

## Project Outline 01 - Discovery of novel antibiotics

### Background to the project





Antimicrobial resistance (AMR) is one of the most significant threats to healthcare systems globally. As pathogenic bacteria increasingly evolve resistance to existing antibiotics, there is an urgent need for the discovery of either new antibiotics, or drugs with potential to restore the sensitivity of pathogens to existing antibiotics by blocking resistance mechanisms.

Historically, the discovery of new antibiotics has been achieved mainly by the large-scale screening of natural products. This approach has also given rise to many of the most widely used and successful drugs in other categories, including anti-cancer, anti-inflammatory, anti-hypertensive and cholesterol lowering drugs.

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 growth of a bacterial strain of interest.



### Project at a glance

-  400 extract library
-  8 weeks lab time
-  BSc / MSc level
-  Antibiotic 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 quantitative microbiology, dose response assays, dataset management and statistical data analysis. Beyond gaining key industry-relevant skills, you will contribute to the search for new antibiotic leads, helping to address the escalating problem of antibiotic resistance.

### 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 growth of a chosen bacterial strain
- 2) Analyse data from the screening experiments to identify hits for follow-up
- 3) Attempt replication of the top hits to confirm their ability to inhibit bacterial growth
- 4) Perform dose curve analysis of validated hits to test if their activity is dose-dependent
- 5) Analyse dose response curves to calculate IC<sub>50</sub> values for your top hits
- 6) Perform disk diffusion assays to confirm anti-bacterial properties of your hit extracts
- 7) 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	
Aseptic technique	✓	Management of large data sets	✓
Standard microbiology methods	✓	Background correction & normalisation	✓
Multichannel pipetting	✓	Calculation of assay Z' factor	✓
Microplate absorbance measurements	✓	4-parameter dose response curve fitting	✓
Preparation of dose response curves	✓	Calculation of IC <sub>50</sub> values	✓
Disk diffusion assays	✓	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 Antibiotic Discovery.

Steps 1 to 6 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 7 to 20 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 microbiology.

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) and your chosen antibiotic. 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: Choose which bacterial strain you will investigate**

Discuss with your supervisor which bacterial strain is most appropriate for your research goals. We strongly recommend selecting an organism from Hazard Group 1 (essentially harmless to humans), as these can be safely cultured in the Containment Level 1 (CL1) laboratories which are typically available to students at UK universities.

**Gram-negative Option:** We suggest *Escherichia coli* strain DH5 $\alpha$ , or other K-12 derivatives. These are ubiquitously available, grow rapidly, and are a commonly used model for studies of Gram-negative bacteria.

**Gram-positive Option:** We suggest *Micrococcus luteus*. This organism is recommended for natural product screening as it often yields a superior "hit-rate" compared to other species, making it an excellent choice for a discovery-focused project.

## Step 5: 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 600 nm \*
- A 37°C incubator with a shaking platform (to prepare overnight cultures)
- A 37°C incubator without shaking (to culture plates overnight)
- Access to a containment level 1 microbiology laboratory and associated glassware

### Reagents

- Autoclaved Luria broth or another bacterial growth medium
- Dimethyl sulfoxide (as vehicle control, about 10 ml)
- A positive control antibiotic (e.g. ampicillin or tetracycline)

### Consumables

- Clear plastic sterile 96-well microplates (at least 30)
- Autoclaved plastic tips compatible with available pipettes
- Plastic reagent reservoirs (sterile, for loading plates)
- 15 ml or 50 ml sterile plastic tubes (for preparation of master-mixes)
- Nitrile gloves

\* If a filter is not available at 600 nm, comparable results can be obtained using a filter between 570 nm and 630 nm.

## Step 6: 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 1-6 before beginning your project in the laboratory.



### Step 7: Prepare a bacterial culture for screening in 96-well format

Ask your supervisor to provide a glycerol stock, or an agar plate with live colonies, of the bacterial strain of interest you have agreed to work on. Then prepare an overnight starter culture by following **Method sheet 02**.

