY your membrane** for one hour first. Consequently, the block step is redundant, and all incubation steps are considerably faster because you are not incubating the “whole” membrane, you are just incubating the “parts” of the membrane that have proteins on them.

THEREFORE all steps much shorter.
PRIMARY Ab -> 40min to 1hr
SECONDARY Ab -> 20min to 30min
WASHES in seconds.

This quick method works. but you should try it first in comparison to the classical technique to convince yourself that the technique is comparible. Some tricks associated with the technique include a quick **20minute block step** should your antibody be prone towards non-specific binding. ALSO, you can speed up the drying process by immersing the membrane in 100% methanol 10min, and then drying 10min.

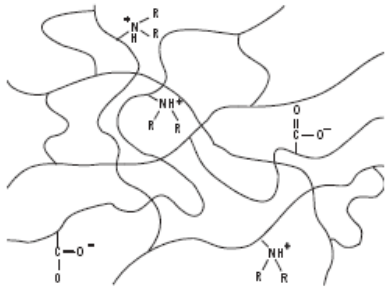
6.4 PROTEINS: 2D gel electrophoresis. DAY 2/5

Essentially, this procedure is now way easier to pull off than even a few years ago, and is largely due to major improvements in providing immobilized pH gradient strips (as opposed to the carrier ampholyte gradient tube gels). As you will see, nowadays this procedure is relatively straight forward if you have access to a set-up that makes life generally easier.

PREPARATION OF PROTEIN SAMPLES: This is arguably the most important part, and the trickiest to advise on. In essense, you want to use a reagent recipe that result in **controlled solubilization, disaggregation, denaturation, and reduction** of the proteins in the sample. Meanwhile, you want to make sure that during this step, you don't have too many things that can (i) interfere with the overall ionic environment of your sample, (ii) affect the visocity of your sample, (iii) promote protein degradation (iv) reverse the reduction process, (v) dilute the sample too much, and of course (vi) mask the pI of your proteins. Basically any and all crap in your sample can seriously make things messy, ugly, and not at all reproducible.

This is why, things like lysis procedure, non-ionic detergents, reducing agents, carrier ampholytes, BUT the bottom line is to do your homework for the latest greatest preparation protocol for your particular type of tissue.

THE RUN: **First dimension**. Isoelectric Focusing essentially takes advantage of settling your protein specis at its pI where it essentially loses its net charge and therefore stays put. Consequently, the big deal is to use a **pH gradient** in your first dimension. Olden days would use a mix of **carrier ampholytes** within an acrylamide mix – here, the amphoyltes would migrate according to their own prefer pI, settling at a particular place in the gel, and thereby provide their specific buffering capabilities at a defined point in the gel. Basically you get a continuous pH gradient. BUT, this methodology is a bit flawed because the amphlytes can drift, are often complicated in content (therefore hard to characterize properly), and also the gel set up had a tendency to break. In the past, this particular step may have been arguably the trickiest part. BUT NOW:



We have **immobilized pH gradient (IPGs)**. Here a gradient is incorporated covalently into a polyacrylamide gel at the time it is cast, using special buffers (one acidic and one basic, in different ratios along the gel).
Furthermore, casting is done on a plastic backing to provide better reproducibility.
There is usually a **rehydration** step with IPGs
NOTE that IEF runs generally have very low current values.

Second Dimension: First dimension needs to be **equilibriated** so that it is amenable for the second dimension. This step, usually involves the following reagents: **Buffer (happy pH), Urea + Glycerol (elctroendosmosis), DTT (reducing agent), SDS (ionic detergent), Iodoacetamide (alkylates thiols), Dye (so you can see the run)**. Once this is done, the rest is easy – basically a normal SDS-PAGE.

DETECTION/STAIN: Radioactive > Silver Stain > Coomassie Blue

7.1 RNA: first steps DAY 3

BASICALLY, RNA work is not fun. Although it can be argued that due to its overall trendiness, they have been a lot of recent developments in making it more fun to work with. As a molecule, it is very similar to DNA (the most notable chemical difference is its flexibility), but suffers because the **nucleases (RNases)** that can destroy it are notoriously **very stable and very difficult** to get rid of.

Oh yeah, and did I also mention that RNases are **EVERYWHERE.** Oh, and they don't require divalent cations as cofactors. If you want a sense of just how stable these suckers are, consider the fact that they are generally unharmed by autoclaving procedures or exposure to temperatures as high as 150C.

Because of this, you have to take the following precautions.

MUST USE ASEPTIC TECHNIQUE. Segregate all your equipment, your space, even yourself if you have do. Different people exhibit different levels of care, which may depend a lot on your specific locale, but a general rule is to be as careful as possible. Wear gloves; keep things cold.

USES DISPOSABLE PLASTICWARE: Essentially, there are different levels of care. There is sterile plastic ware that you take good care of, there are RNase free grade plastic ware that you take care of, and it's also a good idea to use filtered tips if possible.

DECONTAMINATE OTHER EQUIPMENT FROM RNASES: There's a couple different ways of doing this. For instance , with glassware, you can thoroughly clean with detergent, rinse, and then ovenbake at >250C for >4hrs. Things that are not oven friendly (please don't put your gel box in the oven) often use **DEPC**. *(Diethyl pyrocarbonate)*

Show O/H DEPC treated Barbie doll

DEPC is a very useful reagent for making buffers and equipment RNase free. WHAT DOES IT DO? DEPC results in a covalent modification of nucleases rendering them inactive. **Histidine modifier – imine -> carbonate >NH >N-CO3**
IN GENERAL, Work in fumehood when treating water/buffers or equipment. Usually let your buffer + DEPC or equipment/glassware **incubate at ~0.1% DEPC** concentration for **>12hrs**. Then leave it in fumehood O/N with venting (lid slightly ajar) SO THAT FUMES ESCAPE. Or autoclave for faster evaporation (if possible). NOTE that technically, things are "**DEPC treated**" meaning that the DEPC is no longer present.

Some nuances regarding DEPC:
- comes as a liquid/ can buy at different percentages.
- **stinks**. It is an organic solvent. Handy b/c you can tell its presence by its smell.
- DEPC can **explode!** When in contact with water creates CO2 and Ethanol. If not careful and kept in sealed container can lead to excessive pressure buildup.
- **Can't treat Tris solutions** with DEPC . Tris is full of amines that DEPC will spend more of it's time modify the wrong thing

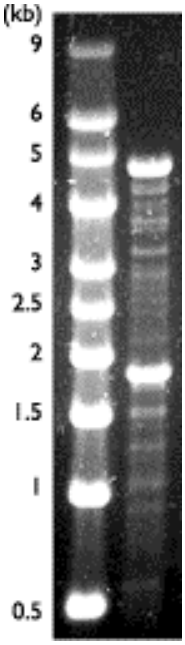
TRICKS:
- Lots of **quickie protocols** out there in the web especially, although be careful.
- **Chloroform** also denatures Rnases, (rinse with chloroform). Can be quite **harmful** to plasticware.
- Fancy reagents like "**Rnase AWAY**." "**RNase ZAP**" Can wipe apparatus clean. Not harmful to plastics.
- **RNase inihibitors**: so many classes of RNases to worry about **(A, B, C, I, T1, T2...)** can get expensive.

7.2 RNA: Isolation and Purification. DAY 3

We are extracting RNA from your cheek cells using a product called Trizol.
Although this reagent is essentially a proprietory product, it is undoubtably based on the familiar Phenol+Guanidium Thiocyanate procedure. HERE, Guanidium thiocyanate is often classed as a denaturant although it is also considered a chaotropic salt (sucks water)
This methods works in an analogous fashion to phenol chloroform extraction except that in this case, you also want to get your DNA to go into the phenol layer (therefore, you are left with just RNA in the the aqueous phase). This Trizol business works because RNA is still water soluble in a high molar guanidium thiocyanate solution whereas proteins and DNA is not. Consequently, the insoluble components will tend to go to the organic phase. Trizol, whilst a bit old school, is arguably still one of the best ways to get a total RNA prep from cellular material.

