Introductory tutorial to bacterial growth and kinetic assays in the Molecular Systems Biology Group

This tutorial serves as a practical introduction to the typical experimental work performed in the Molucalar Systems Biology lab. *E. coli* cells will be grown from freezer stocks, and cell lysates will be prepared and used in a simple lactate dehydrogenase kinetic assay.

It is assumed that the person performing these experiments will have received proper instruction with regard to the safety protocols and equipment booking systems in the department.

1 Sterile technique

When working with microorganisms almost all work is done under sterile conditions to prevent contamination, and solutions prepared under non-sterile conditions are subsequently sterilised by autoclaving. Contaminating bacteria, yeast and fungi live on almost everything and float around in the laboratory air. When using sterile technique, the following minimal guidelines apply:

1. Work in the laminar flow hood which provides positive pressure (make sure the sterilising UV light is turned off whilst working the hood, and on otherwise).

2. Always wear latex gloves OR regularly sterilise hands with a 70 % ethanol solution.

3. Flame bottlenecks with a bunsen burner whenever they are opened or used, and flame inoculating loops before each inoculation. Hockey-sticks should be kept in 70 % ethanol and flamed before use.

4. Only use autoclaved pipette tips.

Note also that when using shakers and centrifuges to observe correct protocol and ensure that flasks or centrifuge rotors are balanced.

2 Growing *Escherichia coli*

E. coli is typically grown in LB medium for bulk preparation of cell matter, however for reproducible physiological and/or kinetic studies a defined medium such as M9 minimal medium has to be used.

To demonstrate the typical growth process of *E. coli* the following steps will be taken:

1. prepare either LB or M9 medium

2. inoculate a 5 ml culture from a freezer stock of *E. coli* and allow it to grow overnight (O/N)

3. use the O/N culture to inoculate a large culture and incubate on a shaker at 37 °C

4. monitor cell growth using a spectrophotometer

The following general guidelines for preparing LB or M9 medium should be adjusted to prepare the desired amount of medium. For the purposes of this introduction a 50 ml overnight culture and a large 500 ml culture will be sufficient.

The general buffer for cell washing, lysis, and kinetic assays used in this introduction is 100 mM TRIS at pH 7.0. 500 ml of this buffer is sufficient and should be prepared beforehand and autoclaved (121 °C for 15-20 min).

2.1 Lysogeny broth (LB) medium

This is a rich medium composed of tryptic digests of casein (peptides), yeast extract (vitamins, trace elements), and NaCl. As the exact composition of constituents in tryptone and yeast extracts is unknown and will vary per batch, LB medium is not recommended for physiological and kinetic studies, but rather for bulk growing of bacteria (e.g. for protein expression).

To prepare 1 l of LB medium, dissolve the following components in water (distilled water or preferably milli-Q) in a beaker with a magnetic spinner:

10 g	tryptone
5 g	yeast extract
10 g	NaCl

As a precaution it is possible at this stage to adjust the pH of the solution but is probably unnecessary. The solution can now be decanted into Erlenmeyer flasks, each with a cotton wool bung and foil covering the mouth (note that for adequate aeration a 1 l culture should be grown in a 3 l flask, and a 500 ml culture in a 2 l flask) and autoclaved (usually at 121 °C for 15-20 min).

2.2 M9 minimal medium

M9 is a minimal medium with clearly defined components making it ideal for reproducible physiological and kinetic studies. Minimal media force the organism to produce the majority of the required metabolites, vitamins, and co-factors endogenously and only provides the bare minimum of components exogenously in the medium. Unlike LB medium, M9 medium is buffered by the two phosphate components HPO_4^{2-} and $H_2PO_4^{1-}$. It is thus imperative that care is taken during preparation to include the correct forms of the phosphate salt!

To prepare 1 l of M9 medium, dissolve the following in 977 ml of water (milli-Q is preferred for reproducibility) in a beaker with a magnetic spinner:

12.8 g	$Na_2HPO_4.7H_2O$ **OR**
6.0 g	Na_2HPO_4
3.0 g	KH_2PO_4
0.5 g	NaCl
1.0 g	NH_4Cl

Also prepare a 20% w/v glucose solution in milli-Q water (typically glucose, sodium gluconate or glycerol are used

as carbon sources); 500 ml is enough for 25 l of M9 medium. Glucose solutions may take a while to dissolve fully, but if left stirring with a magnetic stirrer the clumps will eventually disperse.

