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.

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.

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 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.

2.4 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.

2.5 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 buffer and resuspend the pellet

[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.

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.