Lab 2: Mixing and Centrifuging Solutions - Molecular Biology Basic Skills

Introduction: Molecular Biologists work with extremely small volumes of solutions that are combined in precise ratios in order to carry out various reactions. The starting solutions that are combined are usually made in more concentrated forms that are called 'stock solutions'. When some of the chemical in the stock solution is needed multiple times, a small volume is poured or micropipetted into a *clean, sterile working container* and then the solution used in each individual reaction is taken from that secondary container. That way the original stock solution remains uncontaminated even if you accidentally mix two of your working secondary solutions.

Many common stock solutions can be purchased from suppliers. In making a stock solution yourself, care must be taken to weigh the chemical you are going to dissolve very carefully, deliver all of it to the mixing vessel, bring the volume up to the exact level stated with properly prepared solvent (usually sterile water for molecular biology but various alcohols are common), deliver the mixed solution to a storage bottle and to carry out any additional purification steps, such as autoclaving (heating under pressure) or filtering, as directed. This stock solution should be labeled with its contents, the date, and the name of the person who made it.

The following exercises have you practicing a number of tasks that develop skills you will need when we start working with our samples. They include combining stock solutions to make a reaction mixture, as practice for following the instructions (protocol) in a molecular biology experiment. You will continue to use the micropipetters that we practiced with before. You will practice a variety of methods for mixing these solutions, including inverting, vortexing and pipetting up and down (it is not always easy to mix very small volumes, especially if one is very dense and one is very light).

We will practice making a serial dilution, which creates a set of solutions, each being a common proportion less concentrated than the one before it, created by mixing part of the previous solution and the solvent. This type of series is often needed when using a spectrophotometer or optimizing an assay. You will experiment with the order in which you mix components of a reaction: sometimes the order is very important to having a successful outcome. Finally, you will learn to spin samples in a centrifuge and recover insoluble material as a pellet I nthe bottom of the tube (a common process for recovering protein and DNA).

Preparation: make sure you can identify the following on your bench or nearby

- Blue- or Purple-capped 15ml in a rack that contain solutions A, B, C and D
- Blue- or Purple-capped 15 ml tubes containing ethanol and isopropanol
- Blue- or Purple-capped 15 ml tubes containing MB-H_2O (molecular-biology grade water)
- In a different rack, 1.7ml tubes labeled
    - 10X Buffer F
    - 5X Buffer H
    - 50% Glycerol
    - $CHCl_3$ (chloroform)
    - DNA (10mg/ml)
    - BSA (10mg/ml)
- Empty 1.7ml microfuge tubes (in a box on your bench) that you will use for combining solutions.
- A 100-ml beaker to dispose of any solutions.
- A set of micropipetters in a stand, including
    - 20ul micropipetter
    - 200ul micropipetter
    - 1000ul micropipetter
- A box of tips that fits each micropipetter
- Identify the nearest
    - Vortexer,
    - Low-speed benchtop minifuge

  - High-speed benchtop microfuge.
- Green pipette pump
- Serological pipettes in sizes 2,5 and 10ml

Part 1. Mixing Solutions with different viscosities and densities.

You are practicing your pipetting skills using stock solutions. You have to pipette the stock solutions carefully to get just the right volume, and any small droplets left behind have a big impact on the final results, so you need to rinse the tip with some of the water in the target container in order to get a good result.

1. In a 1.7ml microfuge tube, add 500ul of Solution B to 500ul Solution A
    a. Observe the pipette tips: do any droplets cling to the sides so they are left behind
    b. Observe how the solutions 'layer' – which one is denser (on the bottom)?
    c. If you spin the tube for a few seconds in the minifuge on your bench do the layers separate?
    d. Can you get all of each solution out of the tip and into the tube?
2. Try mixing the two solutions by several methods:
    a. Finger mix (tapping the bottom of the tube gently)
    b. Shake by hand (invert rapidly 9-10 times)
    c. Use the Vortexer to mix them (3-4 seconds).
    d. Pipette up and down 8-10 times.
    e. Are droplets left on the sides of the tube? In the lid?
3. Now add 500 ul of Solution C to the tube – answer the same questions as above.
4. In a fresh tube, place 500ul of solution A, add 500ul of solution D. Try mixing as above.
5. Not add 500ul of Solution C to the tube, try mixing as above.

Part 2 – Creating a Serial Dilution set

Introduction: sometimes you need such a small amount of the concentrated stock that it is hard to measure accurately – in this case you might make a series of dilutions, with the final one having the concentration your need. The idea is not to change the measurement setting on your micropipetter so you always take out the same volume, but you use the first dilution as the new stock for the second one, the second dilution as the stock for the third dilution, and so on in a series. The most common ratios are 2-fold dilutions and 10-fold dilutions. It can take a while to get the hang of the concept, but it is easy to keep track of what you are doing when you always add the same volume and the difference in volume in adjacent tubes makes it easy to tell if you have done the next step. This process is also used sometimes when you do not know how much of a material is in your unknown sample – by making a dilution series and measuring them all you can find the one that your measurement device is able to accurately quantify.

