# Project Outline 03 - Enzyme inhibitor discovery

## Background to the project

Numerous diseases are driven by the dysregulation of enzyme activity. The development of small molecule drugs with the capacity to inhibit a specific enzyme therefore remains a cornerstone of modern drug discovery and development. The enzyme β-galactosidase is a widely used tool in biomedical research, and is also a recognized biomarker for cellular senescence (aging). Developing inhibitors of this enzyme could therefore have useful applications in research.

Many of the most successful drugs used today were discovered by the large-scale screening of natural products. This approach has given rise to drugs targeting diverse conditions, including cancer, inflammatory disease, hypertension and heart disease.

These discoveries were enabled by the vast chemical diversity present in the Natural world, a uniquely powerful resource which retains great potential for further drug discovery. By systematically testing hundreds or thousands of different natural extracts or compounds, researchers aim to discover and isolate a small handful of rare 'hits' which possess the desired biological activity against a target of interest. This method, called **high throughput screening** (HTS), is a critical step in the modern drug discovery process. Your project will involve the screening of 400 natural extracts with the aim of discovering novel agents with potential to inhibit the activity of the enzyme beta galactosidase.

### Project at a glance

- 400 extract library
- 8 weeks lab time
- BSc / MSc level
- Drug discovery

**⚠ Safety First!**

- Read the safety information in section 2 before beginning any experimental work.

## Benefits of choosing this project

Through this project, you will gain experience of the HTS method, and several other techniques which are also in high-demand from life-science employers, such as multichannel pipetting, dose response assays, dataset management and statistical data analysis. Beyond gaining key industry-relevant skills, your work could help identify new leads for the discovery of enzyme inhibitors which may have utility in diverse areas of biomedical research.

## How long will it take?

The basic project outlined below can be achieved typically within an **8 week** period of lab time. However, this can be extended to up to **30 weeks** if necessary by following the suggestions for further experiments and follow-on investigations given in the later sections. Please discuss with your supervisor how to modify the project to achieve your aims while fitting within the available timeframe.

## What will I do on this project?

The core activities you need to complete the basic version of this project are listed below:

1) Screen the *Phytotitre* library for inhibitors of the beta galactosidase enzyme
2) Analyse data from the screening experiments to identify hits for follow-up
3) Perform dose curve analysis of the top hits to test for dose-dependence
4) Calculate $IC_{50}$ values for your validated hits
5) Perform dose curve analysis of established inhibitors to compare their IC50 values
6) Use these data and analyses to prepare your final report or dissertation

## What skills will I gain through this project?

Completing this project should give you experience of the following core employability skills:

| **Practical skills** | | **Analytical skills** | |
|---|---|---|---|
| Multichannel pipetting | ✓ | Management of large data sets | ✓ |
| Microplate absorbance measurements | ✓ | Background correction & normalisation | ✓ |
| Enzyme activity assays | ✓ | Calculation of assay Z' factor | ✓ |
| High throughput screening | ✓ | 4-parameter dose response curve fitting | ✓ |
| Preparation of dose response curves | ✓ | Calculation of IC50 values | ✓ |
| Performing kinetic analyses | ✓ | Statistical analysis of large data sets | ✓ |

## What support is available?

Comprehensive method sheets, which describe how to set up and complete each experiment, are available from the downloads page on our website. These are free to download without registration. Troubleshooting advice to help solve common problems that may arise during the project is also given towards the end of this project guide. Your university supervisor will be your primary point of contact and will be responsible for your day-to-day supervision.

# Instruction list

Complete these tasks in the following order for a basic project in Enzyme Inhibitor Discovery.

Steps 1 to 5 should be performed before you begin in the laboratory. It is best to complete these several weeks or months in advance, to ensure there will be no delays to the start point of your project.

Steps 6 to 16 will occur after you start your work in the laboratory.

# The preparation phase

## Step 1: Read the introduction to the *Phytotitre* library

The *Phytotitre* library is a collection of natural extracts that has been developed to support drug discovery projects in academia and industry. The full-size kit comprises both polar and non-polar extracts of 400 plants, for a total of 800 extracts. This collection has been a useful source of discovery for researchers in diverse therapeutic areas in over a dozen countries.

We now provide a half-size version of the larger collection to support student research projects. The student project version of the *Phytotitre* library comprises only the non-polar extracts of the same 400 natural products present in the larger collection. This carefully curated collection is conveniently arranged in five re-sealable 96-well microplates for ease of use in such projects.