ALTERNATE PROCEDURES:

Another way of getting RNA (at least in eukaryotes) is to utilize the fact that your RNA resides in the cytoplasm whilst the DNA resides in the nucleus (at least for eukaryotes). Consequently, an option is to first treat the cell with a "gentle" detergent to lyse the cell membrane but leave the nuclear membrane intact. Examples of common detergents used for this purpose are Triton X-100 and NP-40 (these two are almost identical). NOTE: The biochemistry and behaviour of detergents is very complicated. CONSEQUENTLY when dealing with a detergent, it is a good idea to follow the procedures given rather than playing around too much. Detergents have many attributes that affect their effectiveness. Dependant on their existence as free form particles or micelle complexes (kinda like a vesicle). Detergents forming micelles don't work as well and micelle formation is very sensitive to both temperature and concentration effects which vary enormously from detergent to detergent.



CHECKING STUFF OUT ON A GEL:
Running RNA on a gel has a few extra considerations to note. Firstly, most systems use a MOPS, MES buffer (getting away from Tris). In addition, RNA is usually treated with a denaturant step (i.e. + formaldehyde, or use DMSO + glyoxal), since RNA loves to form tertiary structures.
NOTE that total RNA preps look decidedly unspectacular. Normally, you see two or three very bright bands corresponding to ribosomal RNA (especially 28S/18S which run at around 5kb and 1.7kb respectively), with lighter bands throughout (mRNA spec)

7.3 RNA: Gene Expression. DAY 3

MORE STUFF ON RNA:
Total RNA is ~ **%80 rRNA**
%15 tRNA
<5% mRNA

most people concerned about mRNA (although some study rRNA as an evolutionary marker since the rRNA moelcules are very conserved from species to species -> use it to gage evolutionary trees).
Generally, the cleaner the mRNA prep the easier your life is gonna be. BUT getting purified mRNA sample requires additional steps.

NOTE: Almost all mRNA have poly A tails, which is a very useful characteristic that many mRNA techniques take advantage of...

5'----------AAAAAAAAAAAAA 3'

Problem is that in this case, RNase degradation is an even bigger concern, since these nucleases tend to chew the ends of RNA first. Point is that whilst the majority of your RNA is fine, you may lose the one region that is important for many subsequent steps.
Examples of using the polyA tail.
YOU can use **oligo dT affinity** chromatography to purify your mRNA.
Can use the polyA region as a primer binding site for reverse transcriptase or PCR experiments.

Reverse transcriptase: some specifics:
NOTE that the procedure itself is very straightforward. Difficulties arise primarily because of the RNA prep.
The enzyme: Number of commercially variants available, that are usually derived from **AMV** (avian myeloblastosis virus), **MMLV** (moloney murine leukemia virus), or **HIV** (human immunodeficiency virus).

RTs generally contain some combination of four basic functions: **(1) RNA-dependent DNA polymerase, (2) hybrid dependant ribonuclease (RNaseH), (3) end point hairpin loop formation, and (4) DNA dependant DNA polymerase.**

For **EST (expressed sequence tag) or mRNA work:** Oligo dT primer usually a minimum of 12 nucleotides in length. Commonly in the 12-20 nucleotide range.

TWO STEP vs ONE STEP RT-PCR:
two step is generally more reliable as you have the option of tweaking your PCR parameters (will talk later). i.e. you can make your PCR go at its optimal best, because the reaction can be fine tuned independantly. Sometimes you have to do two step because the RT you use prefers Mn2+ as a cofactor. More likelihood for cross contamination since there are more steps - this can be a problem if your mRNA is in very low amounts and the sensitivity of your assay is high.

one step is quicker, less work BUT also possibly less reliable because your PCR reaction conditions are constrained by some of the conditions used in your reverse transcriptase assay. i.e. cofactor amounts stay the same. Affect of your PCR primers in RT assay, etc etc etc. One step works because the two enzymes (RT and the heat stable DNA pol) work and can be differentially activated at **different temperatures**.

8.1 POLYMERASE CHAIN REACTION - The basics DAY 1

PCR (polymerase chain reaction) is an excellent example of illustrating how it's often the most simple and elegant ideas that really propel science. A bit of history :**Kary Mullis** is the principle investigator behind the techniques. Interesting (slightly eccentric fella) who won a nobel prize in 1993 for the technique (actually shared the nobel that year with our very own Michael Smith.

Basic premise.
Working with DNA. If you want to manipulate it, or even see it --> needs LOTS of it.
PCR is an attempt to amplify DNA in a test tube environment.

EXCEPT that this amplification occurs via relpication in the natural sense, and replication is a little on the complicated side (in terms of getting it to work in an in vitro setting). So, Dr Mullis essentially tried to MacGyver his way through the experiment.

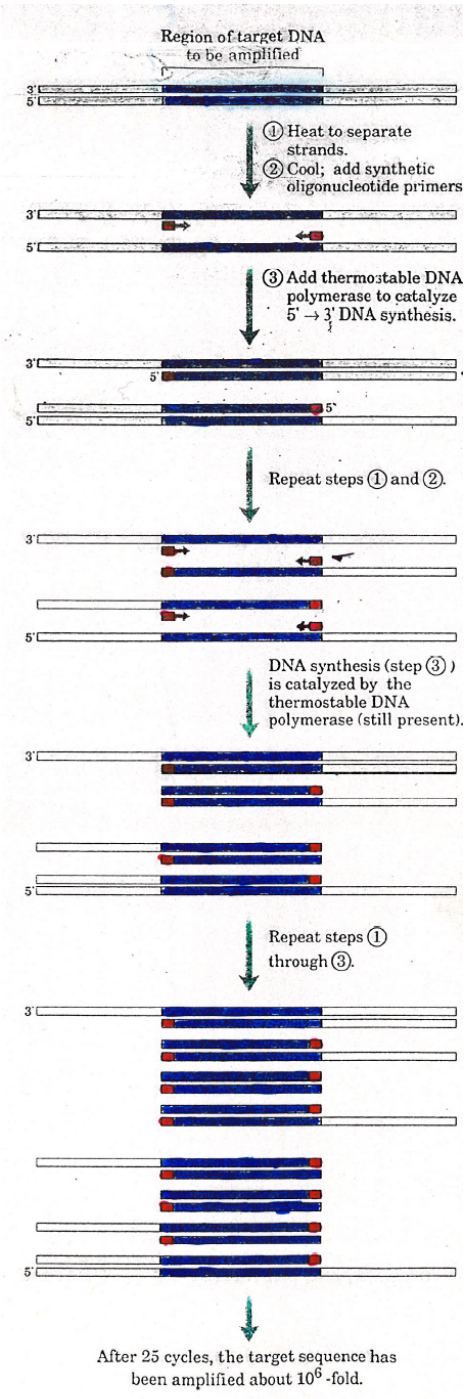
- **POINT ONE:** replication requires open or single stranded DNA. Usually accomplished by many enzymes that unwind DNA (such as helicases). Screw the enzyme - Let's use HEAT to open up our DNA.
- **POINT TWO**: replication also needs a primase enzyme to make primer for the polymerase. BUT, you can make your own! Buy oligo's.
- **POINT THREE**: with the use of a DNA primer system, you don't need DNA pol I.
- **POINT FOUR**: with a forward and reverse primer system, don't need LIGASE.
- **POINT FIVE**: with heat globally opening your DNA, you don't need TOPOISOMERASES either.
- **POINT SIX**: You can get this to work with only a workshorse DNA polymerase (i.e. one enzyme).

HOWEVER, we do have one problem: this high temperature will basically knacker out any protein structures,including our polymerase. TO get around this, let's use a polymerase from a bug that grows in high temperatures (thermophile). i.e. this will be a heat stable polymerase.

HUGE ADVANTAGE #1: PCR is elegantly simple, and extremely forgiving procedure.