Transfer the M9 salts and glucose solutions to Schott bottles and prepare some Erlenmeyer flasks for culturing by inserting a cotton wool bung into the mouth of each flask and cover the mouth with foil (note that for adequate aeration a 1 l culture should be grown in a 3 l flask, and a 500 ml culture in a 2 l flask). Autoclave these solutions and empty flasks (usually at 121 °C for 15-20 min). The reason for autoclaving the salts and sugars separately is that when autoclaved together the sugars are often caramelised, especially when phosphates are present.

While the glucose and salt solutions are being autoclaved, prepare the following two constituents. Once prepared, they should be filter sterilised using a 50 ml syringe and a 0.2 μm syringe filter into 50 ml Falcon tubes and stored at 4 °C (use a new filter for each solution, the solutions can be reused for subsequent cultures as long as they do not become contaminated):

- 50 ml 1 M $MgSO_4$
- 50 ml 0.1 M $CaCl_2$

To prepare 1 l of M9 medium add the following under sterile conditions to the 977 ml M9 salts autoclaved salts in the Schott bottle:

20 ml	20 % carbon source (0.2 % final)
2 ml	1 M $MgSO_4$
1 ml	0.1 M $CaCl_2$

As a precaution it is possible at this stage to adjust the pH of the solution which will be buffered around 7.2 by adding sterilised NaOH/HCl. This is generally unncecessary for growth of *E. coli*.

This M9 medium may now be decanted into Erlenmeyer flasks for culturing.

2.3 Growing cells on agar plates (optional)

Often it is useful to grow bacteria on agar plates. We do this regularly to check for contamination, do cell counts, or simply to have an intermediate source of cells so that we don't have to constantly use freezer stocks.

To prepare plates, either LB or M9 medium is prepared as above with the exception that 15 g of agar are added per litre of medium. This new medium is autoclaved in a Schott bottle and once it has cooled enough to be handled (but still liquid), the medium is poured into petri dishes in the laminar flow hood under sterile conditions. Petri dishes should only be filled halfway and left slight uncovered in the laminar flow hood (ideally with the UV light on) will setting to prevent condensation on the lid. These plates can be stored at 4 °C for several weeks by wrapping them in parafilm to prevent dehydration.

There are several ways to grow bacteria on plates. Typically, bacterial cultures are of far too high an OD_{600} to apply directly to a plate as the density of cells will prevent individual colonies from being identified. Thus it is usually necessary to perform a dilution series (e.g. in pre-sterilised Eppendorf tubes) until individual colonies on the plate are identifiable.

If a simple check for contamination is required, an inoculation loop can be used to inoculate a plate by flaming it, dipping it into a diluted cell suspension, and streaking out several lines in one quadrant of the plate. After flaming the loop again, the cells can be spread out further by streaking out several new lines through the old lines. This should be done four times (once for each quadrant of the plate).

Alternatively, if a cell count is required, the same dilution series of a culture is made and 200 ul of each dilution is pipetted directly onto a plate. After this, a glass "hockey stick" is removed from the 70 % ethanol in which it is kept, and flamed. Once the flames have extinguished, the flat frontal part of the "hockey stick" is used to smear the 200 ul of cells evenly over the surface of the plate. Once colonies have appeared, they are counted and multiplied by the appropriate dilution factor to estimate the number of cells in the original culture (1 colony forming unit or CFU represents 1 cell in the original culture).

Agar plates are incubated at 37 °Cupside down on a shelf (to prevent condensation dripping onto the cells).

2.4 Batch growth – overnight cultures

Bacterial stocks are typically frozen at -80 °C in a 50 % glycerol solution to protect the cells from being damaged by ice crystals. To bring the cells out of the deep stationary phase they experience at such low temperatures we usually start by growing a small O/N culture which is used on the following day to inoculate a larger culture. Inoculating directly from freezer stocks is possible, but typically involves such a long lag period as the cells "wake up" that it is more convenient to start with O/N culturing. Additionally, O/N culturing allows one to predefine the starting optical density of a culture.

Typical aerobic bacterial batch cultures are grown by shaking in Erlenmeyer flasks. *E. coli* are grown in a 37 °C room with sufficient shaking to continuously "fold" air into the medium.