A unique focus of the collection is that it comprises almost entirely of traditional herbs or medicines with a history of oral use in humans. By focussing on such plants, the aim is increase the likelihood of identifying leads with both a high hit rate for biomedical targets, and a favourable safety profile.

To learn more about the background to the *Phytotitre* library, please read **Method sheet 100**.

## Step 2: Familiarise yourself with relevant Health and Safety information ⚠

Working in any laboratory is associated with specific risks that must be understood and mitigated before starting a new project. Please read our '**Safety First**' document to understand the primary risks associated with screening a natural product collection and completing a research project in enzyme activity screening.

Every institution has a slightly different way of training and recording student health and safety inductions. However, the following are some common steps that you may have to complete before starting the laboratory phase of your project:

1) Read the project risk assessment and COSHH documents

Every new project should always begin by writing a **Risk Assessment** document to cover the anticipated risks associated with the project. You should discuss with your supervisor who will prepare this, then read and sign the risk assessment associated with your project. Take note in particular of what the key risks are, and how to mitigate them (e.g. wearing appropriate Personal Protective Equipment (PPE) at all times in the laboratory etc.).

You will also be working with several chemicals in this project, including the natural extracts, the solvent they are suspended in (dimethyl sulphoxide, DMSO), the enzyme substrate (ONPG) and your chosen positive control enzyme inhibitor (galactose). These reagents will have an associated Safety Data Sheet (**SDS**), provided by the manufacturer, that lists all of the hazards associated with that substance. The information from these SDS documents is brought together to prepare a Control of Substances Hazardous to Health (**COSHH**) document, which summarises how the risks associated with use of these substances in your specific project will be mitigated within your institutional environment. Make sure you have read the relevant SDS and COSHH documents for your project before starting.

2) Be aware of and follow your local health and safety rules

In addition to reading the relevant risk assessment, SDS and COSHH documents, there may be other procedures necessary to complete before you are able to work in the laboratory. Please ask your supervisor for advice on what else may be necessary to complete before starting.

3) Receive induction and appropriate training

Many university laboratories require students to attend a mandatory safety and general laboratory induction before starting the project. If this is part of the process at your institution, make sure you attend such induction. You should then ask the relevant lab manager, technical staff or day-to-day supervisor to train you on the basic techniques necessary for success on your project.

## Step 3: If necessary, complete a Research Ethics application

Some universities require students to submit an Ethics Application to address any potential ethical issues the project may raise before you start your project. The format of such applications varies greatly between institutions. However, if you are required to submit an ethics application for your project, we advise that you cover at least the following points in your submission:

1: No use of human samples or personal data

It will be helpful to state near the start of your application that the project will be based on the culture of established, non-pathogenic bacterial strains in the laboratory, with no use of human volunteers or tissue samples. Mention also that there will be no collection or use of personal or identifiable data.

2: No use of animals or animal products

Mention that as your experiments will be *in vitro* only, with no use of animals or animal products, your project aligns well with and strongly supports the modern movement towards reducing animal use in research (3Rs - Refine, Reduce, Replace).

3: Compliance with the Nagoya Protocol

The Nagoya Protocol is an international agreement under the Convention on Biological Diversity (CBD) that ensures the fair and equitable sharing of benefits arising from the use of genetic resources (such as plants) or related traditional knowledge in certain ways. Your ethics application should acknowledge that (i) the Nagoya Protocol is relevant as your experiments will involve the screening of plant extracts from diverse countries and (ii) the experiments as designed are compliant with the Nagoya Protocol, as discussed in more depth on our website.

*Reminder*: Always show your draft ethics application to your supervisor before submission, as they are the 'Principal Investigator' (PI) responsible for your supervision.

## Step 4: Make sure all the reagents and consumables you require are in stock

In addition to the *Phytotitre* kit, you will require the items shown in the following lists. Check to ensure they are available in your laboratory in good time before starting the project. Remember that purchasing necessary items can take some time due to internal approvals, purchasing department paperwork, etc.