8.1 CONTINUED.

DAY 1



PCR cycle is usually composed of three steps: (i) denaturation, (ii) annealing, and (iii) elongation. Essentially, each cycle is responsible for doubling the amount of target DNA. A cycle can take anywhere from 1.5 minutes to 5 minutes long, meaning that after 30 cycles, you have the potential to produce ~1000000000 molecules of amplified product from one molecule of template.

HUGE ADVANTAGE #2: PCR gives you data.

8.1 CONTINUED. DAY 1

ALSO, individuals over the last 15 years have been quite creative with the technique to do some very cool things. For example:

*site-directed mutagenesis
*production of restriction endonuclease site for convenient cloning
*production of single and double strand product for sequencing protocols
*quantitation of rare DNA
*amplification of partial cDNA sequences
*quantitation of mRNA expression
*differential display of mRNA by PCR
*random amplified polymorphic DNA (RAPD)
*cloning dinosaurs from dinosaur blood found in fossilized mosquitos trapped in amber

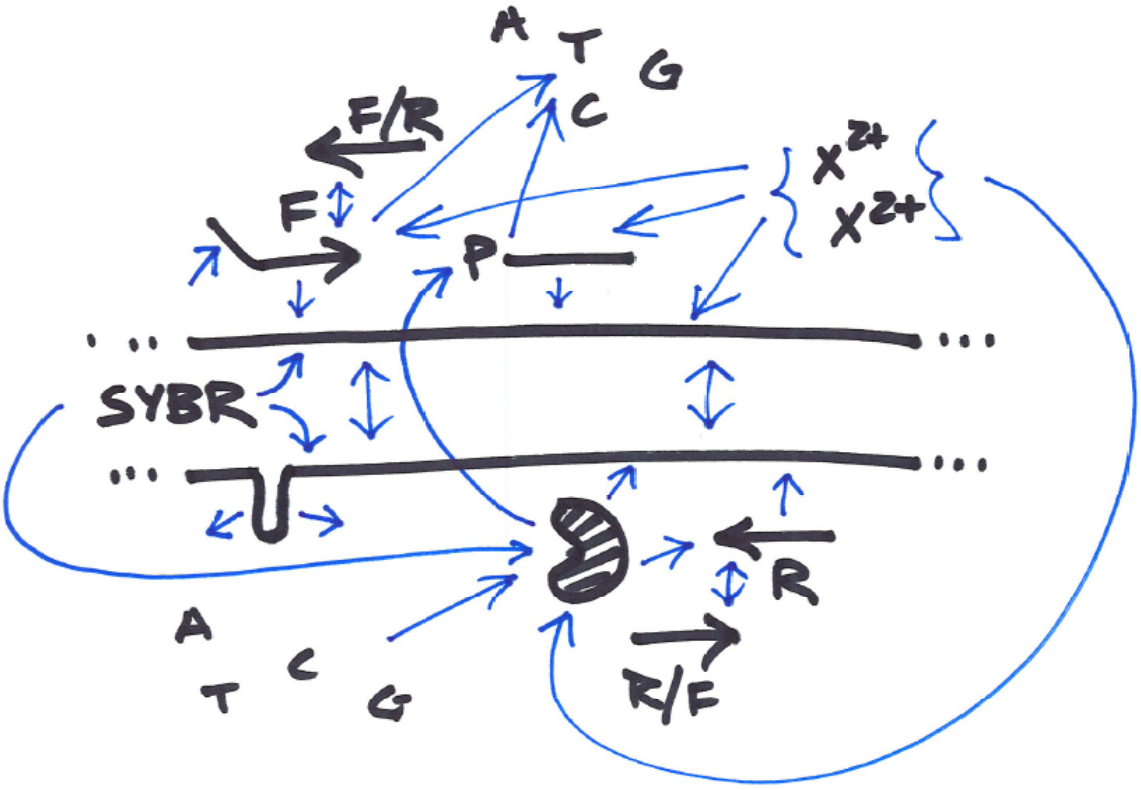
HUGE ADVANTAGE #3: PCR is very versatile procedure with many possible uses.

8.2 PCR - the specifics.

DAY 1

WHAT THE BIG PICTURE ACTUALLY LOOKS LIKE.

NOT SO BASICALLY:



and, as if this isn't bad enough, the kinetics of each of these arrows change with each cycle since the amounts will change after each round of amplication.

Yikes! (and this doesn't even include the reverse transcriptase stuff)

AND OF COURSE, the other big thing to wrap your head around is to understand exactly what it is you are trying to do with your PCR reaction. Here there are a few main things this line of questioning can fit under.

i. Yes or no, please.
ii. I'm very much into amounts.
iii. I need my product for this other thing.
iv. Sorry to tell you this, but I have very little to start with.
v. Hey get this! I have no idea what I'm looking for!

8.2 PCR CONTINUED

DAY 1

O.K. WHAT ARE THE SPECIFICS?
1. General PCR buffer: salt + buffer + detergent.
FOR EXAMPLE: 50mM KCl, 10mM Tris-Cl @ pH9.0, 0.01% Triton X-100
This is simply a recipe that makes the polymerase happy.

2. Need Template DNA to amplify. This is the DNA you will start with. ~1.0ug mammalian genomic DNA/10ul or 0.1ng plasmid DNA/10ul use 10ul per reaction. These amounts, of course, are variable and ultimately have a lot to do with the primers you use. **THE PURER THE BETTER, but PCR is quite forgiving.**

3. Need primers

(Primers tend to be the most strategic portion of designing a PCR reaction. SO MUCH SO, that there is lots of free and commercial software that you can use to help design primers). In this respect, a good representative list of what's available can be found at the following URL:

http://www.bioinformatics.ca/links_directory/index.php

In fact, this resource is useful to browse through a huge variety of different software, specifically scripted for a particular use.

HOWEVER, if we stick to the primer design side of things:
WHAT appears to be the most commonly used, and therefore perhaps most trusted program is the PRIMER3 software. However, different people have different preferences (PRIMER3 doesn't have the most user friendly interface for instance).

To find PRIMER3, google it – you'll come across a variety of different web interfaces for the algorhythm.

ON THE OTHER HAND, it's worth knowing what these programs are doing from a conceptual sense. Here, what I mean, is that there is enormous value is understanding the many aspects that influence strategic primer design. This type of information is especially useful in the context of designing primers where geography is set (i.e. making a product for restriction cloning). Here, your primers are restricted to very specific locations, so the other considerations become much more important.

SO, what are these considerations? LISTED IN NO PARTICULAR ORDER:

1. 3' terminal position is crucial to prevent mispriming, particularly since this is the point of contact with your polymerase. Consequently, it is advantageous to have G's and C's near this end (often called GC clamping).

2. Primers should avoid long runs of single nucleotides (especially A's and T's), this can result in a slipping effect. In a similar manner, avoid direct multiple repeats in the primer itself, which is often a common place for secondary structure interactions.

3. No intramolecular homology - > secondary structure which can interfere with template annealing, or completely block the polymerase from starting.

4. No Intermolecular homology -> kinetically more favourable for two primers to anneal to each other (given their saturating amounts). This is especially prevalent when looking at the 3' ends of your primers.

8.2 PCR CONTINUED DAY 1

5. DNA primers are categorically between 15 and 30 nucleotides, GC content at ~50% but you basically want to aim for a calculated melting temperature of approximately 55C to 60C. Manual way that you can arrive at this number includes using:

   $$Tm = (A+T)2^{o}C + (G+C)4^{o}C$$

   By increasing the primer length, you are also allowed to increase the annealing temperature and thus obtain a higher specificity in your PCR reaction. On the other hand, by increasing the primer length without increasing the annealing temperature correspondingly, you allow for more wobble (that is primer-template mismatch)

6. All primers (and possibly probes) in the process should have similar Tm's. This is because there is one single annealing step in the process, and therefore this should be amenable to all primers used.

7. Primers are normally used at excess, which normally falls within the 100 to 500nM range.

8. AND OBVIOUSLY, choose the sequence of your primer carefully. Mismatches will affect specific binding, and if the mismatch occurs at the 3' end, may affect the ability of the polymerase to read.

