Laboratory 1:
Micropipetting

Micropipetting

Micropipettes are essential tools during the practical course. For accurate measurements it is important to follow the instructions below. Each student does the practical exercises 1-3 to acquire pipetting skills, exercises 4-5 are done together by the group.

Objectives

- To learn how to pipette small volumes of liquid (0.1 µl - 1000 µl)
- Using the vortex to mix and the centrifuge to spin down
- To create a standard curve by making serial dilutions
- To learn how to make and load an agarose gel

1. Measure different volumes using micropipettes

Pipette different volumes of water up and down using the appropriate pipettes 1 µl – 1000 µl and the corresponding tips. Never allow liquid to enter the body of the micropipette. Practice with different pipettes and volumes until you feel it becomes a routine.

Procedure:

If this is your first time using micropipettes, press the plunger a few times to get used to the feel of the 'first stop' and the second stop'. The start

1. Turn the volume adjustment to the desired volume.
2. Attach a new tip and ensure it has a good seal.
3. Press the plunger to the first stop (apply **gentle** pressure). The piston displaces the air (corresponding to the set volume).
4. Hold the pipette vertically with the plunger at the first stop and immerse the tip **a few millimetres** into the sample.
5. Allow the plunger to **slowly** return to the UP position (do not let it snap) and aspirate the set volume (without air bubbles).
6. Press the plunger to the second stop to dispense the liquid (touching the tube sidewall or liquid for accurate pipetting). Remove the pipette tip from the liquid, then allow the plunger to slowly return to the UP position.
7. Discard the tip by pressing the tip ejector button.

2. Pipette small volumes into larger ones

Reliable pipetting of small volumes is critical since errors in the amount of active compound (enzyme, drug, etc) impact the results. When you set up an enzymatic reaction, always add enzymes (small volume) last!

Procedure

1. Label the lid of four 1.5 ml microfuge tubes (for instance 1-4).
2. Add 1 ml of water in each tube.
3. Add 10 µl, 5 µl, 1 µl, or 0.5 µl of 6X loading buffer (which is of higher density and blue), close the tubes and observe the behaviour of the dye.

4. Vortex briefly to mix. Observe the behaviour of the dye.
5. Centrifuge for a few seconds to collect the liquid. Assistants will show you how to use the microcentrifuge. Always balance the tubes in the rotor of the centrifuge with equal weight (same tube and volume)!

Repeat same procedure with 0.2 ml microfuge tubes:

1. Label four 0.2 mL tubes.
2. Add 0.1 ml of water in each tube.
3. Add 10 µl, 5 µl, 1 µl, or 0.5 µl of 6X loading buffer, close the tubes and observe the behaviour of the dye.
4. Vortex briefly to mix.
5. Centrifuge for a few seconds to collect the liquid.

3. Create a standard curve by making serial dilutions

A serial dilution is the stepwise dilution of a substance in solution. Serial dilutions are commonly used to accurately create highly diluted solutions of a biological sample (e.g. drugs, microorganisms or cells) and standard curves.

Usually, the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion. For example the 10-fold serial dilution of a 1 M stock solution will have the following concentration: 0.1 M, 0.01 M. 0.001 M etc. A X-fold serial dilution means that you pipette 1 volume of the solution to be diluted and mix it with (X-1) volumes of buffer, X being the final dilution volume. Thus, 1 volume solution is added to 9 (=10-1) volumes buffer for a 10-fold serial dilution.

The concentration and volume of the sample can be determined using the **dilution equation**:

$$\mathbf{C_1V_1 = C_2V_2}$$

C_1 = initial concentration (of the stock solution)
V_1 = volume of the stock solution to use
C_2 = final concentration (of the diluted solution)
V_2 = final volume of the diluted solution.

Materials

- Salmon sperm single-stranded DNA (ssDNA; 500 µg/ml)
- Nuclease-free water
- 1.5 ml microfuge tubes
- 96 well UV plate
- Microplate reader

Each student will make a dilution series of DNA and the concentration will be measured using a spectrophotometer. The results show how precise your manipulations are. By comparing your data to the ones from the other group members you can see how much variability arises.

Procedure:

1. Make a 1/2 serial dilution series according to the table below. To reduce the amount of tips used, first add the water to tubes 2-8 without changing the tip. For all subsequent steps remember to change the tip for each pipetting step.