Equipment

- General pipette set, capable of dispensing 1 µl to 1,000 µl
- Multichannel pipette capable of dispensing 1 µl
- Multichannel pipette capable of dispensing 50 - 200 µl
- Microplate reader capable of measuring at 420 nm *
- A 37ºC incubator

Reagents (not supplied with the *Phytotitre* kit)

- Recombinant beta-galactosidase enzyme (1 mg/ml, frozen aliquot)
- Ortho-nitrophenyl-β-D-galactopyranoside (ONPG, at least 60 mg, the substrate)
- Galactose (as a positive control for enzyme inhibition)
- Phosphate buffer, in which to perform the reactions (see **Method sheet 15**)

Consumables

- Clear plastic sterile 96-well microplates (at least 30)
- Plastic tips compatible with available pipettes
- Plastic reagent reservoirs (sterile, for loading plates)
- Nitrile gloves

* If a filter is not available at 420 nm, comparable results can be obtained using a filter between 405 nm and 450 nm.

## Step 5: Plan strategies for Good Laboratory Practice, Data Management and Integrity

A key employability skill for those seeking work in the life-sciences is knowledge and experience of working to the standards of **Good Laboratory Practice (GLP)** and **Data Management and Integrity**. You should therefore aim to practise these principles throughout your project. Begin by reading **Method Sheet 17**. This document will give you an overview of how to maintain your laboratory notebook and data files to comply with GLP and Data Management standards, which are essential for workers in the life science industries.

# The laboratory work phase

Please make sure you have completed all necessary activities in sections 1-5 before beginning your project in the laboratory.

## Step 6: Prepare assay plates for screening

The first experiment will be to explore whether any natural extracts exist within the *Phytotitre* collection which have the capacity to inhibit the breakdown of the substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) by the enzyme β-galactosidase. To do this, prepare 96-well plates containing assay buffer and substrate as described in **Method sheet 15**. Please read the box below to decide whether you will begin by preparing one plate only, of all five simultaneously.

**Choosing how many plates to screen**

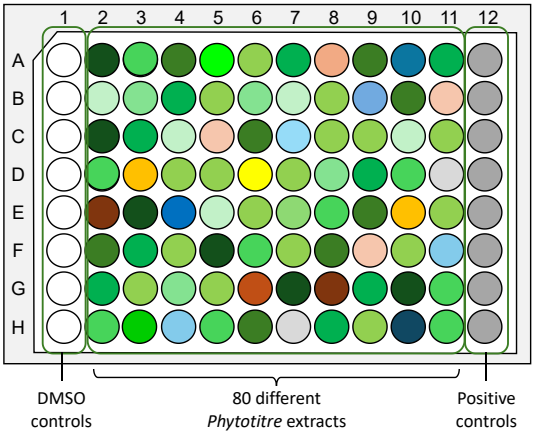
Please note that as the standard project involves screening all 5 plates of the *Phytotitre* collection, the following steps give instructions on how to a screen all 5 plates simultaneously. However, your supervisor may prefer you to focus your efforts on only one of the 5 available library plates throughout your project. If this is the case, you should only defrost and use that particular plate. Alternatively, if you intend to follow the standard plan of screening all 5 plates, and it is your first time working with 96-well plates in this way, you may also prefer to begin by challenging only one plate at a time while you are starting out. Once you become more confident with the necessary techniques, you can progress to challenging all 5 plates on the same day as shown below. Simply adjust the protocol as necessary to fit your comfort level until you are ready to process 5 plates in one experiment.

## Step 7: Defrosting, opening and re-sealing the *Phytotitre* or *Puretitre* kits

⚠ Make sure you keep all *Phytotitre* stock plates **facing upwards** at all times - never place them upside down. Be careful also to not knock or shake the library plates after defrosting. Any of these actions can result in loss of stock solutions and cross-contamination between wells if some of the extracts stick to the resealable cap mat. If this has occurred, please refer to the troubleshooting section towards the end of this document for advice on how to proceed. Please follow the guide on how to defrost, open and reseal the kits given in **Method sheet 01**.

## Step 8: Screen the *Phytotitre* library for inhibitors of β-galactosidase

You should supplement every well of the first column the 96-well plate(s) with 1 µl of DMSO (these wells will be your negative, or vehicle controls). You should then add 1 µl of the positive control (1 M galactose) into every well of the twelfth column of every plate. Columns 2 to 11 should receive 1 µl of each of the respective *Phytotitre* extracts according to the plate map shown at right, and the advice given in **Method sheet 15**.



After adding *Phytotitre* extracts to every well of the assay plates, work with only one plate at a time, and supplement every well with enzyme mix as described in **Method sheet 15**. As soon as pipetting of the enzyme mix is complete, measure the absorbance of the plate at 420 nm using a microplate reader to obtain the baseline absorbance values for every well. Take a note of the time and place the plate in a 37°C incubator. Incubate the plate for 30 minutes to allow the reaction to develop, then read the absorbance values of every well in the plate once again. For advice on how to get the most from your plate reader, follow **Method sheet 16**.