9. Make sure your primer doesn't bind elsewhere, or has the potential to bind elsewhere. Use BLAST to query available databases for this problem, or use software like PUNS.

10. Consider post-replication variants when possible - i.e. things like splice variants.

11. Deliberate mismatches (i.e. for introducing restriction sites, or point mutations) should always occur at the 5' end (away from the polymerase).

12. Distance between forward and reverse primers will need to coordinate with polymerase used. i.e. how big is that amplified product? GENERALLY SPEAKING: <3kb = easy whereas >10kb can be very challenging. For real time experiments, the smaller the better (this is why they are often between 50 and 100bp in size).

13. Please tell me you're not doing an experiment with **degenerate** or **RAPD primers**, unless, of course, you have no choice.

Alright. Go buy some primers now. They cost about 40c to 50c a base for a 40nmole prep. (i.e. 20mer will only cost about $10).

4. need dNTPs ~0.2mM mix. don't mess around -> enough to make 12.5ug of product!!!!!

5. Heat Stable DNA Polymerase: Enzyme usually comes in a stock of about 2-5Units/ul. need ~2.5units per reaction. NOTE, there used to be two main subtypes: Taq (cheaper + higher error rate), and Vent (more expensive + lower error rate). Take care to note nuances of the polymerase you use.

i.e. - Taq can do this wierd thing where an ATP is added to the 3' ends of the PCR product.
- Some polymerases don't like SYBR green.
- Some polymerases better at incorporating nucleotide analogs.

8.2 PCR CONTINUED

DAY 1

THE POLYMERASE SO MANY CHOICES

Really really confusing stuff. If I had to pick an analogy. choosing the appropriate polymerase is not unlike **buying your first house**. In other words, there are a multitude of factors that you can take into consideration when selecting your enzyme.

KEEP IN MIND HOWEVER, that because of the large selection of different polymerases, a company will have no problems with giving you a free sample of said product for you to try out. Here is a general table of some of the products available and why you're thinking about these particular traits. (NOTE that these values were access independantly by Stephen A. Bustin and Tania Nolan from "A-Z of Quantitative PCR")

Enzyme	Half life 95°C (h)	Elongation rate (nt/s)	Processivity (nt)	Fidelity	dUTP o.k.	5'-3' exo	3'-5' exo
KOD HiFi	12	100-130	>300	+++++	?	N	Y
Pfu	6-18	25	10-20	++++	N	N	Y
Tth	0.3	25-33	30-40	+	Y?	Y	N
Pwo	?	40-50	40	+++	N	N	Y
Tgo	2	?	?	+++++	Y?	N	Y
Vent	6.7	67	10	++	N	N	Y
Deep Vent	23	23	<20	+++	N	N	Y
9°Nm	7.7	?	?	?		N	5%
Taq	1.6	60	150	+	Y	Y	N
Tfl	0.6	40	50-60	+	Y	Y	N

1. **Half Life**: generally accesses the heat stability of said enzyme. Since the average PCR reaction will likely take no more than 4 hours, the values stated here are really not that important in the grand scheme of things.
2. **Elongation rate**: This is more of a consideration when dealing with amplicons of significant size. i.e. not in the case of real time experiments where your amplified product is quite small. Current literature suggests maximum size of fragment that can be produced efficiently is about 40kb in size (as stated by BioRad's iProof).
3. **Processivity**: This term applies to the general idea of how long can the polymerase hold on for. Because these polymerases are working at inordinately high temperatures, you have a situation where they are literally continually falling on and off. High Processivity is most advantageous with large fragment sizes.
4. **Fidelity** is key when producing amplicons that ultimately have an auterior destination (i.e. for cloning). In general numbers will hover around $1x10^{-4}$ to $1.6x10^{-6}$ errors per nucleotide. (NOTE that native Taq can have an error rate as high as 1 in 400). However also keep in mind that if you are doing a reaction with high numbers of cycle, the chance of a mutation possibly happening within the primer binding sequence (and most notably at it's 3' end) could be an issue.
5. **5'-3' exonuclease** activity: Absolutely crucial for TaqMan probes. Functions overall with regards to excision repair within a cell (as well as to deal with the Okazaki fragments), which is not so much an issue here. Therefore, logistically, it is an attribute that slows the polymerase down.
6. **3'-5' exonuclease** activity: This is primarily associated with proof reading ability.
7. **Does it like dUTP**. Can be an issue when dealing with amplicon contamination.

OTHER ISSUES NOT LISTED ABOVE:

8. **RT activity**: curiously enough, you'll find that some polymerases can also function as reverse transcriptases. This is something that is occassionally taken advantage of in one

step reverse transcriptase PCR procedures (will touch on this later, but the main one here is Tth).

9. **Compatibility with SYBR reagents**.
10. **A overhang nuance**. Some polymerases have the propensity to finish off each 3' end of the amplicon with an additional A (resulting in a single A overhang).

Beyond the above, the crazy thing is that all of these "attributes" (especially when described by the company pushing the product) can vary significantly depending on the components within the reaction buffer. In addition, comparitive studies advertised by companies to make claims are always done under non-standardize conditions making true comparisons practically impossible. Add to that, many proprietary versions of said polymerase (mutated, genetically engineered etc), which have altered abilities tends to only complicate the analysis further (for instance KlenTaq is a Taq derivative with the 5' exo region has been "toughed up", for better performance overall).

Chemical Name	Final Concentration
BSA	No inhibition
NH4	No inhibition
NP-40	No inhibition
Propidiurn Iodide	No inhibition
Spermidine	No inhibition
Ca 2+	> 3,5 mM
Chloroform	>50 mM
Dimethylformamide	>50 mM
DMSO	>10%
DTT	> 1 mM
EDTA	>50mM
Ferric ion	>10uM
Formamide	>50 mM
Hemoglohin/heme	heme will interfere with PCR
NaCI	>50mM
Phenol	>50 mM
KCI	>50mM
SDS	>50mM
Siliconized tubes	inhibition
TritonX-l00	inhibition

Mg2+ concentrations: Still one of the most important attributes affecting polymerase (as well as template/primer). +1A at high Mg2+

In any event, the following is some useful info regarding some specific polymerases (almost like personals ads)

1. Taq (thermus aquaticus)
Not looking for surprises or flashiness. Just something trustworthy and predictable. Must appreciate some eccentric nucleotides. Note Fidelity is great at certain pH, which may not correspond specifically to best processivity (at high pH's).

2. KlenTaq: (Stoffel Fragment)
Is your 5' exo region thermally tough? If so call on me, and we'll do wonders with TaqMan systems, and general robustness overall.

3. Tth: (Thermus thermophilus)
Are you into organics or RNA? Not a problem, give me a call.

4. Tfl: (Thermus Flavus)
Tth too wussy for you. Then I'm your polymerase.

5. Pfu: (Pyrococcus furiosus)
I may be slow but if its longevity and fidelity that counts, then give me a buzz – I'll be waiting.

6. Vent: (Thermococcus litoralis)
Taq too boring for you, but the others are still a bit too crazy, even though fidelity is important to you. Vent is probably what you're looking for.

8.2 PCR CONTINUED

DAY 1

7. Deep Vent: (Pyrococcus species)
Hot damn! I'm super stable and extremely low maintenance (love formamide and DMSO). Also very good fidelity, but still with high elongation rates.

pTaq = I'm free baby, all free baby.
iProof = call me top of the line baby, top of the line baby (but not compatible with SYBR)

6. Thermal Cycler: can range from CAN$2000 to over CAN$90,000. Depends on capabilities, reliability, reproducibility, and precision (as well as special features like the optical unit on a real time PCR machine)

7. The Cycle: Generally speaking:
Start with something called a "**cold**" or "**hot**" start. 95C for an extended period of time **(2-4minutes).**
These are tricks to **optimize the first round of the PCR reaction** which is crucial to avoid diluting the amplification process. i.e. sample prep at room temperature can lead to non-specific priming events, under the auspices of a functional (albeit slow) polymerase.

