Ministry of Higher Education
King Abdul Aziz University
Faculty of Science
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Laboratory Manual of Introduction to Biotechnology

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Lab. #	Date	*Exp/Practical title*	Reading Assignment	What is Due?
1	3/4/1432 8/3/2011	Safety & Regulations	Practical movie	
2	10/4/1432 15/3/2011	Introduction of biotechnology	Lab hand out practical	explain the machine and tools in lab
3	17/4/1432 22/3/2011	Buffers	Lab hand out practical	Preparation of different buffers
4	24/4/1432 29/3/2011	Extraction of Total RNA and mRNA from different types of samples Restriction enzymes	Lab hand out practical	video for extraction of RNA practical for restriction site of gene and vector
5	1/5/1432 5/4/2011	RT-PCR and cDNA libraries		Explain the of cDNA libraries techniques
6	8/5/1432 12/4/2011	VACATION		
7	15/5/1432 19/4/2011	The Mid Exam		
8	22/5/1432 26/4/2011	Quantitation and diagnosis of microbial	Lab hand out practical	Describing the technique of Real time PCR DNA and RNA viral quantitation
9	29/6/1432 3/5/2011	DNA Fingerprinting and Forensic analysis	Lab hand out practical	describes the technique of DNA fingerprinting (video and demo)
10	7/6/1432 10/5/2011	Protein separation and quantitation (Bradford gene expression)	Lab hand out practical	Explain the technique of SDS and Explain Bradford
11	14/6/1432 17/5/2011	Different method for protein purification	Lab hand out practical	different method for protein purification
12	21/6/1432 24/5/2011	The Final Exam		

Lab 1

Safety and regulation

What is afety?

Elimination of potential threats to human health

Hazards and risk assessment:

- **Hazard** means the equipment,chemicals and conditions that have a potential to cause harm such as chemicals ,electricity,animals and infectious agent .
- **Risk** is the probability that a hazard will cause harm.
- **Risk assessment** is an attempt to estemate the potential for human injury or property damage from an activity.
- **Safety guideline and standards** are procedures that are designed to prevent accidents by reducing the risk of hazards in situation where the hazards cannot be eliminated entirely.

Laboratory Safety Mangement

1- Regulatory agencies

Regulations and Standards.

Categories of Regulations and Standards.

- Worker safety

- Environmental protection

- The use and handling animals

- Regulation of radioisotopes.

2- **Institutional Responsibility.**

- Worker safety regulation

- Environmental Protection

Laboratory Responsibility:

- Risk reduction

-Labeling and Documentation

Material Safety Data Sheet (MSDS)

Job Safety analysis (JSA)

Housekeeping

Emergency response

PERSONAL RESPONSIBILITY:

- Be sure that you are informed about the hazard in the laboratory.
- When in doubt about hazards material or procedure ask.
- Use a personal protective wear such as lab coat and free powdered gloves all the times.

- Do not eat, drink or chew in the laboratory.
- Avoid practical jokes or horseplay.
- Wash your hands regularly before leaving the laboratory.
- Read the labels of chemicals carefully .
- Read procedures before performing them and visualize hazards steps.
- Minimize use of sharp objects and be sure that you properly dispose of them.
- Clean up any spills and pickup any dropped items.
- Label every thing clearly.
- Use fume hood for any chemicals or solvent that you can smell.
- Record every thing in your lab notebook.
- Always report accidents .

Lab 2

Introduction to biotechnology

What is Biotechnology?

Is a broadly based field integrating the use of biochemistry, microbiology, immunology, genetics, cell and tissue culture, physiology and engineering sciences to produce a useful product or service.

Often involves transfer of a gene from one organism to another, hence the term “recombinant DNA technology.”

Biotechnology and the Workplace:

- Academia
- Research Laboratories
- Industry
- Testing Labs
- Clinical Settings
- Regulatory Settings
- Other Work Environments

Techniques for detecting proteins, DNA ,RAN in the lab

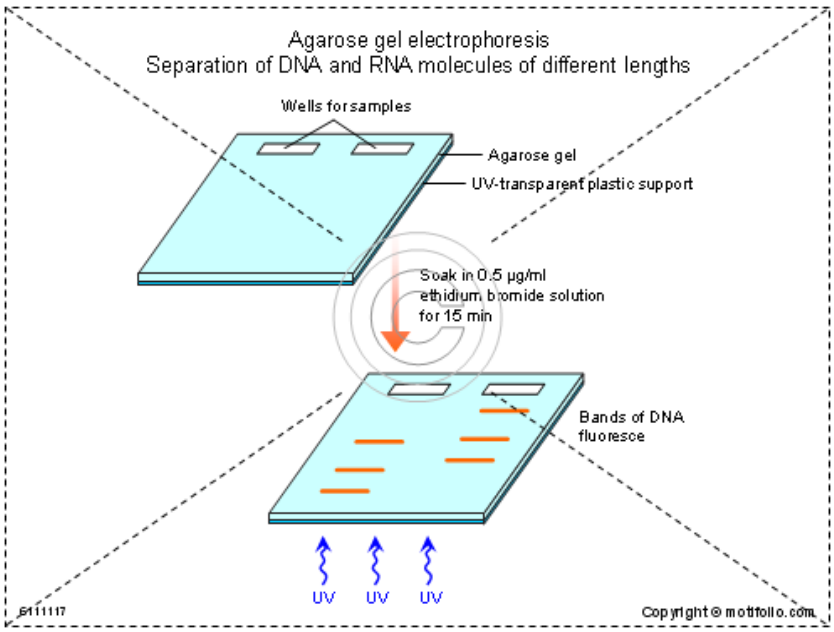
- Western blot, Eastern blot > protein
- Northern blot> RNA
- Southwestern blot>DNA

The Electrophoretic system

Agrose gel :

Agarose is extracted in the form of agar from several species of red marine algae, or seaweed, found in California and eastern Asia. Agar, a term derived from the Malay word *agar-agar*, meaning jelly, is typically derived from the *Gelidium* genera of seaweed. It is composed of bothagarose and agaropectin molecules and provides support to the cell walls within the marine algae. Removed from the plant, the agarcan be used as a food thickener, much like gelatin, a laxative, or a medium for growing bacteria, fungus, or other microorganisms, when purified.

It is fairly easy to separate agarose from agaropectin in agar because the agarose molecules bond strongly to one another, while the agaropectin gels poorly. There are several methods to achieve the isolation of agarose gel.



FIVE (5) FACTORS THAT EFFECT ELECTROPHORETIC MOBILITY OF DNA IN AGAROSE GELS:

1. Size of the DNA:

- Linear double-stranded (ss)DNA migrates through gels at rates that are inversely proportional to the $\log_{10}$ of the their molecular weight (MW).
- For different sizes of ssDNA, the smaller the MW (shorter lengths) the faster the fragment will migrate through the same gel. In other words
- , a mixture of DNA fragments will be separated by size, from the largest to smallest as to their distance from loading wells in the gel.

2. Concentration of the Agarose (% of agarose in gel):

- The same fragment will migrate at different rates through gels containing different concentrations of agarose. As the concentration of the gel increases the rate of migration of same DNA fragment decreases.
- The mathematical relationship is: $\log\mu = \log\mu_o - K_\pi$,
- where μ_o is the free electrophoretic mobility
- K_π is the retardation coefficient (a constant related to the physical properties of the gel and the size and shape of the separating molecules.
- The higher the concentration of the agarose, the K constant becomes larger and the electrophoretic rate (how fast the DNA

moves through the gel) of the same DNA fragment is reduced. Tables showing the % agarose concentration to use for a defined range of DNA fragments are very helpful.