#### 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 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 8: Plate bacterial cell cultures for screening with the *Phytotitre* kit

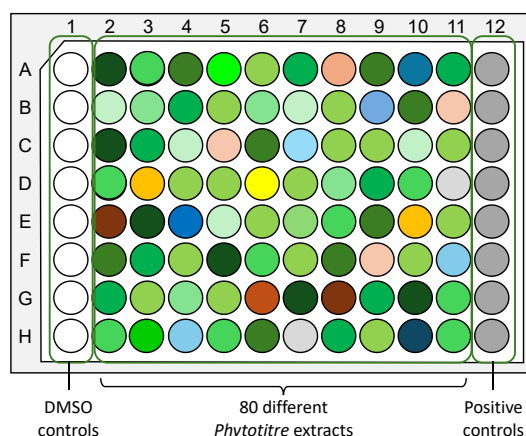
You will have to prepare 5 separate microplates containing bacterial cultures per screen in this project. For instructions on how to do this, follow **Method sheet 03**.

### Step 9: 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 10: Screen the *Phytotitre* library for inhibitors of bacterial growth

You should challenge the bacterial cultures in the 96-well plates with 1  $\mu$ l of DMSO in each well of the first column of every plate (these wells will be your negative, or vehicle controls). You should then add 1  $\mu$ l of your chosen antibiotic into every well of the twelfth column of every plate (the positive controls). The stock antibiotic concentration should be 100-fold higher than that necessary for complete prevention of bacterial growth. For example, a stock solution of ampicillin at 100 mg/ml is sufficient. Columns 2 to 11 should receive 1  $\mu$ l of each of the respective *Phytotitre* extracts according to the plate map shown at right, and the advice given in **Method sheet 04**.



## Step 11: Use a microplate reader to measure bacterial growth by absorbance

Before you place your plates in the incubator to grow the cultures overnight, you must take a “before” reading of the absorbance of every well. Read all plates once at this timepoint using your microplate reader. Then, after incubating your plates for 18-24 hours, measure the absorbance again. If possible, we recommend that you also measure the plates at a third timepoint, such as 6 hours or 8 hours, to gain additional data relating to the inhibition of early growth, returning the plate to the incubator before measurement at the final timepoint (i.e. 18-24 hours). For advice on how to get the most from your plate reader, follow **Method sheet 05** and **Method sheet 16**.

## Step 12: 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 18**. 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 cultures between different experiments. You can do this by following the advice given in **Method sheet 18**.

## Step 13: 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 reveal 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 kit 3 times each.

## Step 14: 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 bacterial growth.

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 15: 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, 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 16: 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 IC<sub>50</sub>, is a key metric in drug discovery as it gives an indication of how potent your extract or compound is. IC<sub>50</sub> 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 17: Perform disk diffusion assays of individual hit extract(s)

It is good practice in drug discovery to further validate the findings of replication experiments by performing additional, orthogonal assays. This means checking that the result of the previous experiment is correct by testing the bioactivity of the sample using a different kind of assay. You can attempt to do this for your hit extract(s) using a simple disk diffusion assay for inhibition of bacterial growth. For instructions on how to do this, please follow **Method sheet 26**. Remember to repeat this experiment at least 3 times to enable statistical analysis of the data.

### **Step 18: Perform statistical analysis of disk diffusion assay results**

You should have measured the diameter of the zones of clearance (which may or may not be present) around each of your paper disks from all of your disk diffusion assay experiments. To test whether these measurements are significantly different between the DMSO control and your hit extract treatment, or if the extract significantly enhances the activity of an existing antibiotic, please perform an ANOVA statistical analysis following the advice given in **Method sheet 27**.

### **Step 19: Examine effects of hit extracts on kinetics of bacterial growth**

As a further validation of your hit extracts, you should explore whether they alter the growth kinetics of your bacterial strain of interest. Please follow the advice given in **Method sheet 29**. Repeat this experiment at least 3 times to enable statistical analysis of the data.

### **Step 20: Perform statistical analysis of bacterial growth kinetics data**

Analyse the results of the growth kinetics experiments using the advice given in **Method sheet 30**.

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 33**.