Denaturation STEP: 95C 15sec to 1minute. Time more dependant on size of template, (i.e. genomic DNA may want to give more time to this step) but particularly is dependant on size of amplicon.

Annealing Step: Temperature is very important. Can be specific (dependant on Tm calculation), can be generally determined **(i.e. GC <50% use 55C, if >50% use 60C).** Usually about 15sec to 1 minute
NOTE that some machines allow the option of doing a **gradient within your multiwell** sample set up, so that you can check multiple annealing temperatures in a single experiment.
NOTE that some machines will allow you to program a **gradient ramp within the annealing step**, should you be stuck with a primer set of very different Tm (i.e. the annealing step goes from high to low)

ALSO: **Touchdown PCR**, which is where annealing temperature will get lower as the number of cycles increase. Idea being that this facilitates specific priming initially, but as amplified product is generated, the stringency of the annealing temperature can be relaxed to allow greater amplification efficiency.

Elongation Step: Temperature is usually around 72C (Favourite for Taq), time will depend on elongation rates, but can vary between 30sec and 3-5minutes.

TWO-TEMPERATURE PCR: this is where the annealing and elongation steps are combined into one step. This method works optimally when primers have a Tm > 60C. Particularily useful with Taq derivatives since this polymerase is still very active at 60C.

NOTE: that total time for the PCR reaction also includes RAMP time which is the speed at which the temperature changes can occur.

8.3 PCR – Troubleshooting DAY 1

Generally, PCR is a standardized procedure but due to differences in primer and template, evitably you may come across a PCR that won't go. SO,.. when you're doing a particular PCR for the first time, it is worth figuring out the best conditions.

WHAT are the conditions to play around with? BASICALLY, this has to do with things that affect the ability of the primers to go to the right place (hybridization paramaters) – it's a kinetic thing.

OPTION ONE: MgCl2:: VERY COMMON thing to play around with, although exact reasons why are a little vague. In essense, Mg ions seems to do a number of things.

- it binds DNA: may affect primer/template interactions.
- it binds the DNA polymerase -> required as a cofactor
- it influences the DNA polymerase's ability to interact with primer/template sequences.

BOTTOM LINE is that more Mg = less stringency in binding.
DO a titration. generally people will check between 1 and 6mM final concentration.

OPTION TWO: Kinetic parameters. Template, Primer, Enzyme concentrations. What's the likelihood of things interacting appropriately, not appropriately, not at all, etc. Annealing Temperatures.are a common option, especially with machines that can easily provide temperature gradients in the PCR.

OPTION THREE: Template quality. Said this before, but why introduce caveats by having a sample that is not high quality. That being said, one of PCR's great appeal is that with a well designed strategy, you may not need to clean your template up.

OPTION FOUR: BONUS REAGENTS:
+/- DMSO denaturant ability. good at keeping GC rich template/primer strands from forming secondary structures. Doesn't seem to generally affect the reaction. Most people will include it regardless (use at 5%)

+/- glycerol increases apparent concentration of primer/template mix. Therefore may help in getting good primer/template interactions at high temperatures. (use @ 10%)

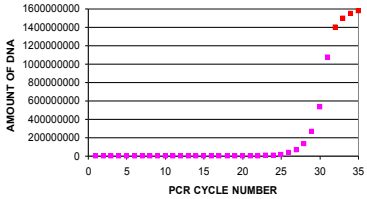
AND... OPTIMIZATION of the first round of amplification. (IMPORTANT)
General idea is that whilst you are setting up your reaction, the taq pol. may start replication when the primer is binding at an inappropriate site. This will lead to "pseudo" bands and a diluting out effect of your real template. SO,.. there are a couple tricks to optimize your first cycle.
-"hot start" + polymerase after first denaturation and annealing step -> go to RT.
-ice cooling after annealing temperature -> go to ice temp.
-taqstart antibody. Includes addition of taq antibody which will get denatured upon 95C step.

FINALLY CHECK THAT THERMAL CYCLER IS WORKING. TEMPERATURE NEEDS TO BE SPOT ON FOR THIS WHOLE THING TO WORK PROPERLY.

8.4 REAL TIME PCR.

DAY 4

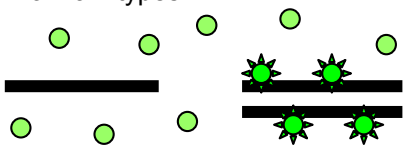
This is, essentially, **the use of chemistry and optics to detect and evaluate production of PCR products in real time**. i.e. conventional PCR is based on **end-point analysis** -> you look at a band on a gel, you look at your data at one point in your reaction which happens to be when it's all finished.
WHICH is flawed in principle, **if quantitation** is something you are interested in. With endpoint data representation you are looking at a tired reaction (enzymes going off, reagents becoming limiting etc).



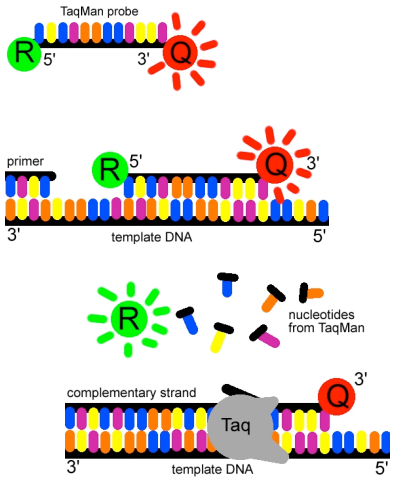
So, real time enables you to watch the kinetics of the reaction, which ultimately means your ability to quantify the reaction is significantly improved.

HOW do you do it: FANCY CHEMISTRY:

Two main types:



SYBR systems: a reagent that selectively binds to double stranded DNA, and flouresces under this interaction. CHEAPEST way – by far the most common.
ALSO extremely useful for melt curves, which provide a mechanism to look at your amplification products.



TAQMAN systems: a two component system that relies on something known as the FRET (Fluoresence Resonance Energy Transfer) phenomenon. Here, there are reagents that can donate energy (R) at specific wavelengths when excited, AS WELL as acceptor reagents that can quench (Q) this energy if located in close enough proximity. i.e. the two chemistries are located on the same TAQMAN probe.

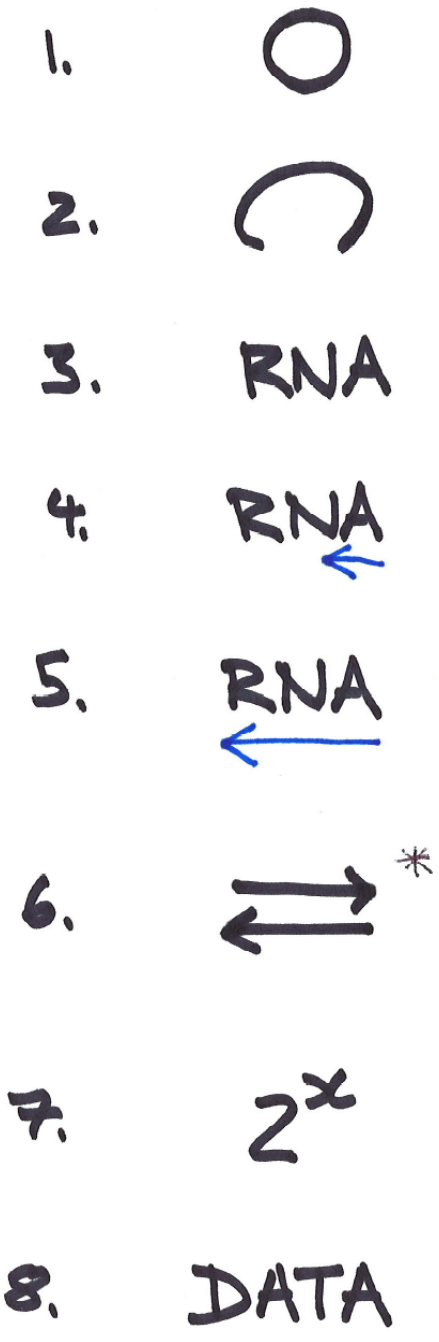
Because of Taq's 5' exonuclease activity, one can separate the two components during amplification. Therefore monitor PCR reaction as a consequence of TAQMAN probe degradation -> Emitter is released from quencher, so that energy can now be detected. Because there are a variety of different FRET systems, available, TAQMAN methods are useful for multiplexing experiments (especially in Single Nucleotide Polymorphism determination

Overall advantages, include increase sensitivity, increased speed. Often products being observed are deliberately small, since we are more interested in an effective PCR reaction, that the band of DNA at the end of the procedure.