3. Conformation of the DNA:

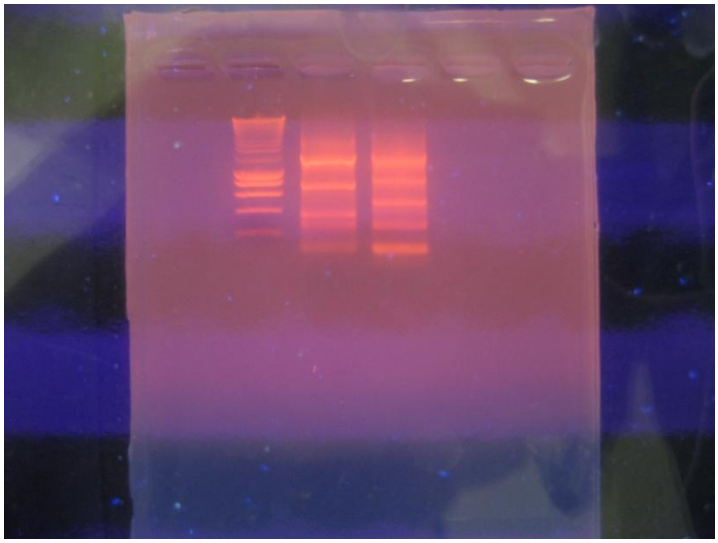
- Closed circular, nicked circular, and linear DNA of the same molecule will migrate at different rates through agarose gels.
- Closed circular > nicked circular>linear or the closed circular fragment will be found the furthest from the starting well when run on the same gel.

4. Applied current (voltage) to the electrophoretic system:

- At low voltages, the rate of migration is proportional to the voltage applied.
- As voltage increase, the rate of the mobility of larger DNA fragments increases differentially relative to the smaller and the effective range of separation decreases. (No longer proportional and fragments will be closer together on the gels; possibly giving decreased resolution. If the current is too high the gel will show artifacts and band smearing.
- For maximum resolution (2 close bands being seen) gels should run at no more than 5 V/cm (of gel). So mini-gels = 80 – 100 Volts; large gels = 125 - 150 Volts.

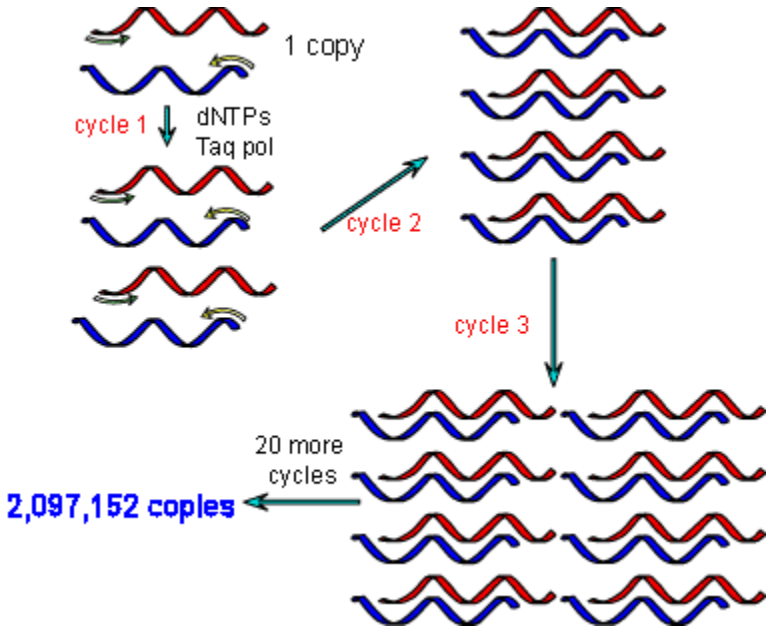
5. Base composition and temperature:

- The electrophoretic mobility in agarose gels is not significantly affected by temperature at which the gel is run (for a the range of 4-30degrees C) or by the base composition of the gel in contrast acrylamide gels.



PCR :

PCR (**P**olymerase **C**hain **R**eaction) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).



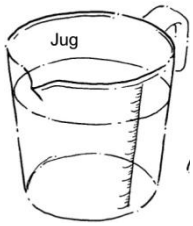
lab 3

Buffers

Solute: the stuff being dissolved. For example, in sugar water, it would be the sugar.

Solvent: the stuff doing the dissolving. For example, in sugar water, it would be the water.

Solution: a mixture of solute + solvent. For example, sugar water is a solution. By definition, solutions need to be clear.



Molarity, M

Molarity is the most common unit of solution concentration. Concentration means how many parts there are in the whole thing. Molarity is defined as the number of moles of solute in a total volume of a liter of solution.

To make a molar solution, calculate how many grams of solute (powdered stuff) you need, weigh it out, put it in a beaker, add water, mix until the solid is dissolved, then put in a graduated cylinder and add

more water until the volume reaches the total volume you need. You can also use a volumetric flask instead of a graduated cylinder.

$$\text{Molarity} = \frac{\text{moles of solute}}{\text{Liter of solution}} = \frac{\text{mol}}{\text{L}}$$

You can use dimensional analysis to determine how much solute you need for a particular molarity, or what the molarity will be given a certain amount of solute.

For example, what is the molarity of 25.5 grams of NaCl dissolved in water, total volume of the solution being 100. mL?

First calculate molecular weight of the solute, NaCl. Recall that the atomic mass on the periodic table is the same number as how much a mole of the substance would weigh, in grams. So:

Na: 23.0 g/mol Cl: 35.5 g/mol therefore NaCl: 58.5 g/mol

Write down what you are given in grams and convert into moles.

$$\frac{25.5\ \text{g}}{58.5\ \text{g}} \times \frac{1\ \text{mol}}{} = \text{mol}$$

$$= 0.436\ \text{mol}$$

This 0.436 mol is dissolved in 100. mL, so:

$$100.\ \text{mL} \times \frac{1\ \text{L}}{1000\ \text{mL}} = \text{L}$$

$$= 0.100\ \text{L}$$

now set up the fraction so it is in terms of mol/L:

$$\frac{0.436\ \text{mol}}{0.100\ \text{L}} = \text{M}$$

$$4.36\ \frac{\text{mol}}{\text{L}} = 4.36\ \text{M}$$

Example 2: Prepare 800 mL of 2 M sodium chloride

MM_{NaCl} = 58.5 g/mol (The molecular weight or molar mass of NaCl)

Volume in mL x mL to L x Molarity x Molecular weight = grams needed

$$800\ \text{mL} \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{2\ \text{mol}}{1\ \text{L}} \times \frac{58.5\ \text{g}}{\text{mol}} = \text{g of NaCl needed}$$

93.6 g of NaCl is needed; bring the total volume up to 800 mL.

Example 3: Dilution with molarity:

$$M_1V_1 = M_2V_2 \qquad \text{Or} \qquad C_iV_i = C_fV_f$$

M_1 = starting molarity ,M_2 = ending molarity ,C= concentration

V_1 = starting volume ,V_2 = ending volume ,i = initial ,f = final

In this equation, the volume goes with its molarity on the same side of the equals sign.

Prepare 100 mL of 1.0 M hydrochloric acid from concentrated, 12.1 M, hydrochloric acid.

So to solve this problem, you would put 100 mL and 1.0 M on the same side of the equals sign because they go together. 12.1 M would go with a variable on the other side of the equals sign. We need to figure out how much of the really concentrated acid we need to only have 100 mL of a 1.0 M solution of acid.

$M_1V_1 = M_2V_2$

$(12.1\ \text{M})\ V_1 = (1.0\ \text{M})(100\ \text{mL})$

divide both sides by 12.1 M

V_1 = 8.26 mL of concentrated HCl

Percent Solutions

Mass-volume percent solution (w/v %)

These are the most common percent solutions made. These are made when the molecular weight of the substance is very large or difficult to figure out. You don't have to know the molecular formula or molecular weight of the solute when you do mass-volume percent solutions. It makes things easier.