## Step 9: Perform background correction on the primary screen data

It is essential that you do not leave data analysis until the end of the project! You must chart the results from every experiment as soon as they are complete to be able to plan the next experiment. Begin by collating all of your data from the first screen into one spreadsheet file. You should then perform **background correction** of the data from every plate according to **Method sheet 20**. This is necessary to account for any effects of coloured plant compounds on absorbance measurements. After that, you should **normalise** the data from every plate to transform the raw absorbance values into a percentage of the negative control values (i.e. the DMSO controls in column 1 of the plate). This is necessary to enable easy correction for day to day variation in the maximum absorbance reached by assays between different experiments. You can do this by following the advice given in **Method sheet 20**.

## Step 10: Calculate Z' factor values for each of your plates

Now that you have normalised the data from each plate, the next step is to test how reliable and reproducible the results of the screening assay were. This is a necessary step in the drug discovery process, as it helps to establish whether the observed results reflect real differences, and are not just artefacts or "chance events". The industry standard approach is to calculate a metric called the Z' factor (pronounced "Z-prime factor") for every plate tested during the primary screen.

You should calculate the Z' factor value for every plate you measured by following **Method sheet 22**. If your Z' factor values are greater than 0.5, you can have confidence that your assays have been set up well and are likely to revel genuine hits. If they are much lower than this, explore potential reasons for low reproducibility (see Troubleshooting Guide below), then attempt to solve these before repeating the experiment.

Once most of your Z' factor values are within range, repeat the whole screening experiment at least twice more. In other words, you should aim to screen all 5 plates of the *Phytotitre* kit 3 times each.

## Step 11: Identify hits from the primary screen

Now that you have data from at least three repeats of the full screen experiment, the next stage is to identify "hits" from these data. For your project, these will be the extracts that show the greatest potential to inhibit the activity of β-galactosidase.

The first step in this process is collating the normalised results from your experiments and aligning them correctly with the extract ID numbers in a single column format. To do this, you should follow the advice for mapping experimental data to plate maps given in **Method sheet 44**. Once you have completed this task, you should define your hits and identify them, following the advice given in **Method sheet 23**.

## Step 12: Perform dose response assays using individual hit extract(s)

The next step in drug discovery after completion of a screening experiment is to attempt **replication** of the top hits. This is the process where the highest scoring extracts only, not the full collection, are tested in further experiments to confirm that they have biological activity against the target of interest. These tests aim to give further confidence that the hits are genuine, and not false positives, which may arise from artefacts in the screening process, a statistical error arising from variability in the assay, or mis-calculation during analysis of the original dataset.

You should choose at least 1 (we recommend up to 3 for a basic project), of your highest scoring “hit” extracts to explore further in these replication studies. You will require larger quantities of extract than are available in the kits to perform these experiments. You have two main options to obtain them:

(i) You can attempt preparation of the herb extract yourself (please see **Method Sheet 28** for how to do this).

(ii) Alternatively, you can ask your supervisor to arrange purchase of a larger quantity of the specific hit extract(s) from our company. These ship quickly and are supplied in a ready-to-use, DMSO solubilised format.

Your first replication experiment should be to perform a dose response assay using doubling dilutions of the fresh extract. For advice on how to set up your dose curve experiments, please follow **Method sheet 11**. Repeat this dose response experiment at least 3 times to enable statistical analysis of the data.

## Step 13: Perform statistical analysis of dose response data

Once you have obtained data from your dose curve experiments, you will have to calculate the concentration of extract necessary to achieve 50% inhibition of the response in your experiment. This value, referred to as the IC50, is a key metric in drug discovery as it gives an indication of how potent your extract or compound is. IC50 can be calculated by fitting a 4 parameter logistic (4PL) curve to the dose response data. This can be easily achieved using specialised data analysis tools such as GraphPad Prism or R. It can also be calculated using Microsoft Excel, as explained in **Method sheet 24**.

You should also perform a two way ANOVA to test if any differences in cell growth seen between the extract dose curve and the DMSO dose curve are statistically significant, as explained in **Method sheet 25**.

## Step 14: Establish the type of inhibition mediated by galactose

When developing drugs to target specific enzymes, it is essential to learn which of inhibition these drugs employ to reduce enzyme activity. Enzyme inhibitors can be of three major types: competitive, non-competitive and uncompetitive. These major classes of inhibitor can be distinguished by measuring the initial reaction rate of an enzyme in the presence of varying concentrations of substrate, with or without the inhibitor, and plotting the data using a Lineweaver-Burk plot. To set up these experiments, please follow **Method sheet 39** using galactose as your inhibitor. Remember to repeat these experiments three times.