To inoculate a 50 ml O/N culture, fetch a freezer stock of *E. coli* cells in an ice-box. After allowing the sample to thaw, using sterile technique in the laminar flow hood, inoculate the 50 ml culture with 20 μl of cells. Return the freezer stock to the -80 °C freezer, and place the O/N culture on a shaker in the 37 °C room. This culture will be ready tomorrow!

It is a good practice to leave the uninoculated large culture on a shelf in the 37 °C room over night as well so that in the morning it will be at the correct temperature for growth.

2.5 Batch growth – the large culture

On the day following the growth of the O/N culture, retreive it (and the uninoculated large culture) from the 37 °C room. Typically bacterial cultures are inoculated to a final optical density at 600 nm (OD_{600}) of 0.05-0.1. To be able to do this one has to determine the OD_{600} of the O/N culture and inoculate the larger culture with enough to achieve the correct OD. Note that any steps in which cells are either removed or added to a culture are to be performed under sterile conditions. OD_{600} determinations themselves do not need to be sterile as the contents of the cuvettes are discarded afterwards.

After a night of growth the 50 ml culture should be at an OD_{600} of about 1.2. As the linear range of most spectrophotometers is 0.1-1.0, the O/N culture will need to be diluted to measure the OD_{600} (a 1:2 dilution should be sufficient). Once

the OD_{600} has been determined, inoculate the large culture to a final OD_{600} of 0.1, and place it on a shaker in the 37 °C room. It may be useful to double check that the culture is in fact at an OD_{600} of 0.1 after inoculation.

Bacterial cultures undergo four phases of growth in a batch culture:

1. Lag phase – this is a phase in which no growth takes place and are traditionally thought to be adjusting the new medium[1]

2. Log phase – at this stage the cells are growing exponentially

3. Stationary phase – exhaustion of nutrients and the accumulation of toxic metabolic products causes the cells to stop growing in this phase

4. Death phase – continued lack of nutrients and exposure to toxic compounds causes the cells to gradually die off

For kinetic assays cells are typically harvested in mid-log phase to ensure that all the cells are configured similarly and are exhibiting maximal growth without being subject to external effects from nutrient limitation or the accumulation of toxins.

To determine when the cells have hit mid-log phase an OD_{600} determination (using 1 ml of culture, retreived under sterile conditions) should be made every 30 min. Plotting OD_{600} on a semilog axis will result in a straight line in log-phase. *E. coli* growing aerobically usually reach mid-log phase after about 3-4 hrs and for the purposes of this introduction can be harvested at an OD_{600} of 0.8.

2.6 Cell harvesting

Cells will be harvested for subsequent experiments by centrifugation. Sterile technique is no longer necessary from this stage onward.

Retreive the 500 ml culture from the 37 °C room and decant it into a Beckman 500 ml centrifuge bottle. Weigh the complete bottle including the plug and cap on a laboratory scale, and prepare a second bottle for balancing the centrifuge rotor by filling it with water to the same weight (centrifuges typically have a ±1 g tolerance).

Centrifuge the two bottles using a JA-10 rotor at 7000 rpm for 10 min at 4 °C. After centrifugation a large cell pellet will have formed on the bottom of the bottle with cells. Pour off the supernatant, add about 20 ml of 100 mM TRIS buffer and resuspend the pellet by holding the centrifuge bottle on a vortexer. After the pellet is resuspended, transfer the cell suspension to a 50 ml falcon tube and place it on ice. Divide the cell suspension into about 20×1 ml aliquots in Eppendorf tubes.

Centrifuge the 20 Eppendorf tubes in a benchtop centrifuge at 13,500 rpm for 7 min, pour off the supernatants and store the tubes with cell pellets at -80 °C. At this temperature cell pellets are stable for years.

3 Kinetic assay

As this is an introduction to kinetic assays a simple lactate dehydrogenase (LDH) assay will suffice. LDH converts pyruvate to lactate whilst reoxidising NADH to NAD^+. Assays involving the light spectrometer usually measure the consumption or production of NADH. This often means that a series of enzymes must be included in an assay to couple the reaction of interest to NADH. This is not necessary with LDH as it directly consumes NADH.

3.1 Preparing a cell lysate

Many methods are available for cell lysis with viable proteins involving glass beads, sonication, french-pressing, bead-milling, and lysozyme. Grinding the cells with glass beads is gentle enough to retain the activity of the enzymes whilst yielding a large amount of protein.