These solutions are indicated by w/v% and are determined by the grams of solute per 100 milliliters of solution. NOTE: I have found references that state that it is per 100 mL of solvent and references that say it is per 100 mL of solution. Since I don't know what the rule book for solution making is, I don't know for sure if it is per solvent or per solution. Per solution is easier, so we'll do it as per solution.

$$\text{Concentration of solute (w/v \%)} = \frac{\text{mass of solute (g)}}{\text{Volume of solution (mL)}} \times 100$$

For example, suppose we dissolve 1.2 g of NaCl in enough water to make 160 mL of (saline) solution, what is the w/v % of NaCl?

$$\frac{1.2 \text{ g of NaCl}}{160 \text{ mL}} \times 100 = 0.75 \text{ w/v \% solution}$$

We interpret this number as 0.75 g of NaCl in 100 mL of solution.
(Example borrowed from website:
http://www.iun.edu/~cpanhd/C101webnotes/aqueoussolns/wvpcnt.html)

Mass percent solutions (w/w %)

Grams of solute per 100 grams of solvent.

For example, 20. g of sodium chloride plus 80. g of water is a 20% by mass solution.

To make a mass percent solution you weigh both the solute and the solvent, then mix.

Volume percent solutions (v/v %)

Milliliters of solute per 100 mL of solution.

For example, 10 mL of ethyl alcohol plus enough H_2O to bring the total volume to 100 mL is a 10% by volume solution.

Calculating Molarity from Percent Solutions

These can be done a couple of different ways. One example is given here. You may be able to think through a different, but equivalent way to solve this problem.

To determine the molarity of a weight percent solution, the density of the solution is required.

volume of solution x density of liquid solution = *mass of solution*

mass of solution x mass percent of solute = mass of solution due to solute

mass of solution due to solute x molecular weight of solute = moles of solute

$$\text{Molarity} = \frac{\text{moles}}{\text{Liter}}$$

so, take your moles of solute and put over the volume of solution

Example: Determine the molarity of 12.5 % hydrochloric acid. (density 1.19 g/mL).

$$1000 \text{ mL} \times \frac{1.19 \text{ g}}{1 \text{ mL}} = 1190 \text{ g}$$ (this is the *mass of solution*)

(arbitrary number ,works well)

If we had 1.000 L, or 1000. mL of 12.5% HCl, the solution should weigh 1190 g. We want to know how much of these 1190 g are due to HCl molecules.

1190g x 12.5/100 (rewriting percent as a fraction) = the number of grams of HCl that make up the 1190 g

= 148.75 g This means that of the 1190 g of solution, 148.75 g are due to HCl molecules.

Convert g to moles

$$148.75\ \text{g} \times \frac{1\ \text{mol}}{36.5\ \text{g}} = 4.08\ \text{mol}$$

4.08 moles in a total volume of 1000 mL (that arbitrary number chosen in the beginning) = 4.08 M

Molality, m:

Number of moles of solute divided by the number of kilograms of SOLVENT (not of solution.)

Note for water, 1 kg of solvent is 1L.

To make a liter of a molal solution with water, you would add one liter of water because 1 L of water weighs 1 kg.

The difference between molar and molal is that when making 1 liter of a solution, with molar, the total volume is 1 liter, whereas with molal the total volume is slightly larger than 1 liter because you added 1 liter of water to the solute. It is a small difference, but for some reason chemists think it is important.

Normality, N

The number of equivalents of solute per liter of solution.

For example, 1 M HCl is 1 N, but 1 M H_2SO_4 is 2 N because 2 hydrogen ions are given off per mole of H_2SO_4.

I've only seen normality used in reference to acids and bases. Essentially it is the number of hydrogens that can come off in solution as the acid dissociates.

So, a 3 M H_2SO_4 solution would be 6 N

$$\frac{3 \text{ mol}}{\text{L}} \times \frac{2 \text{ H+ ions released}}{\text{mole}} = \frac{6 \text{ equivalents of H+ ions being released}}{\text{liter}}$$

= 6 N

Serial Dilutions

There are three main types of serial dilution ratios:

1:2 1:5 1:10

These ratios are part:whole. So a 1:2 dilution is 1 part something: *2 parts something plus buffer*

(where the *2 parts something plus buffer* = 1 part something + 1 part buffer).

A 1:5 dilution is 1 part something : 5 parts something plus buffer
(where the 5 parts something plus buffer = 1 part something + 4 parts buffer).

A "part" is a volume measurement. It could be 10 mL, 100 mL, or even 1000 mL. It does not matter what the exact volume is, but what does matter is that 4 parts of something is 4 times the volume of what 1 part would be. For example if 1 part is 100 mL, then 4 parts is 400 mL.

Serial dilutions can be done all the way from 1:2 to 1:1,000,000 in easy, logical steps. For example, to get a 1:1000 dilution, what someone does is a succession of three 1:10 dilutions.

To make a 1:2 dilution, you take equal parts of your substance and buffer and mix them together.

To make a 1:5 dilution, you take one part of your substance and four parts buffer and mix them together. The sum of the parts is 5, thus the second number in the ratio.

To make a 1:10 dilution, you take one part of your substance and nine parts buffer and mix them together. The sum of the parts is 10, thus the second number in the ratio.

To get a 1:500 dilution, you would do a 1:5 dilution and two 1:10 dilutions. The order in which you do them is not important.

Sample problem: Tell how to make a 1:500 dilution:

1. Take one part of your substance and add 9 parts buffer.
2. Mix.
3. Take one part of this 1:10 dilution and put in a new container.
4. Add 9 parts buffer.
5. Mix.
6. Take one part of this 1:100 dilution and put in a new container.
7. Add 4 parts buffer.
8. Mix.

You now have a 1:500 dilution made out of two 1:10 dilutions and one 1:5 dilution.

You will be expected to draw and tell how to make any serial dilution scheme that involves a 1:2, a 1:5, and/or a 1:10 dilution.

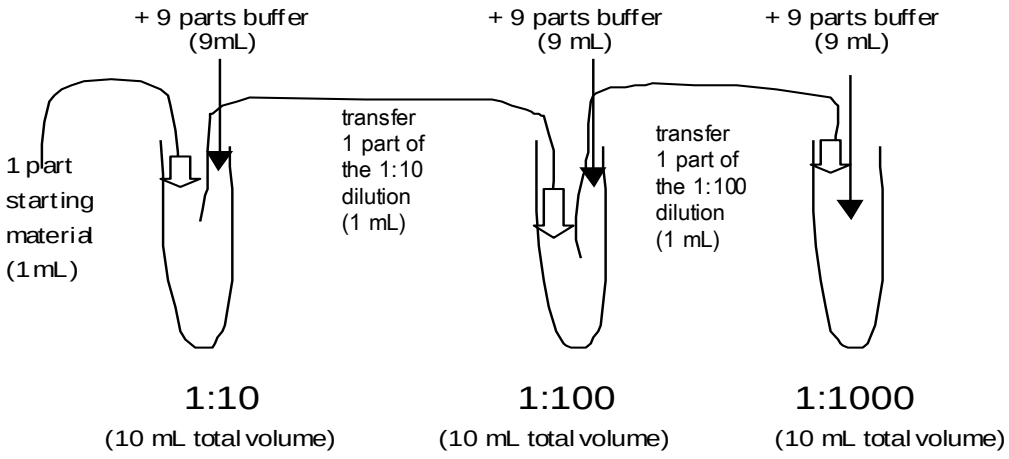
One thing to consider when doing serial dilutions is how much volume you are transferring from one tube to the next. For example, a 1:1000 dilution could, in theory, be 1 uL of starting material + 999 uL of water to make 1000 uL of solution. The amount of error in mixing 1 uL in 999 uL of water is much greater than if you were to :