## Step 15: Plot a Lineweaver-burk chart of the assay data

Once you have obtained the results from step 14, proceed to plotting a Lineweaver-Burk chart of the data. Use the information on this chart to establish if the tested inhibitor is a competitive, non-competitive or uncompetitive inhibitor. To do this, please follow the advice given in **Method sheet 40**.

## Step 16: Establish the type of inhibition mediated by hit extracts

Now that you have experience of the Lineweaver-Burk method, repeat steps 14 and 15 using your hit extract(s) as the inhibitor. Perform a separate experiment for each hit you are testing. To do this, follow the advice given in **Method sheet 39** and **Method sheet 40**.

Got this far and still have time left in the lab? Please see the following section for suggestions of optional, additional experiments you can try to extend your project if you'd like to do so.

# Advice on data analysis

## Don’t leave analysis until the end of the project

The number one tip for your project data analysis is that you must not wait until the end of the project to start analysing your data! Chart the data from every experiment as soon as it has been completed. This is essential to be able to plan the next stage of your experiments. Science progresses in a step-wise fashion, with the next direction always being based on the results of the previous experiments. Analysing your results as you obtain them in this way allows you to progress with a solid foundation of logical choices (the study rationale) - a key hallmark of quality that examiners are looking for in your final dissertation.

## Don’t corrupt the original data files

Remember to keep the original raw data file in a separate folder unmodified from the point it was collected from the instrument (in this case, the microplate reader). To perform your analyses, first copy and paste the data from the original file into a separate spreadsheet before working on it. This helps minimise the risk of corrupting the original source of information, and keeps your work compliant with data management standards.

# Writing up: Advice for your project dissertation

Congratulations on the completion of your data collection phase! Now comes the writing up. Giving as much care and attention to this process as your experimental phase is necessary to help you score the highest possible marks in your dissertation. If you would like some advice on how to begin writing up your project report or dissertation, please refer to our notes in **Method Sheet 35**.

# Suggestions for project 'Extension' or upgrading to 'Level 7' (MSc) work

It should be possible to complete the experiments suggested above for the basic antibiotic discovery project within about **8-10 weeks** of lab time. However, if you have managed to complete all of these experiments, and still have more time available in the laboratory, please discuss with your supervisor whether it would be appropriate for you to attempt one or more of the additional experiments shown below to further explore your hits and extend your project.

If you are completing a project at Masters level (i.e. Level 7), or have much more time available in the laboratory, you should aim to complete at least two of the additional experiments shown below. These additional experiments do not have to be performed in any particular order, and you are of course free to modify them with the approval of your supervisor to better fit your own project aims.

## Project variation 1 - Perform replication studies on additional hits

The instructions for the basic project described above suggest you should take forward between 1 and 3 hit extracts from your primary screen for further analysis in dose response and disk diffusion assays. A simple way to expand the scope of your project and create more charts for your dissertation is to increase this to 4 to 7 of the top hits using the same methods given above.

## Project variation 2 - Repeat the screening project with a different enzyme

If time and funding permits, it may be possible to repeat the above processes shown in steps 6 to 16 with a different enzyme. Suitable additional enzymes with examples of substrates and positive control inhibitors to try are shown in the table below:

| Suggested enzyme | Substrate | Positive control inhibitor |
|---|---|---|
| Alkaline phosphatase | p-Nitrophenyl phosphate | L-Phenylalanine |
| Horseradish peroxidase | Tetramethylbenzidine | Ascorbic Acid (vitamin C) |

## Project variation 3 - Compare your results with existing *Phytotitre* screen datasets

Complete the first part of the data analysis (dry) projects available on our website to find inhibitors of growth of the bacterium *Escherichia coli*. This organism naturally expresses the enzyme β-galactosidase, which supports bacterial growth in the presence of certain nutrients. Attempt a correlation analysis to compare the published findings with your own findings. Calculate the r-values and p-values for these correlation analyses. Are any of your hits also able to inhibit growth of *E. coli*? If not, why do you think this may be? Collate information from the literature to support your hypothesis to explain for any similarities or differences.