8.4 CONTINUED

DAY 4

Quantitation: THE REALITY.



So what's the problem? The problem is that at its heart reverse transcriptase Real Time PCR is all about quantitation. In fact, it is currently considered the gold standard technique for answering this type of biological question.

The reality, however, is that your final readout value, whilst theorectically provides a quantitative reflection of your answer, is actually derived from a lot of steps, where at each point, that confidence in that correlation goes down.

If you take the following diagram as an example, you can see multiple points where things can unduly affect your final data point (and this isn't even considering the actual experiment on the tissue source itself!).

For example, step 5 questions the efficiency of the Reverse Transcriptase itself. In experiments done with internal RNA controls, it has been shown that efficiency values of commonly available RTs can fit anywhere between 14 and 85% (!!!). This means that already, your cDNA amounts will not reflect your actual RNA amounts (Invitrogen Platinum, by the way, won this contest).

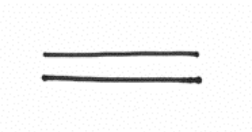
A QUESTION OF PRECISION VS ACCURACY...

Then, there is the question of just what is an appropriate control to generate a standard curve.

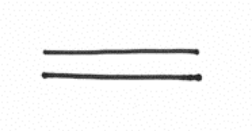
- A housekeeping gene?
- Cell numbers?
- RNA controls? (univeral, T7 prepared)
- synthetic Armoured RNA controls?
- cDNAs / sense strand oligos?

APPENDIX A: Replication.

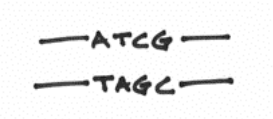
(Use magnetic board)



To begin with, we'll start with a chicken scratch drawing of a DNA molecule, which you know is double stranded. My poor pathetic attempt at illustration is therefore going to look like this:

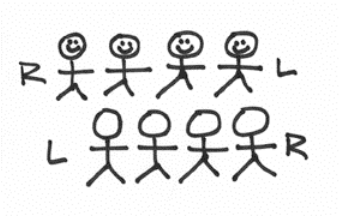


You also know that each strand of DNA is composed of building blocks called nucleotides, and that these nucleotides are always interacting in a complementary manner. For example, A's are always with T's, C's are always with G's, Beavis is always with Butthead, etc etc etc. Let's draw them in like so:

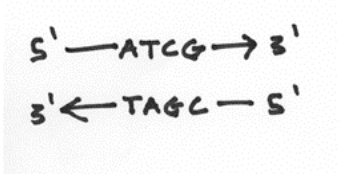


What you haven't been told at this point is that chemically speaking, the two strands are going in opposite directions. The correct term for this is actually known as *anti-parallelism.* To denote this, I'll draw some arrowheads on the DNA strands:

Although, this may seem a little confusing at first, try to picture two lines of square dancers facing each other. In this circumstance, you notice that when focusing on the left or right hands of the row of dancers, the two lines are going in opposite directions. This picture should help:



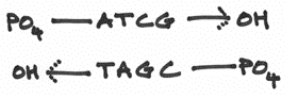
Your DNA strands are doing something very similar in a chemical sense. The difference, of course, is that instead of dancers, you have your choice of four nucleotides. Furthermore, like the situation of left hands versus right hands, the ends of the DNA strands are also different. One end is known as the *3'* (pronounced 3 prime) end and the other is known as the *5'* end. To the layman, these rather stoic terms are an unfortunate consequence of chemical labeling. So now, our picture should look like this:



I should reemphasis that the 3' and 5' ends are very different from each other. To be more specific, we say that they are chemically distinct from each other. They are as different from each other as apples and oranges. In fact the 3' end is composed of a *hydroxide group* and the 5' end is composed of something known as a *phosphate group*. These groups look a little like this:

Hopefully, it's easy to see that they are indeed distinct from each other —even more so than apples and oranges. The hydroxide group being comparatively small and meek, whereas the phosphate group is prominent, overbearing even. This turns out to be a crucial factor because replication is carried out by the activities of a variety of different enzymes which *all* function by focusing on one DNA end or another or both.

So now, the picture looks like this:



It should also be pointed out that DNA is not really like this flat goofy looking cartoon. As mentioned in a previous chapter, the two DNA strands are actually intertwined around each other in a rather pretty helical fashion. This is where the two strands are wound around each other, sort of like two elastic strings twisted and coiled together. Sort of like this:

Now that the stage is set, it's time to introduce the proteins or the *enzymes*, which are responsible for the actual process of replication. Enzyme is just a fancy word for a protein that is able to facilitate a chemical process. What I'll do here is to focus on terminology associated with a simple organism like the bacteria, *e. coli*. However, all organisms, even those as complicated as humans, do more or less the same thing when it comes to doubling their DNA — the principle difference being that unfortunately, the enzymes have difference names and labels.

That aside, the first enzyme for replication in *e. coli* that we should introduce is, of course, the most important enzyme in the entire process. In *e. coli*, this

enzyme is called *DNA polymerase III* (or DNA pol III for short), and is essentially the one that is responsible for the actual business of making more DNA. If this entire exercise was analogous to a movie, then this enzyme is the marquee player. It is the Tom Cruise, the Julia Roberts, the proverbial bread and butter of replication. It is, quite simply, the star of the entire process. Instead of drawing a picture of Tom Cruise or a picture of Julia Roberts, I think a picture like this should suffice:

Problem is, if we were to draw this enzyme to scale with a helical DNA molecule (like this),

you'll notice that the DNA pol III is actually too big to get inside the DNA strands. It can't go about its business of copying the DNA, because the strands are all coiled up in the helical structure. In other words, there is a serious issue of accessibility. Even our star enzyme, despite its importance, can't do its job without access to the molecules of DNA it wants to copy. Consequently, the enzyme that inevitably has to act first is one that is responsible for opening up the DNA strand. This enzyme is known as a *helicase*, and its role is to essentially unwind the DNA molecule, which would look like this:

The net effect being the production of a "bubble" of opening where the two DNA strands are pried apart and are subsequently accessible to the whims of the replication machinary.

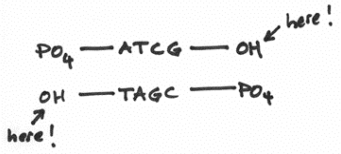
Curiously, the DNA pol III, which after the unwinding event, can now interact with the DNA molecules, does so whilst attached to a bunch of other enzymes. This attachment is a little like a bunch of buddies hanging out together. The complex actually looks a little like this:

You'll notice it has the following... (i) two DNA polymerase III's: which kind of makes sense given the fact that there are two strands of DNA that need to be copied; (ii) one helicase molecule: which also sort of makes sense, because as this replication complex is doing its thing along the DNA molecule, wouldn't it be handy to have the built-in ability of opening up the DNA molecule as it moves along; and (iii) one new enzyme which is known in *e. coli* as the *primase*. However, the purpose of the primase molecule is a little complicated and so to fully comprehend the role of this enzyme, we need to switch gears a little and tell you a bit more about the DNA pol III molecule.

What actually needs to be done, is for us to go over a few mechanisms that all DNA polymerases seem to use. In fact, it's apparent that every DNA polymerase that has been discovered on this planet:

In fact they all (without exception) seem to follow a two basic rules.