Take 10 uL of starting material and add 90 uL of water. Mix (1:10 dilution)

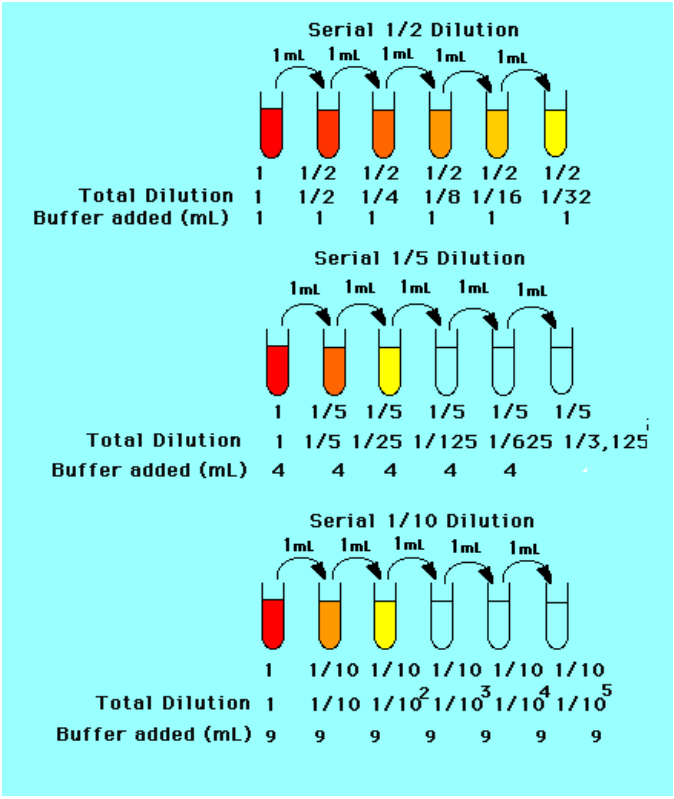
Then take 10 uL of the 1:10 dilution and add 90 uL of water. Mix (now you have a 1:100 dilution)

Finally take 10 uL of the 1:100 dilution and add 90 uL of water. Mix. You now have 100 uL of a 1:1000 dilution.

If you needed 1000 uL of a 1:1000 dilution, you could take all 100 uL of the 1:100 dilution and add 900 uL of water to get 1000 uL of a 1:1000 dilution.



A diagram of 1:2, 1:5, and 1:10 serial dilutions. Diagram stolen from: http://ntri.tamuk.edu/dilutions/dilution.html



X solutions

Stock solutions are stored anywhere from 5x to 50x concentrations. Most working solutions are at 1x, although over time, some protocols will say that a 0.5x concentration is good to use. Knowing how to dilute a stock solution is important because most of the solutions you use in the lab are stored in a concentrated form. For example, we have 10x TBE as our stock buffer for electrophoresis. We use it in a 1x concentration. We need to go from 10x to 1x, a dilution of 10 times. So we need to do a 1:10 dilution. If we made a liter of 1x solution, we would take 100 mL of the 10x solution and add it to 900 mL of water.

If we were to use TBE as a 0.5x solution, then we would have to dilute a 10x stock 20 times. (10 divided by 0.5 = 20) This means for 1 liter of 0.5x TBE we would take 50 mL of 10x stock and add 950 mL of water. (50 mL is 1/20 of 1000 mL)

pH

pH by definition is –log [H+]. In words this is: pH equals the negative log of the concentration of hydrogen ions in solution. Acids add hydrogen ions to solution. Bases remove hydrogen ions from solution.

[formula] means concentration of whatever the formula is. The concentration is in terms of molarity.

The pH scale ranges from 0 -14. If a pH reads from 0-6.9, the solution is acidic. If the pH is 7.0, the solution is neutral. If the pH is 7.1 -14, the solution is basic.

At first the pH scale seems backwards. The higher the pH, the fewer H+ ions are in solution. A lower pH is actually more acidic….has more H+ ions in solution.

This backwards situation is because pH only measures the amount of ions in solution if the Molarity is less than 1.

Why? Looking at the equation: -log [H+], if the H+ = 1.0 (because a 1 M solution of HCl has 1 mole of H+ ions per liter of solution)

The equation becomes – log 1

Put in your calculator: 1, then log (unless you have a graphing calculator which lets you put it in directly as –log #)

The log of 1 is 0.

The negative of 0 is 0.

A number larger than 1 would give you a negative pH. Scientists decided that a negative pH has no meaning. (Technically measuring pH involves an electrical gradient so negative values would have no meaning.)

A molarity smaller than 1 will give you a positive pH. Scientists like a positive pH so they allow it to have meaning.

Example:

What is the pH if the molarity of HCl is 0.1 M?

$0.1\ M = 10^{-1}\ M$

$pH = -\log [H+]$

$pH = - \log 10^{-1}$

$pH = - -1$

$pH = +1$

So, as the concentration of hydrogen ions (acid) gets smaller, the pH gets larger. Another example:

What is the pH if the molarity of HCl is 0.001 M?

$0.001\ M = 10^{-3}\ M$

$pH = -\log [H+]$

$pH = - \log 10^{-3}$

$pH = - -3$

$pH = +3$

Why is pH of 7 considered neutral?

Neutrality happens when the amount of acid equals the amount of base. Put another way:

[H+] = [OH-]

With water, the H_2O dissociates into H+ ions and OH- ions. HOH < == > $H^+ + OH^-$

For each water molecule, there is one hydrogen ion and one hydroxide ion. Since water is the foundation for studying science, scientists decided that water is neutral. When there are more hydrogen ions in solution than in pure water, you have an acid. When there are fewer hydrogen ions (more hydroxide ions) in solution than in pure water, you have a base.

There is a mathematical way to write the concentration of hydrogen and hydroxide ions showing that they are in equilibrium. At equilibrium the rate that water falls apart to make hydrogen and hydroxide ions equals the rate that hydrogen and hydroxide ions find each other to make a water molecule. The following notation is used:

$$K_{sp} = \frac{[\text{products}]}{[\text{reactants}]}$$

Using a balanced equation, the coefficients become exponents. Numbers are substituted and the equation is solved. Fortunately with water, all of the coefficients are 1, so the math is easy.

For water, the Ksp becomes Kw meaning the equilibrium constant for water.

$$Kw = \frac{[H+][OH-]}{[H_2O]}$$

The $[H_2O]$ is assumed to = 1 because it is in a liquid form. The concentration of liquids remains constant so $[H_2O]$ will always be a constant. The [H+] and [OH-] ions can fit between water molecules, so their concentration can change without really changing the volume very much. Since you can put in more H+ ions and OH- ions without changing the volume, their concentrations are included in the equilibrium constant expression.

Some scientist figured out that for water, $Kw = 1.0 \times 10^{-14}$

The "14" is where the "14" for the pH scale comes from.

Solving for the concentration of H+ and OH-, we do the following:

$K_w = [H^+][OH^-]$

$K_w = (x)(x)$ because the concentration of H^+ = concentration of OH^-

$K_w = x^2$

$K_w = 10^{-14} = x^2$

Square root both sides to get $10^{-7} = x$

This means that the concentration of hydrogen ions and hydroxide ions in pure water is 10^{-7}.

$pH = -\log [H^+]$

$pH = -\log 10^{-7}$

$pH = - -7$

$pH = 7$

You do not have to know how to solve equilibrium constants, but you should be able to go from concentration to pH and from pH to concentration.