1. Use Solution A as the 'stock', the original solution that you are diluting with water, then make 2-fold serial dilutions:
    a. Label five tubes: 1, 2, 3, 4, 5
    b. To each tube add 500ul (or 0.5ml) of water.
    c. Add 500ul (0.5 ml) of Solution A to the water in tube 1 (the volumes are equal, so this is 2-fold dilution).
        i. Cap the tube, then vortex to mix thoroughly
        ii. Briefly centrifuge the tube to make sure no droplets are on the sides (be sure to balance it with another tube that contains 1000ul of water, Label Balance, or with another student's sample → label tubes with your initials or team ID!)
    d. Remove 500ul from Tube 1 and add it to the water in Tube 2.
        i. Repeat the mixing and centrifuging steps.
    e. Repeat step d, removing 500 ul from Tube 2 and adding it to the water in Tube 3.
        i. Repeat the mixing and centrifuging steps.
    f. Remove 500 ul from Tube 3 and add to the water in Tube 4, mix and centrifuge as before.
    g. Remove 500 ul from Tube 4 and add to the water in Tube 5, mix and centrifuge as before.
2. Now you should check that you did each dilution correctly. You can line up the tubes and see if the volumes look the same, except for Tube 5, which should have twice as much as the others.
3. Other ways to verify that you have the expected volume in each tube
    a. Re-measure each volume with the micropipette set at 1000ul.
        i. If you appear to have too much or too little you can 'dial' the volume-set on the micropipette until you have just pulled up all of the liquid or just pushed out all of the air.
4. Tare an empty tube, then weigh the tube containing the sample on an appropriate balance
    a. Remember that you will need to first check the mass of 500ul of Solution A (or 1000ul if you want the gm/ml), since it might not have the same density as water.

Part 3 – Preparing a mixture of solutions, where the order of addition matters.

Introduction*: in molecular biology reactions you are often combining 5-10 chemical solutions to get a final mixture that works for the reaction you want to do. Each chemical is available as a concentrated stock, but you use different volumes of each one. Some chemicals will not stay in solution if they are in the presence of a concentrated solution of another chemical, so the order in which you add the solutions is very important. Another important factor is that some solutions are frozen – you thaw them right before you use them – then you must mix them (usually by flicking them, also called finger-mixing, pipetting up and down, or vortexing), then centrifuge briefly (2- 3 sec)to collect everything at the bottom of the tube and then measure out the amount you need to use for your reaction.*

Carry out the following instructions as carefully as possible – make a note wherever you have a question about why the order is written in the way we have it, or if you notice anything odd about your solution – especially if you see anything that looks like particles or dust coming out the solution when you mix two solutions.

1. Label a 1.5 ml microfuge tube that you are going to use as the final reaction mixture, into which you will measure the stock solutions.
2. Our goal is to make a total final volume of 200ul of a 'reaction mix'.
    a. Add 20 ul of 10X F-use the 100ul micropipetter, set at 20
    b. Add 40ul of 5X H- use the 100ul pipetter, set at 40
    c. Add 10ul of 50% G- use the 10ul pipetter, set at 2
    d. Vortex the mixture, then spin briefly in the microfuge (balance with a tube having an equal volume containing water),
        i. Do you see any particles? (this is called precipitate)?
    e. Check the volume using the 100ul micropipetter set at 70 (if there a bubble of air at the bottom of the tip, your volume came up short, if there is a bit of solution left in the tube your volume is too big).
    f. Add 130 ul of water with the 200ul micropipetter, vortex and centrifuge to collect all of the liquid.
    g. If you did have precipitate, did it disappear?
    h. What is your final volume?
        i. What is the total volume supposed to be?
    i. Repeat the process, but put the water in your tube FIRST, then add the solutions in steps a,b,c.
        i. Was there a difference in volume?
        ii. Did a precipitate still form?
        iii. What is the FINAL concentration of Solutions 1,2 and 3 in your reaction mix?

Part 4. Recovering Pellets and Separating Layers of Liquids.

Introduction*: in many molecular biology applications you want to concentrate a material. One way to do this is to make it insoluble, so it forms a solid that precipitates, or collects, in the bottom of the tube. To collect it much faster, you can spin the solution in a microcentrifuge (this spins much faster than the bench-top minifuge) it will collect in the bottom of the tube – this is called the pellet. You then remove as much of the liquid part (solution) as you can without disturbing the pellet. Usually you air-dry the pellet for a few minutes, and then you can dissolve the pellet in a small volume of buffer, so the material is more concentrated than when you started. This is also a way to change what solvent the material is dissolved in, you could precipitate out of a NaCl salt solution and re-dissolve in a Tris buffer solution. Another common task it to combine two liquids that do not mix (think oil and water in salad dressing), so that one type of material will dissolve in one liquid and something that contaminates it that you do not want will dissolve in the other liquid. You then spin the sample to separate the two types of liquids. Then you remove the top layer to another tube. Now each material is in a different solvent and in different tubes – you could concentrate them next by precipitation.*

Concentrating DNA by pelleting it out of a solution

Making DNA insoluble and pelleting it:

1. To the DNA tube add 300ul of 100% ethanol.
2. Vortex 10-15 seconds
3. Centrifuge for 15 minutes.
4. Remove the liquid from the pellet – use a micropipetter set at 200ul and remove as much of the liquid as you can.
    a. If your pipette tip touches the pellet, put the material back in the tube (rinse the tip up and down in the remaining solution)
    b. Vortex the pellet with 200ul of ethanol
    c. Centrifuge for 15 minutes and try again.

Making a bi-phasic solution, pulling off the top layer

Separating Liquid Layers

1. In one 1.7ml tube combine
    a. 300 ul of the solution in Tube $CHCl_3$
    b. 300ul of the solution in Tube BSA.
2. Vortex for 10-15 seconds
3. Centrifuge for 15 minutes
4. Remove the top layer to a new tube.
    a. Did you get all of the top layer off?
    b. Did you get any of the bottom layer in the top layer tube? How do you know?
    c. Is there a thin white layer in between? What do you think it is?
    d. Is the top layer from Tube CHCl3 or from Tube BSA and what does this tell you about the density of each solution?
5. Measure the volume of each layer and record it.