To perform a glass bead extraction, cells are resuspended in a solution with tiny glass beads and vortexed. Retrieve a cell pellet from the -80 °C freezer and resuspend it in 1 ml of 100 mM TRIS buffer. The ideal ratio of glass beads is 1 g/ml of cell suspension, so add 1 g of 0.1 μm glass beads to the cell suspension, and transfer the whole suspension to a 1.5 cm diameter glass test tube.

Perform the extraction by vortexing the test tube on full power for 6 minutes in total whilst resting once every minute by placing the tube on ice for 15 s to prevent heating of the sample. Note that typically it is advised to include protease inhibitors during this stage to prevent the proteases released from the cell's periplasm from degrading the enzymes of interest (e.g. PMSF). For our purposes this is unnecessary as LDH is a relatively stable enzyme and shows little degradation.

Once the grinding process is complete, pour the slurry into an Eppendorf tube and centrifuge it using the benchtop centrifuge at 13,500 rpm for 5 min (remember to balance it). Transfer the supernatant to a new tube and keep it on ice; discard the tube with the glass beads.

3.2 Lactate dehydrogenase assay

pyruvate + NADH → lactate + NAD^+

The LDH assay will be performed by varying the concentration of the substrate pyruvate until the enzyme is saturated with substrate, and measuring the rate of the reaction at each of these steps.

Prepare the following solutions in 100 mM TRIS:

- 1 ml 200 mM pyruvate
- 1 ml 4 mM NADH

This assay will be performed using a 5-fold dilution series of the substrate pyruvate in a 96-well plate with each of the five reaction wells having a final volume of 100 μl. Prepare the following mix which excludes pyruvate and is sufficient for ten reactions. Then pipette 90 ul into each of five wells on a 96-well plate:

[1] It has been shown that in fact a small persister population is growing exponentially in the background during this phase but the growth is masked by the presence of a large static population which results in what appears to be a "lag" in growth; eventually the persister population grows large enough to take over the culture.

10 µl	cell lysate
10 µl	4 mM NADH (final conc. = 0.4 mM)
70 µl	100 mM TRIS

Now, prepare a 1:2 dilution series of pyruvate. This can be done in Eppendorf tubes or conveniently in a series of adjacent wells on the 96-well plate by diluting each step with 50 % TRIS (bear in mind that the maximum volume of the wells is 250 µl). The final concentrations in each tube/well should be 200, 100, 50, 25, 12.5 mM.

After inserting the plate and initialising the plate reader it is always good practice to monitor the levels of NADH at 340 nm to ensure that they are stable, and to allow the wells to equilibrate with the set temperature (25 °C is fine). The other benefit of making the substrate dilution series in adjacent wells is that the temperature is the same in the substrate and reaction wells. The reaction can be initiated by pipetting 10 µl of pyruvate into each reaction well. If a the substrate is in a series of wells then a multipipette may be used, otherwise pipette each well individually starting at the lowest concentration (to avoid losing too much data with the faster high concentration reactions). Start the experiment which should measure NADH at 340 nm every 6 s for about 10 min. Note that these assays are typically performed in triplicate to be able to report a standard error.

3.3 Protein determination – the Bradford assay

One final step is necessary: to determine the amount of protein present in the cell lysate. This will be done using the Bradford assay. The Bradford reagent contains Coomassie Brilliant Blue G-250 dye, which binds to protein and forms a blue colour that can be measured at 595 nm. The following solutions will be necessary for the assay:

- 1 ml 1 mg/ml bovine serum albumin (BSA)
- a bottle of Bradford reagent stored in the laboratory refrigerator

To perform the assay, a 4-fold 1:2 dilution series of the BSA must be made either in Eppendorf tubes or in adjacent well of a 96-well plate. This will be the protein standard with final concentrations of 1, 0.5, 0.25, 0.125 mg/ml. Likewise a 4-fold 1:4 dilution series of the cell lysate must be made in Eppendorfs or in the wells of a 96-well plate. 295 µl of the Bradford reagent is pipetted into eight wells (four for the BSA standard, four for the lysate), to which 5 µl of each protein solution is added (four concentrations of BSA, four concentrations of lysate). After allowing the reaction a few minutes to complete, measure the absorbance on a plate-reader at 595 nm.

By fitting a straight line function to the BSA standard points, the concentration of the original cell lysate can be extrapolated back from the cell lysate dilution series. A lysate dilution should be selected for extrapolation that produces a reading within the range of the calibration curve produced by the BSA dilution series. Note that these assays are typically performed in triplicate to be able to report a standard error.