From concentration to pH

What is the pH of a 0.023 M solution of HCl?

pH = -log [H+]

pH = - log (0.023)

pH = - -1.6

pH = 1.6

From pH to concentration

What is the concentration of HCl whc

pH = - log [H+]

6.3 = - log [H+]

- 6.3 = log [H+]

Anti-log both sides

5.0×10^{-7} M = [H+] (We assume that the units that go on the concentration are Molarity units.)

Buffers:

A buffer helps stabilize the pH of a solution. We often use a buffer called Tris. Tris is a common buffer used in electrophoresis because it works well to withstand pH changes under an electric current. The pH of a solution changes according to room temperature, but for our purposes, we will act like our room temperature is a constant 25 °C.

Tris base is often combined with boric acid or acetic acid to make electrophoresis buffers. The electrophoresis buffer we use is TBE: Tris, Borate, EDTA.

EDTA is added as a preservative.

TBE is most often made up as a 10x concentration. We run gels at 1x or 0.5x concentration of TBE depending on where we get the agarose from and if it has already been dissolved in a buffer.

10x TBE is: 89 mM Tris-borate and 2mM EDTA, pH 8.3.

one liter of 10x TBE is made by adding together:

108 g of Trizma base

7.5 g of EDTA

55 g of boric acid

bring up the volume to 1 L

Dilute 1 to 10 for a 1x buffer; the pH should already be 8.3. Do not adjust the pH.

lab 4

Extraction of RNA

Nucleic Acid Preparation

Application?

RNA

• Amplification methods (RT-PCR)

• Hybridization methods (Northern analysis)

Choosing an Isolation Method

Important factors are:

- Processing speed
- Ease of use
- Yield of DNA or RNA
- Quality of DNA and RNA prepared

  (amplification performance)
- Shelf life/storage conditions
- Quality assurance criteria
- Cost of preparation

Precautions for Working with RNA

in the Laboratory RNA is not a stable molecule! It is easily degraded by RNase enzymes.

- Use sterile, disposable plasticware (tubes, filter tips) marked “For RNA Use Only”.
- Always wear gloves and work in a hood whenever possible/practical.
- Treat liquids with DEPC, except Tris-based buffers.

RNA Isolation Methods:

Cesium Chloride Gradient

Used mainly to get clean RNA for Northern blots

- Homogenize cells in guanidinium isothiocyanate and b-mercaptoethanol solution.
- Add to CsCl gradient and centrifuge for 12–20 hours; RNA will be at the bottom of tube.
- Re-dissolve in TE/SDS buffer.
- Precipitate RNA with salt and ethanol, then rehydrate.

Advantage: high quality

Disadvantages: extremely time-consuming, hazardous materials disposal issues

Guanidinium-based Organic Isolation

Phenol/guanidinium solution disrupts cells, solubilizes cell components, but maintains integrity of RNA.

- Add chloroform, mix, and centrifuge.
- Proteins/DNA remain at interface.

- RNA is removed with aqueous top layer.
- RNA is precipitated with alcohol and rehydrated.

Advantage: faster than CsCl method

Disadvantages: fume hood required, hazardous waste disposal issues

Nonorganic Salt Precipitation

Cell membranes are lysed and proteins are denatured by detergent (such as SDS) in the presence of EDTA or other RNase inhibitors.

- Proteins/DNA are precipitated with a high concentration salt solution.
- RNA is precipitated with alcohol and rehydrated.

Advantages: fast and easy, nontoxic, produces high quality RNA.

DNA or RNA is characterized using several different methods for assessing quantity, quality, and molecular size.

- UV spectrophotometry
- Agarose gel electrophoresis
- Fluorometry
- Colorimetric blotting

Storage Conditions

Store RNA in Rnase-free ultrapure water at –70 °C.

Troubleshooting Nucleic Acid

Problem 1 : No or low nucleic acid yield.

1. Make sure that ample time was allowed for resuspension or rehydration of sample.

or

2. Repeat isolation from any remaining original sample (adjust procedure for possible low cell number or poorly handled starting material).

or

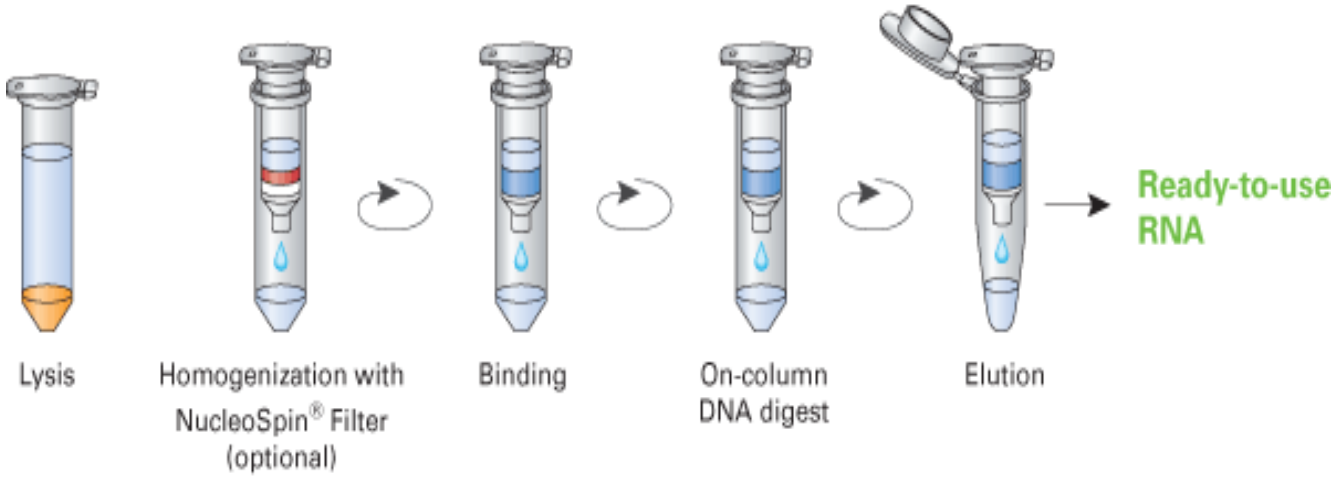
3. Concentrate dilute nucleic acid using ethanol precipitation.

Problem 2: Poor nucleic acid quality

1. If sample is degraded, repeat isolation from remaining original sample, if possible.

or

2. If sample is contaminated with proteins or other substances, clean it up by re-isolating (improvement depends on the extraction procedure used).



functions of some elemnts in DNA and RNA Extraction

Glucose and salt are : added to increase the osmotic pressure outside the cells.

Tris is a buffering agent used to maintain a constant pH (= 8.0).

EDTA protects the DNA from degradative enzymes (called DNAses); EDTA binds divalent cations that are necessary for DNAse activity.

NaOH and SDS (a detergent) : The alkaline mixtures ruptures the cells, and the SDS detergent breaks apart the lipid membrane and solubilizes cellular proteins. NaOH also denatures the DNA into single strands.

The acetic acid neutralizes the pH, allowing the DNA strands to renature.

The potassium acetate also precipitates the SDS from solution, along with the cellular debris.

Isopropanol effectively precipitates nucleic acids, but is much less effective with proteins. A quick precipitation can therefore purify DNA from protein contaminants. It also make the DNA form in the fiber form.

Ethanol helps to remove the remaining salts and SDS from the preparation.> it precipitate the DNA .

Protinase K digest the proteins.

Phenol: $CHCl_3$ extract DNA.

The solid support e.g. (silica , pharmaceia ,clonetech ,Qiagen) : binding DNA and then the elute will be with low salt buffer.