# Troubleshooting FAQ

Science does not progress without challenges! It is very unusual for a student project to reach completion without facing some difficulties along the way. A key element of your training is learning how to identify problems and apply solutions as you encounter them. The FAQ section below lists some issues that are commonly seen in such projects, and approaches you can try to remedy them.

## 1) Some extract has come out of the *Phytotitre* kit wells onto the resealable cap mat - how do I fix this?

**Probable Cause:** The plate was either inverted or knocked sharply after defrosting.

**Suggested Action:** Do not invert or knock the plate after defrosting. If contamination is seen on the inside of the cap mat before removing it from the plate, place the whole plate with the cap mat still sealed into a centrifuge with swing-out rotor and carriers compatible with 96-well plates, then centrifuge gently (100 g for 1 minute), to settle the contents back into the wells. If contamination is seen on the inside of the cap mat after it has been removed from the plate, use a paper towel soaked in a small amount of 70% ethanol to carefully clean the areas where extract has stuck to the cap mat. Reseal the cap mat onto the plate after use and return to the freezer gently to avoid any further spillage.

## 2) The colour changes to yellow too quickly - why is this?

**Probable Cause:** The concentration of enzyme is likely too high.

**Suggested Action:** Some preparations of β-galactosidase have higher enzyme activity than others. If the reaction turns strongly yellow as soon as you add the enzyme, or if the colour reaches an absorbance (OD) of greater than 1.5 within 30 minutes in the negative control (DMSO) wells, repeat the experiment using a lower concentration of enzyme (e.g. diluting by a factor of 10 from the original concentration).

## 3) The colour does not change to yellow - why is this?

**Probable Cause:** Defective enzyme preparation.

**Suggested Action:** If you have prepared the reaction buffer and substrate properly (see **Method sheet 15** for advice), the problem is likely due to defective enzyme. This can happen if the enzyme has been left out of the freezer for an extended period of time between uses, or if it has been subject to too many freeze-thaw cycles, both of which can result in denaturation of the enzyme. Do not use a temperature higher than 37°C (body temperature) to defrost the enzyme.

## 4) Why do some of the extracts seem to increase assay activity?

**Probable Cause:** Impact of coloured compounds in some extracts.

**Suggested Action:** Some of the extracts may contain compounds that absorb light at the same wavelength used to monitor production of reaction products. Perform background correction of the data to compensate for this, according to **Method sheet 20**.

## 5) Why are some of my absorbance results negative numbers?

**Probable Cause:** Some plant pigments lose colour during incubation due to degradation, resulting in a paradoxical reduction in absorbance over time. After performing background correction, this can result in a value below zero.

**Suggested Action:** Extracts yielding such low values are still likely hits, so you should take them forward for further study. Plot them as normal on your screening charts, but explain the possible reason for any values below zero in the discussion section of your report.

## 6) My Z' factor values are often below 0.5 suggesting low reproducibility - why is this?

**Probable Cause:** Pipetting errors.

**Suggested Action:** Check for bubbles in the wells after pipetting, make sure the tips fit well to your pipettes, ensure you pipette only to the first stop when setting the volume, when using a multichannel pipette always check by eye that the aspirated liquid reaches the same level in all of the tips, pipette gently by moving your thumb up and down on the plunger more slowly.

## 7) I did not find any "hits" that completely block enzyme activity - why is this?

**Probable Cause:** This is quite normal.

**Suggested Action:** It is actually quite common to complete a screen and find no compounds or extracts that completely inhibit your target of interest. It is much more common to find several hits that inhibit, for example, enzyme activity or bacterial growth, by perhaps only 50%. These are actually seen as good results. Remember, the screening stage is only the first step in the drug discovery process. We do not expect the compounds identified here to be the most potent possible molecules. They are a starting point that medicinal chemists will work on to improve, and derivatives they provide will eventually form the basis of much more potent drug leads. Remember also that the extracts are complex mixtures of hundreds of different compounds. This means the active compound is present at a very low concentration in your assay well. Further work to isolate and concentrate the active compound could result in much higher biological activity. If you find no hits that reach 70% inhibition, look again with the threshold set at 50%, or even lower. Remember that the mark for your project will not be impacted by whether or not you find a strong hit in your screen - what matters is that you complete your experimental work, analysis and write-up to a high standard.

## 8) Why is there a kick upwards in growth at the highest concentration of extract in dose curves?

**Probable Cause:** Low solubility of active compounds at high concentrations.

**Suggested Action:** When examining responses to a dilution series of different doses of natural compounds or extracts, it is not uncommon to see a paradoxical uptick in enzyme activity at the highest concentrations. This is because the non-polar molecules that comprise your extracts may be less soluble at the highest tested concentrations, which may cause them to precipitate into clumps, leaving space in between where the enzyme can be more active.