Tube #	dilution	µl H_20	µl ssDNA	from	A260	A260 - bg*	µg/ml
A	undiluted	0	150	stock			500
B	2-fold	150	150	stock			
C	4-fold	150	150	tube B			
D	8-fold	150	150	tube C			
E	16-fold	150	150	tube D			
F	32-fold	150	150	tube E			
G	64-fold	150	150	tube F			
H	bg *	150	0	-			

* bg = background (blank)

2. **Mix** samples by pipetting up and down or vortexing **at each dilution**.
3. Centrifuge for a few seconds to collect the liquid.
4. Transfer 100 µl of each tube into a 96 well UV plate. Dispense slowly on the side of the well to prevent bubbles, they create artefacts in the measurement. Two groups / plate (group 1 into column A-D, etc.).
5. Label the plate (group number and initials) and record the order into your lab notebook.
6. Nucleic acids have an absorption maximum at 260 nm (A260). We will measure the absorbance at 260 nm using a platereader (TECAN). Results will be on Moodle.
7. Create a graph with standard curve of your dilution series using Excel (or another suitable program):
    - Subtract the background absorbance (bg, tube 8) from each A260 value.
    - Plot absorbance (A260) versus DNA concentration (µg/ml).
    - Select chart => Choose XY (Scatter) and the unconnected points icon.
    - X axis with independent variable/ input (here: concentration)
    - Y axis with dependent variable/ output (here: absorbance)
    - From the Chart menu, add a regression line (Trendline) to the chart.
    - Choose the Options tab and select Display equation on chart.
    - Use the regression equation to calculate concentrations.

8. Create a second graph and plot the data from all three group members with three trendlines (groups with two members chose one dataset from any other group). Then calculate the mean for each dilution and display the mean with trendline (use a different color for the fourth trendline). You will see later that it is important to have replicates when doing experiments.
9. Paste raw data and both charts into SLIMS and label the data (show units!) with an adequate description.

Data Analysis

The following questions should help you to analyze your results. If relevant, use for interpretation of the data.

Q1 Do your A260 values correlate with your serial dilution? Do you see aberrant values (outliers) in the data? Mention the aberrant data points and how you identified them.

Q2 Is your standard curve usable to determine the concentration of an unknown DNA sample? Compare to the results of the other group members. Justify your answer.

Q3 Why is a blank measurement important? What is the source of background absorbance?

Q4 What is the concentration of a DNA sample with a measured A260 of 0.7 (remember to subtract the bg absorbance from the A260 value)? Use your own serial dilution and background to answer the question.

Q5 Troubleshooting question- mistakes happen, let's look into how to handle them!

You are preparing a series of 1:10 serial dilutions of a bacterial culture to estimate colony-forming units (CFU).

Intended Procedure:

- Add 1 mL of culture to 9 mL of sterile saline in Tube A (this should give a 10^{-1} dilution)
- Mix Tube A thoroughly, then transfer 1 mL into a new tube containing 9 mL saline to make Tube B (10^{-2})
- Repeat this process until Tube E (10^{-5})

What actually happened:

- In step 1, instead of adding 1 mL of culture, 2 mL of culture was accidentally added to the 9 mL of saline in Tube A
- The rest of the dilutions (Tube B → Tube E) were carried out correctly (1 mL into 9 mL each time)

a) What is the actual dilution factor in tube A?
b) Given this error, what are the actual dilution factors for tubes B–E?
c) Can the dilution series still be used to estimate the original bacterial concentration? Why or why not?
d) If a student was unaware of the mistake and assumed Tube E was 10^{-5}, how would their calculated bacterial concentration differ from the true value?

4. Making an agarose gel

Agarose gels are usually prepared freshly (same day) for analysis of nucleic acids. To save time the gel was prepared in advance by the assistants or the previous lab group. You make a gel for the next group and store it in the fridge.

Material

- agarose
- Tris-acetate-EDTA (10xTAE)
- Laboratory grade water
- Gel tray and combs
- (GelRed to detect nucleic acids- not used here since we do not run the gel)

Preparation of agarose gel

1. First prepare in a cylinder 250 ml of 1X TAE buffer (Tris-acetate-EDTA) buffer: mix 25 ml of 10X TAE buffer, fill up to 250 ml of laboratory grade water. Cover with small piece of parafilm, mix well. Transfer into a 250 ml bottle, label tape with 1X TAE buffer and date.
2. Prepare 50 ml of 1% agarose in 1X TAE buffer in a 200 ml Erlenmeyer. Melt the agarose (0.5 g /50 ml) in a microwave oven (no aluminium foil or Parafilm!). Mix until completely dissolved. Let cool down for a few minutes.
3. (Not needed for this demo: Add 1:10'000 parts GelRed dye (5 µl /50 ml). Mix well.)
4. Set up the gel-casting chamber into the frame. Place **two combs** onto the frame at the top and the middle of the chamber.
5. Pour the melted agarose into the gel-cast (don't overfill) and wait until the gel solidifies.
6. Store at 4°C for the next group.

5. Pipette a DNA sample into the slots of an agarose gel

Each group member loads two wells or more. To save time the gel was prepared in advance by the assistants or the previous lab group. The assistant will show you how to remove the comb and cover the gel in the tray with 1X TAE buffer.

Procedure

1. For the groups prepare a mastermix: add 12 µl of 6X loading buffer to 60 µl DNA sample (1X final concentration). Gel loading buffer contains glycerol, which increases the sample density, and facilitates loading the gel.
2. Each person loads each 10 µl sample into two slots of an agarose gel, covered with running buffer. Samples contain 5% (w/v) glycerol and will therefore sink into the slots, if you pipette carefully and don't create bubbles. Observe the blue dye.
3. We will run other gels in the next lab sessions, this exercise is for warm-up!