Rule number one states that all DNA polymerases function by adding nucleotides to the 3' end of the DNA strand. What this means exactly is that a DNA strand can be extended by the addition of new A's, T's, C's or G's. However, the new nucleotides can only be added to one particular end, namely the 3' hydroxide group. This is a molecular restraint in that the DNA polymerase can only join nucleotides via this smallish chemical group. This rule can be drawn out like this:



Rule number two states that all DNA polymerases require a *primer* to function properly. This is probably the most challenging concept that needs to be addressed. If you get through this, then you consider yourself home free.

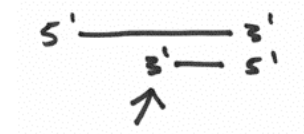
To simplify the notion of a primer, let's look at a single strand of DNA, complete with its 5' and 3' ends. It should look a bit like this:

Now according to rule number one, a DNA polymerase can extend this single strand chain but only by adding nucleotides to the 3' end. In effect, you can argue that all of the relevant chemical groups are present for making more DNA. However, the problem lies in the fact that under these circumstances, the DNA polymerase doesn't actually know what to add. How does it know, whether to add an A, a T, a C or a G? It can't exactly be a random event, because replication is all about making sure cells receives an identical copy of the DNA code.

Take the following picture:

Under this layout, it should be clear that now, the DNA polymerase has the required 3' group, AND it also has a *template* to read and ascertain what those nucleotides should be. For instance, if the nucleotide in the opposite strand is a G, then the DNA polymerase knows it should add a C. If the nucleotide in the opposite strand is a T, then the DNA polymerase knows it should add a A. Hopefully, at this point, you'll at least agree with the following statement. A DNA polymerase can not do anything with a single strand of DNA. True, it has the right chemistry, but in effect, it does not have the template or instructions needed to define how the chain is extended.

If we redraw the picture. Say like this:

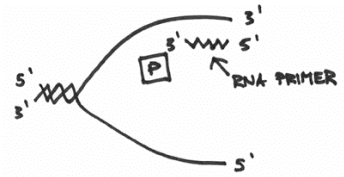


What you'll notice are two strands of DNA, one long and one short. You'll also notice that the strands are anti-parallel as discussed earlier. If you focus on the arrowhead, you'll find yourself focusing on a perfectly situated 3' group. Here is the end of a DNA strand that is chemically ready to have nucleotides attached. Furthermore, it is also a 3' end that is located where a template is present on the opposite strand. In other words, everything is in place. The right chemistry, and a means for instructing which nucleotides to add. Again, taken at the simplest level, we can conclude that in order for a DNA polymerase to do its thing, it needs an area of double strandedness.

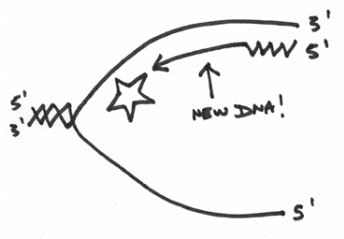
So,.. the small sequence of nucleotides that has been circled here...

... which makes an area of double strandedness is technically known as a primer. With this all sorted out, hopefully the rule about requiring this primer makes a little more sense, and you can probably guess that the enzyme called the primase may have something to do with this nuance.

Which turns out to be exactly what this primase enzyme is all about. In a nutshell, it is an enzyme capable of making a short sequence of nucleic acids which functions as a primer. A key point that needs to be emphasized, however, is that this primer is made up of RNA, which if you recall, is a molecule that is very similar to DNA in that it is also composed of the representative four nucleotide code. This is actually due to a biological technicality whereby it is possible to make a complementary strand of RNA without the use of a primer (hmmm, think about this for a second). Taken together, the function of the primase should end up looking like this:

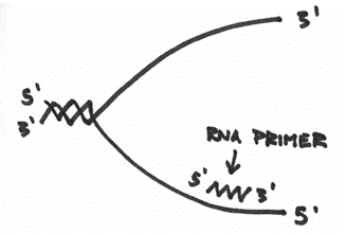


If you've been following along, then hopefully you can see that replication from this RNA primer can proceed in a manner that can be drawn like this:



However, it's wise to pause here for a second, because you have to understand that whilst this top strand is being replicated, the lower strand is also being worked on simultaneously (There are two DNA polymerase III's attached together afterall). The lower strand is actually a bit messier for reasons that will become clearer as we proceed in this discussion.

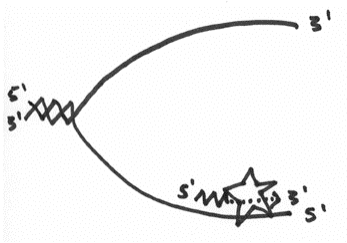
Basically, the primase enzyme will also go about preparing a primer for the lower strands. However, if we draw this primer and label the ends in the anti-parallel manner, you can hopefully see a logistical problem in this set-up. Take a look at the following picture, and see if you can find the problem (remember, the DNA polymerases, the helicase and the primase all move as a single unit in one direction, and remember that all DNA polymerases must add to the 3' end):



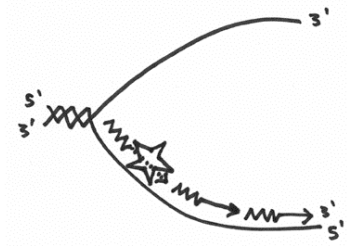
Do you see the problem? Do you see a problem with the direction of the primer? Do you see that the 3' end of the lower primer is facing the wrong direction?

This is obviously a problem, and it turns out that in order to overcome it, the DNA polymerase will still add nucleotides to the 3' end, but

can only do so for a short distance. To keep it simple, think of it as being able to replicate as far as the enzyme is big, which should look a little like this:



Unfortunately, this doesn't inherently solve the direction problem, so what ends up happening, is that with this lower strand, the primase has to continually make a primer, and the DNA polymerase III has to continually replicate a little bit. In the end, it should look like this:



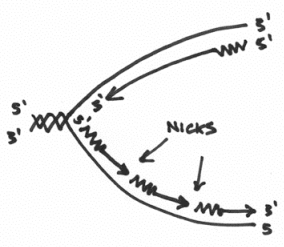
The difference in how each strand gets copied is reflected in why some people call them the *leading* and *lagging* strands of replication. One strand is obviously fairly straight forward whereas the other is quite labour intensive.

Anyhow, after this is all said and done, hopefully, you'll agree with the following statement. That is, we have finally doubled or copied our genetic sequence. However, it should also be clear that the whole thing is a bit messy. For instance, there are bits of RNA everywhere, and the lagging strand is composed of pieces. To address these problems, we have to introduce a few more enzymes.

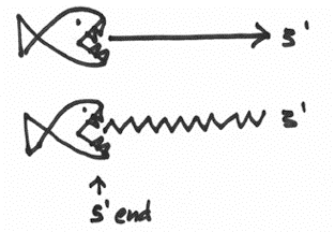
The first of which is *DNA polymerase I*, which I will draw as a fish with sharp teeth. This enzyme is special in that, in a nutshell, it is responsible for dealing with the RNA. In a nutshell, its job is to somehow replace it with DNA. In a nutshell, I'll draw it like this:

DNA polymerase actually has two distinct functions. Firstly, as its name implies, it is a DNA polymerase, meaning that it is capable of extending the DNA chain, but in doing so must follow the same two rules that govern these enzymes. In other words, it must add nucleotides to the 3' end and it must use a primer as a springboard. Ironically, it is a shitty DNA polymerase. Whereas DNA polymerase III can replicate for several hundred nucleotides, DNA polymerase I has difficulty getting past a few dozen.