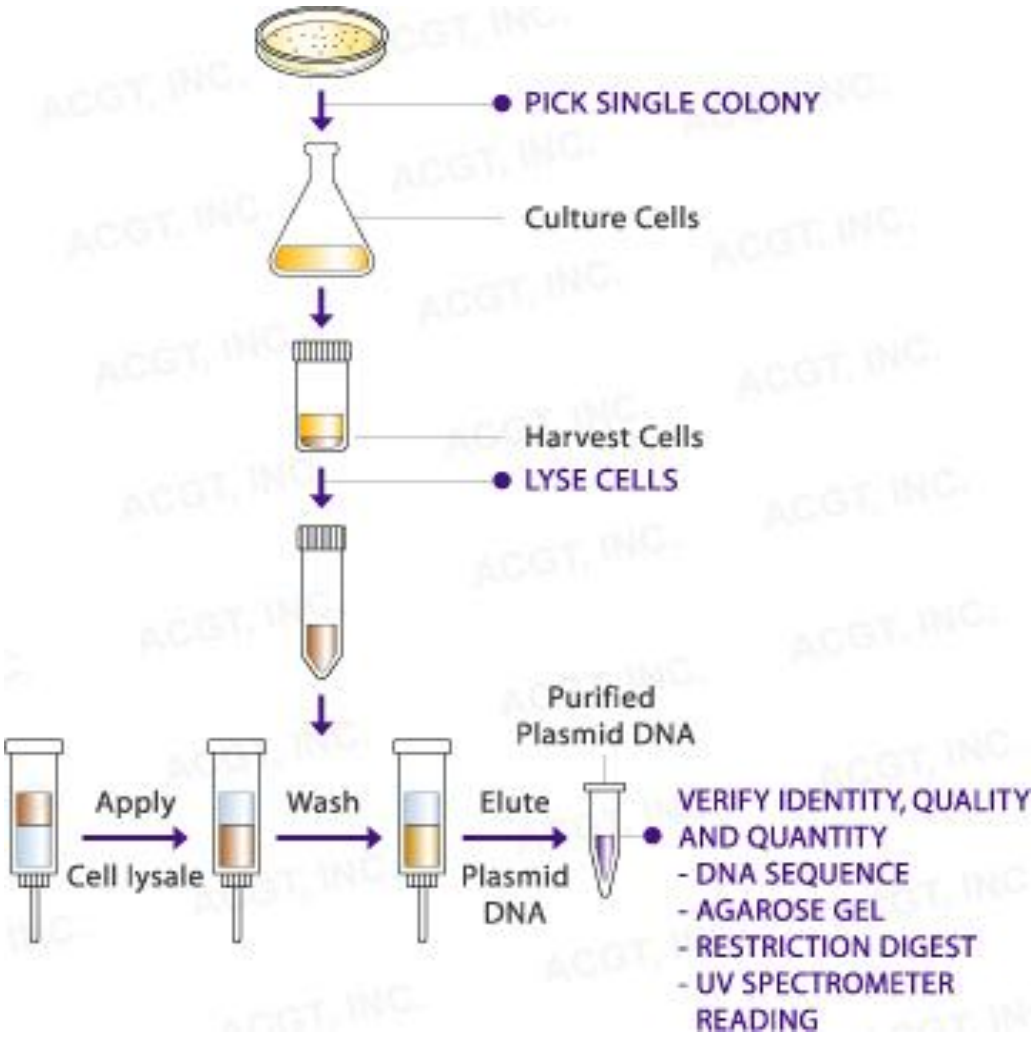
RNA Extraction

Guanidinium thiocyanate : is used to lyse cells and virus particles in RNA and DNA extractions, where its function, in addition to its lysing action, is to prevent activity of RNase enzymes and DNase enzymes by denaturing them. These enzymes would otherwise damage the extract.

b-mercaptoethanol solution : is used to reduce disulfide bonds and can act as a biological antioxidant.

salt and ethanol Precipitate RNA.

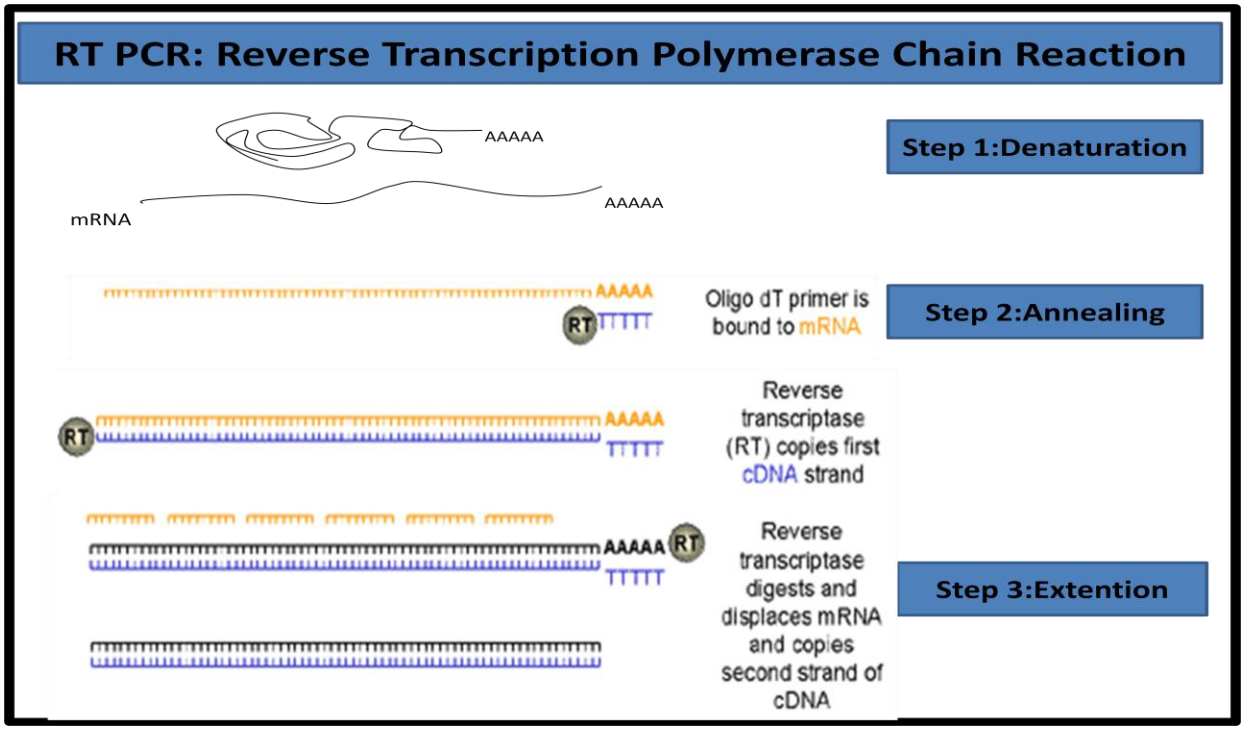
SDS : Cell membranes are lysing and proteins denaturing .



lab 5

Reverse transcription polymerase chain reaction (RT PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) is a method used to amplify cDNA copies of mRNA. It is an extremely useful technique in RNA semi-quantitation, cloning, and probe synthesis. The technique consists of two main steps. In the first step (“first strand reaction’’), complementary DNA (cDNA) is made from an mRNA template using dNTPs and reverse transcriptase enzyme. After the reverse transcriptase reaction is complete and cDNA has been generated from the mRNA, standard PCR (‘’second strand reaction”) is performed with Taq DNA polymerase and gene-specific primers.