## 9) I could not replicate one of my hits in a dose response assay - why is this?

**Probable Cause:** Natural variability in the screening process.

**Suggested Action:** Because you are screening 400 extracts, and there can be significant variability in absorbance values due to pipetting, measurement and gas exchange variation, it is possible that some of your "hits" may arise not because of genuine biological activity, but because of random fluctuations in these parameters during the screening process. This is an example of a false positive, and is the reason why we always check whether hits can be replicated in follow-on dose-response assays. If one of your hits does not replicate in such assays, this is the most likely cause, and you should try taking the next hit in the list forward.

## 10) When I plot my data from dose response curves, why is the curve back to front?

**Probable Cause:** Microsoft Excel data handling error.

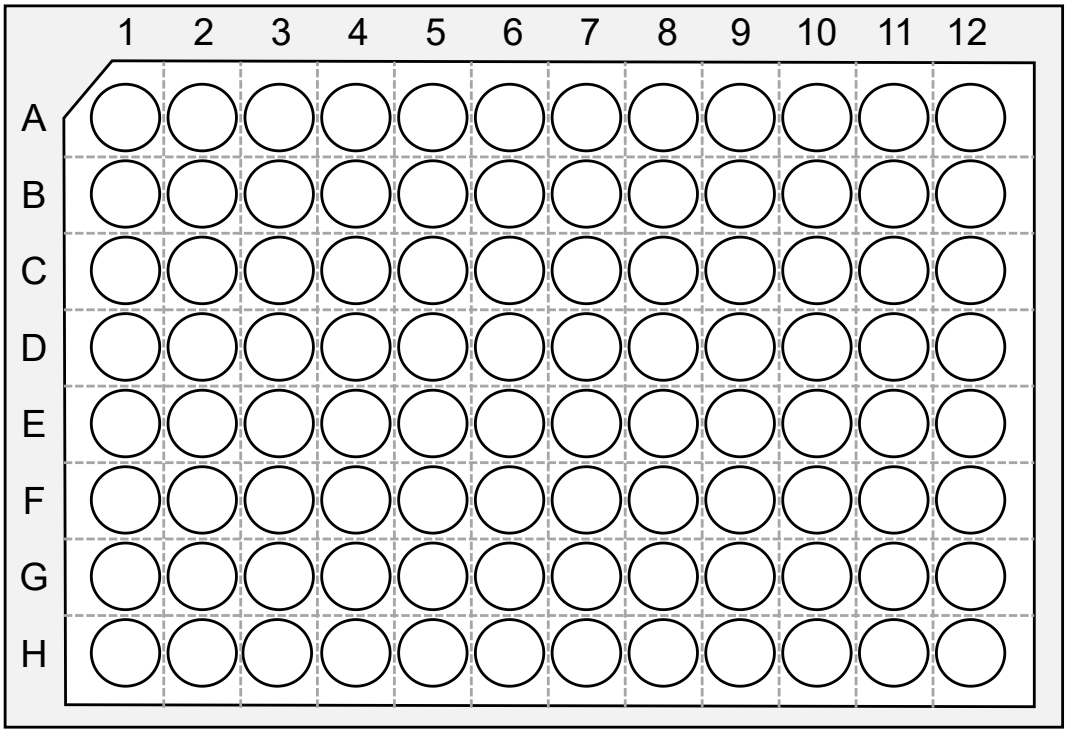
**Suggested Action:** If your assay plate had the highest concentration at the left-hand side of the plate, and the data are copied into your spreadsheet in the same orientation as the plate, make sure you type the concentrations in the correct (descending) order from left to right, so that