Secondly, DNA polymerase I is also an *exonuclease*. This means it's capable of degrading or chewing up nucleotides. Which is another reason why I drew a fish with teeth. And not only does it chew stuff up, it does so in a fairly specific manner. To begin with, it likes to start at areas, which are termed as *nicks* in the DNA. In our picture, this is where the nicks would be:

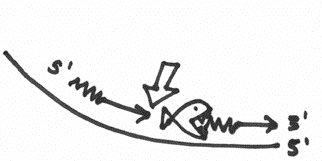


Furthermore, this exonuclease is picky in that it always chews from the 5' end. Basically it is gunning for that big phosphate group. So that you don't forget this, I've drawn this picture to help you visualize this:



Now, if you take all of this into consideration, you come up with the following mechanism. DNA polymerase I will come in on our replication picture, and zone in on a nick in the strands. Once there, it will begin chewing on the 5' end, which should look a bit like this:

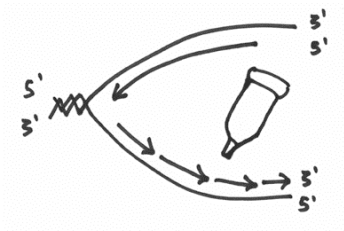
Don't forget that this enzyme is also a DNA polymerase, and if you look at the other side of the nick, you will hopefully realize that there is this beautiful 3' end ready for action. This beautiful 3' end is right here:



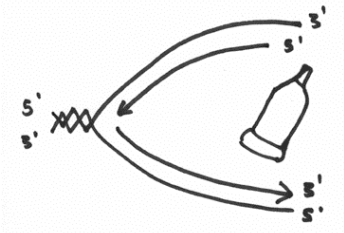
Let's say that the fish's ass happens to contain the DNA polymerase function. What therefore happens is that DNA polymerase I will start replicating from that 3' end, which incidentally fills up the gap that was created by the exonuclease activity. This should nicely demonstrate how DNA pol I achieves its function of replacing the RNA with DNA. This whole step should kind of look like this:

Hopefully, this puts the shittiness of this DNA pol I in perspective. It's quite biologically pretty because, I hope you can appreciate that DNA pol I doesn't need to be very good. It's only responsible for replicating the small region encompassed by that RNA primer.

So,.. after this enzyme has done its thing, you should now agree with the following statement — that you have now doubled your DNA. Of course, it's still a bit untidy because the strands (especially the lagging strand) are still in bits and pieces. Enter the next and final enzyme, which is called the *ligase*. This enzyme has only one job and that is to seal all of the bits and pieces together. It fairly analogous to a glue job and essentially your picture will go from something like this:



To something like this:



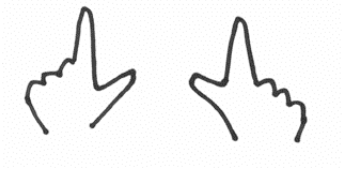
And (drum roll please) VIOLA! You have *doubled* your DNA. You have made two copies of the same genetic code - which during the process of cell division, will enable each of the two new cells to receive a copy of the genome.

One of the nuances that should be mentioned is that if you examine the entire process, you will notice that each of the DNA sequences is derived from one old strand and one newly synthesized strand. Because of this, replication is often termed *semi-conservative*, whereby each of the original two strands is read individually to synthesize a new and complementary strand.

* * *

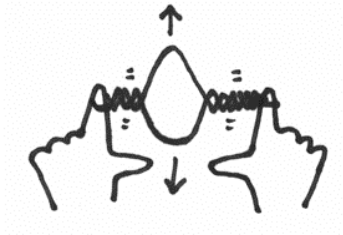
Actually, I lied. It's not quite over. Before, I finally put this whole replication thing to rest, I think it's also worth talking about one other enzyme, or a family of enzymes, known to scientists as *topoisomerases*. I like mentioning these enzymes, because I think they do a wonderful job of illustrating just how complicated and elegant nature is, when confronted with a specific job.

What we'll need to do here is undergo a visual exercise. Let's say I tell you to hold two fingers up like this:

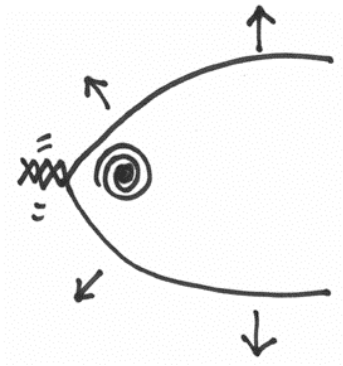


And let's say that I have an elastic band. With this elastic band, I will twist and coil it and then place it around both of your fingers. Essentially, this will represent the double helix and will look a bit like this:

If you recall, the first thing that had to happen was for a helicase enzyme to come in and open up that helix structure. Let's say that I am the helicase, and I come in and grab hold of the two strands of your elastic band and pry them open. It should make a little bubble and should look a little like this:



Can you see that under these circumstances, the helix on either side of the opening will be actually twisted even more. It would be like taking your replication fork, grabbing hold of each strand, and like the helicase forcing an opening like this:



Do you see that this will cause a further tightening of the coil along the helix?

This is actually very bad for the DNA molecule, as this twisting can cause a lot of structural stress. So much so, that the DNA molecule is in very real danger of snapping - which you can imagine would be a very bad thing to happen during replication.

?Topoisomerases are enzymes that are designed to take care of this problem. These enzymes can actually detect these areas of high structural stress, and zone in on them. Not only that, but whilst they are at these areas, they will then cut both strands in the DNA complex. Remarkably, they will then hold

on to all four ends of the cut, and in a very controlled fashion, unwind to alleviate the stress. Finally, they will also behave like ligases and stick back the correct ends together again.

?This is nothing short of amazing, but hopefully you can see that these enzymes play an important role. As the DNA is opening up for replication, there will always be an issue of structural stress, which is always addressed by the actions of these remarkable enzymes.

APPENDIX B: Hybridization/Stringency DAY 1

HYBRIDIZATION: Term defining the act of a single strand piece of nucleic acid, finding its complement.

Very important concept, because at the end of the day, any procedure that relies on probe or primer binding is basically a challenge of getting the hybridization to work properly.

BUT WHAT EXACTLY IS GOING ON DURING HYBRIDIZATION?
Well you have two very negatively charged, very hydrophobic, potentially flexible, one possibly immobilized to a charged/hydrophobic matrix, things coming together AND at the heart of it, you want that interaction to occur because of specific H-bonding between the two molecules.

WHIC BRINGS US TO...

STRINGENCY: Need to get comfortable with this concept. High stringency means the binding conditions are such that specificity is promoted , although too high stringency infers the incubation conditions are too string such that nothing will interact. Low stringency means that the binding conditions are such that binding is more easily facilitated. But, if incubation conditions are too weak, your probe may bind to sequences that are close, but not necessarily specific. Stringency is the difference between perfect data, dirty data or no data.

THINGS THAT EFFECT STRINGENCY: or why solutions are just so.

1. **SALT CONCENTRATION**. Salt will neutralize the net negative charge of nucleic acids. **More Salt = Lower Stringency**. This is a condition that is especially played around with southern/northern,microarray type of experiments. In PCR, modification of Mg ion amounts is a reflection of this.
2. **TEMPERATURE:** Higher Temperature = Higher Stringency
3. **DENATURATION REAGENTS**: (like formamide or DMSO), essentially help ensure that non-specific interactions do not occur because of tertiary structure formation. More = Higher Stringency.
4. **DETERGENT CONCENTRATION:** alleviate hydrophobic interactions More Detergent = Higher Stringency.
5. **NUCLEIC ACID AMOUNTS**: (as in the primer/probe and template). Saturating binding conditions can lead to non-specificity. Conversely, extremely low concentrations, may not meet the binding kinetic criteria for an interaction to occur in the first place. NOTE that in PCR, this parameter effectively changes with each cycle (i.e. TEMPLATE amount doubles each cycle). More stuff = Lower Stringency.
6. **THE PRIMER/PROBE ITSELF**: Obviously the length and specificity of the primer/probe will impact on your stringency values. Furthermore, if labeled, the sensitivity of the type of label will impact.

BOTTOM LINE. Is that stringency conditions will vary from situation to situation. But hopefully the above will give you some guidelines to pursue. Follow these guidelines, make an educated guess, look at your data and adjust the conditions accordingly…