One step and two step prosedures

- One step :RT and PCR performed consecutively

In a single tube

- Two step: RT and PCR performed in seperate tubes

Advantages of one step procedure:

- Minimize time requires
- Reduces risk of contamination
- Improve sensitive and specificity

Advantages of two step procedure:

- Allows optimal reaction conditions
- Provide maximum flexibility
- Amplifies long sequences

Choosing the RT-PCR enzymes:

- Maximum lenght of tamplet that can be

transcribed into full-lenght cDNA

- Temperature optimum
- Snsetivity

Choosing the primers for TR:

- Oligo (dt)n
- Anchored oligo (dt)n
- Random hexamer
- Sequence-specific

Before beginning:

(1) Set up reverse transcriptase (RT) reactions at the bench labeled RNA ONLY.

(2) Make sure to use a new set of gloves while working with RNA so that you avoid RNAse activity. RNAses degrade RNA and are found everywhere, especially on your hands. use DEPC H20. DEPC water contains Diethyl Pyrocarbonate, which degrades any trace of RNAses.

(3) When setting up the RT reaction use filtered RNAse free tips.

(4) You will need buffer, oligo d(T) or random oligomer, dNTPs, DEPC water, Superscript II reverse transcriptase, MgCl2, DTT, RNAse Out, and RNAse H.

(5) The entire procedure may be done in the icycler PCR machine or you can use water baths/heatblocks to incubate the samples for the various incubation steps.

Setting up Reverse Transcriptase Reaction:

(1) In the first step of the reaction, we typically use 2 mg of total RNA. The source of RNA can be from tissues or from cultured cells. Oligo dT is the primer of choice. If you need to isolate 5'end of the cDNAs or longer cDNAs you can use a random oligomer to facilitate the reverse transcriptase enzyme to proceed all the way to the 5'end of the mRNA. The reaction is incubated at 65oC for 5 min to allow Oligo dT to anneal to the mRNA.

(2) In the next step of the reaction, additional reagents are added. The 10 buffer contains Tris, KCl. MgCl2 is added to the reaction in order to stabilize ATP and aid in the transfer of the phosphate group during extension. Also, DTT (dithiothreitol) is added, which is a reducing agent that breaks disulfide bonds. Lastly, add RNase OuT, which is a potent non-competitive inhibitor of ribonucleases such as RNase A, B, and C, but not H. The reaction is incubated at 42oC for 2 min to allow the reaction to get to optimal temperature for reverse transcriptase.

(3) Next, Superscript II Reverse Transcriptase is added and the reaction is incubated at 42Oc to allow extension. This is the step where cDNA is synthesized from the RNA template.

(4) The reaction is then terminated at 70oC. RNase H is added. RNase H is an

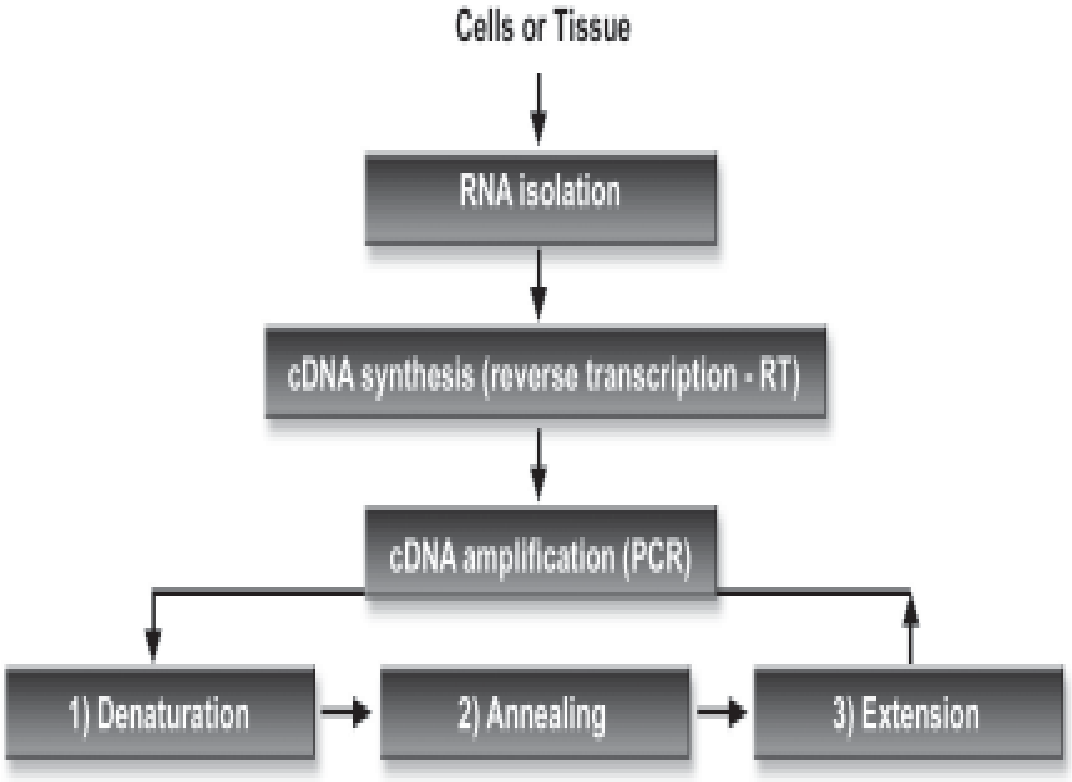
endoribonuclease that specifically hydrolyzes the phosphodiester bonds of RNA in RNA:DNA duplexes to generate products with 3’ hydroxyl and 5’ phosphate ends. It will not degrade single-stranded or double-stranded DNA or RNA.

(5) There are three types of DNA primers commonly used to facilitate reverse transcriptase binding and function: random primers, oligo (dT) primers and gene specific primers. Oligo (dT) primers consist of short strands (12-18 bases) of thymine deoxynucleotides that hybridize to the mRNA poly A tail and prime reverse transcription. These maximize the presence of long cDNAs with many mRNA 3’ ends in the final cDNA population.

(6) Wherever possible, oligonucleotide primers that bind to sequences located in different exons of the target RNA should be used as sense and antisense primers for amplification of the cDNA product. In this way, amplification products derived from cDNA and contaminating genomic DNA can be easily distinguished.

(7) Once, the cDNAs are generated, proceed with PCR for housekeeping gene

and then the gene of interest. Products should stored at -20oC for long term use.



some links:

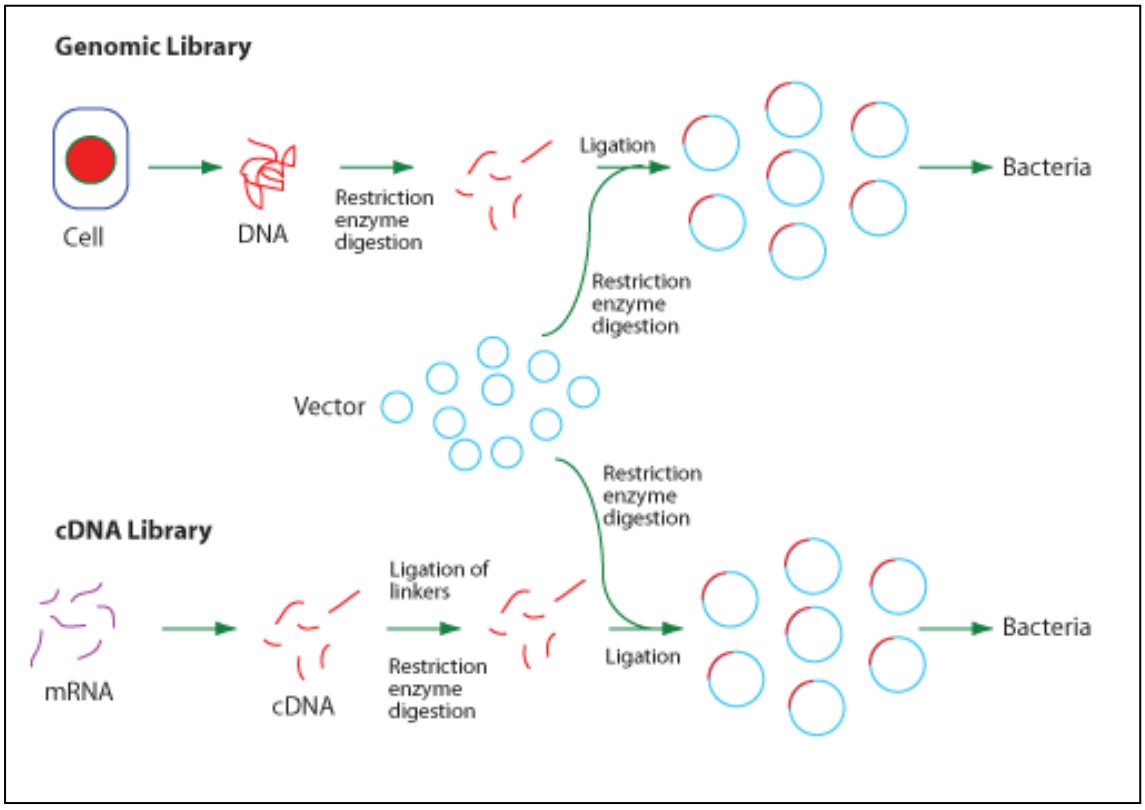
- http://www.youtube.com/watch?v=HMC7c2T8fVk&feature=related
- http://www.youtube.com/watch?v=4FLThf7r3ZE
- http://www.youtube.com/watch?v=qmACKSvFZpM

DNA library

is a collection of cloned DNA fragments. There are two types of DNA library:

-Genomic library contains DNA fragments representing the entire genome of an organism.

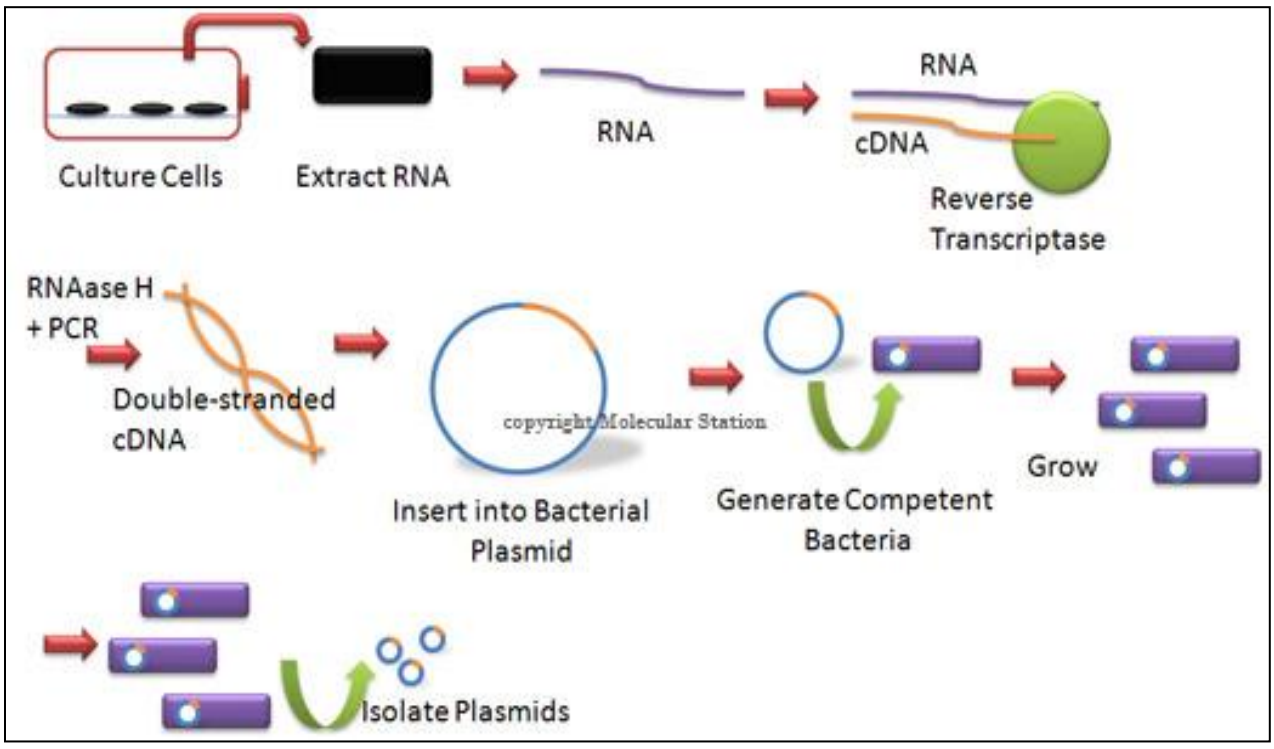
-cDNA library contains only complementary DNA molecules synthesized from mRNA molecules in a cell. It contain all the coding information (the genetic code that produces proteins) from a given cell population



A cDNA library

is a combination of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which together constitute some portion of the transcriptome of the organism. cDNA is created from a mature mRNA from a eukaryotic cell with the use of an enzyme known as reverse transcriptase. In eukaryotes, a poly-(A) tail distinguishes mRNA from tRNA and rRNA and can therefore be used as a primer site for reverse transcription.

cDNA Library Technique:



1- After the cDNA is synthesized, it is cloned into expression vectors or plasmids.

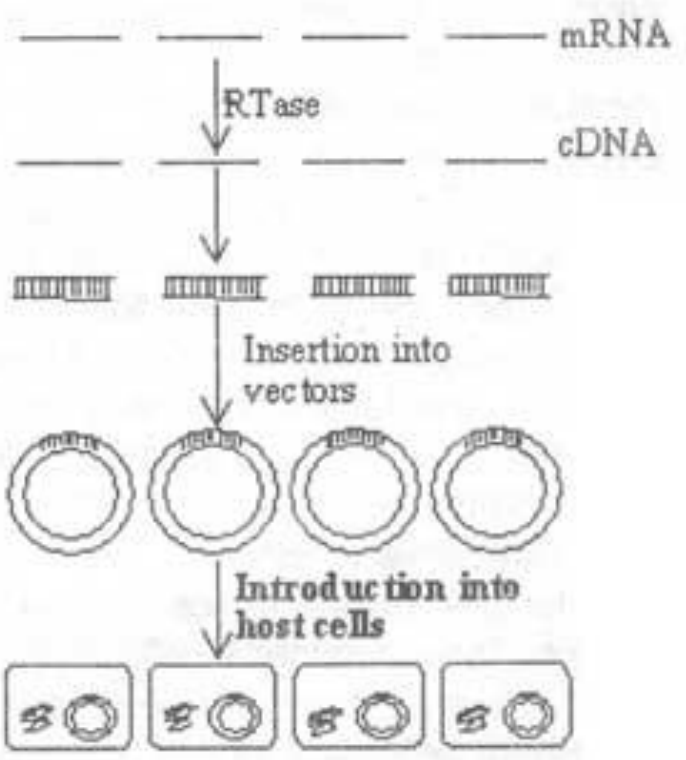
2- These plasmids each containing one cDNA are transformed into bacterial competent cells .

3- These plasmids are amplified in the growing bacteria.

4- The bacteria clones are then selected so that only bacteria containing the plasmid will survive.

5- This is commonly done through antibiotic resistance selection.

6- Once the bacteria are selected, stocks of the bacteria are created which can later be grown and sequenced to compile a cDNA library.



Construction of cDNA library