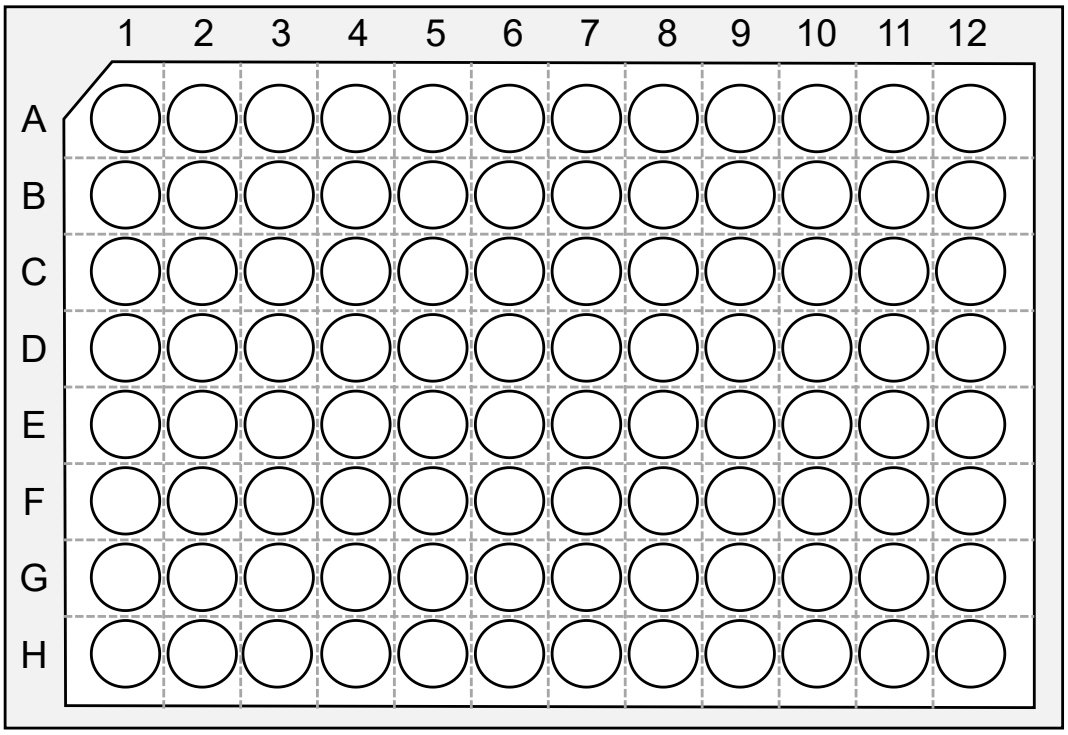
the scatter plot lines up the correct result with the correct concentration. A less common problem is that the plate may have been inserted into the reader in the incorrect orientation (i.e. turned by 180$^{o}$ such that well H12, not A1, was in the top left position).

# Appendices

## 96-well plate template

Printing these plate outlines and drawing a map of what you intend to dispense into which sets of wells can help you plan your experiments.



## Project Progress Checklist

The following checklist table will enable you to keep track off the tasks you should aim to complete for the basic project before entering the writing-up phase.

| Step | Task | ☑ |
|---|---|---|
| 1 | Read the introduction to the *Phytotitre* library (Method sheet 100) | ☐ |
| 2 | Familiarise yourself with relevant Health and Safety information | ☐ |
| 3 | If necessary, complete a Research Ethics application | ☐ |
| 4 | Make sure all the reagents and consumables you require are in stock | ☐ |
| 5 | Plan strategies for Good Laboratory Practice, Data Management and Integrity | ☐ |
| 6 | Prepare assay plates for screening (Method sheet 15) | ☐ |
| 7 | Learn how to defrost, open and re-seal the *Phytotitre* kit (Method sheet 01) | ☐ |
| 8 | Screen the *Phytotitre* library for inhibitors of β-galactosidase (Method sheet 15) * | ☐ |
| 9 | Perform background correction on the primary screen data (Method sheet 20) | ☐ |
| 10 | Calculate Z' factor values for each of your plates (Method sheet 22) | ☐ |
| 11 | Identify hits from the primary screen (Method sheet 23) | ☐ |
| 12 | Perform dose response assays using individual hit extracts (Method sheet 11) * | ☐ |
| 13 | Perform statistical analysis of dose curve data (Method sheets 24 & 25) | ☐ |
| 14 | Establish the type of inhibition mediated by galactose (Method sheet 39) * | ☐ |
| 15 | Plot a Lineweaver-burk chart of the assay data (Method sheet 40) | ☐ |
| 16 | Establish the type of inhibition mediated by hit extracts (Method sheets 39 & 40) | ☐ |
| 17 | Writing the dissertation - hints, tips and advice available in Method sheet 35 | ☐ |

* Remember to perform each experiment a minimum of 3 times to enable statistical analysis of the results.

**Found a Bug?**
We care greatly about the utility of our resources for customers, and are very responsive to user feedback. If you spot an error, incorrectly numbered method sheet, or a broken link, please feel free to report the issue by sending an email to: contact@caithnessbiotechnologies.com.