## 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 - Test whether your hit extracts enhance the activity of existing antibiotics

Another relatively simple way to further explore the antibacterial potential of your hit extracts is to test whether they enhance the capacity of existing antibiotics to kill the bacterial strain you have been studying. Choose an antibiotic that is already available in the laboratory, and perform a dose response experiment using the antibiotic alone to establish its minimum inhibitory concentration (MIC) and IC<sub>50</sub> in your strain of interest. Then prepare a 10 point series of doubling dilutions with the MIC in the middle of the series, and supplement the cultures with either your extract or DMSO, at a final concentration of 1% vol:vol. Follow **Method sheet 31** for advice on how to perform this experiment. Repeat this experiment at least 3 times to enable statistical analysis of the results. Finally, you should test whether the results from these assays reveal statistically significant enhancement by following the advice given in **Method sheet 32**.

## Project variation 3 - Repeat the screening project with a different micro-organism

Simply repeat the above processes shown in steps 7 to 20 with a different micro-organism. Is there any correlation between the extent to which the extracts inhibit growth of the two organisms, across the entire collection? Are any of the hits the same?

## Project variation 4 - Screen the collection for enhancers of existing antibiotics

Repeat the experiments you did for the primary screen of the *Phytotitre* collection, with the minor alteration that all growth medium used to plate the cultures should contain a single antibiotic of interest at a concentration of one quarter (i.e. 0.25x) the MIC for that antibiotic in your strain of interest. Compare the hits from this screen with the screen conducted earlier in the absence of antibiotic. This will allow you to identify extracts with capacity to increase the sensitivity of your bacterial strain of interest to an existing antibiotic.

## Project variation 5 - Compare your results with existing *Phytotitre* screen datasets

Complete the first part of the data analysis (dry) projects available on our website. Attempt a correlation analysis to compare the published findings with your own findings. Calculate the r-values and p-values for these correlation analyses. If your hits differ from those for the organisms tested in the published projects, 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 bacteria did not grow in the negative control (DMSO) wells - why is this?

**Probable Cause:** The inoculum was too old or non-viable, the incubator was at the wrong temperature, or there was a problem with preparation of the media.

**Suggested Action:** Ensure your "starter culture" is in the log phase (usually 16-18 hours old) before inoculating plates. Verify that the incubator is set to 37°C (or the appropriate temperature for your strain). Allow at least 18 hours for *E. coli* to grow. *M. luteus* is slower growing so will take longer, up to 36-48 hours.

### 3) The positive control antibiotic did not prevent bacterial growth - why is this?

**Probable Cause:** Antibiotic resistance of the strain, or degradation of antibiotic.

**Suggested Action:** Prepare a fresh stock of antibiotic solution. Check if your strain of *E. coli* DH5 $\alpha$  carries a plasmid (common in molecular biology labs). If it does so, it may be resistant to at least one antibiotic and you should repeat with a strain lacking the plasmid.

### 4) There was growth in some wells that I did not put bacteria in - why is this?

**Probable Cause:** Contamination.

**Suggested Action:** Check your aseptic technique. Ensure media and tips were properly autoclaved before use. Wipe pipettes clean with tissue soaked in 70% ethanol. If the contamination recurs, discard the stock media and repeat with fresh, sterile stocks.

### 5) Why do some of the extracts seem to increase bacterial growth?

**Probable Cause:** This is normal and common.

**Suggested Action:** No action required. Some of the extracts offer the bacteria a very rich carbon and nutrient source, enhancing their growth instead of inhibiting them.

## 6) I see more (or less) growth around the edges of the plate - why is this?

**Probable Cause:** This is called the “edge effect”. Evaporation of media in the outer wells increases the concentration of solutes/salts and causes stress to the cells in those wells.

**Suggested Action:** When performing screening assays where we must use all the wells of the plate, seal the plates with a breathable film, or alternatively place them inside a secondary sealable container (e.g. Tupperware) with a damp paper towel to maintain humidity. When performing dose response assays, avoid using the outer wells of the plate.

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

**Probable Cause:** Some plant pigments lose colour during incubation due to degradation or metabolism by the bacteria, 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.

## 8) 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.

## 9) I did not find any “hits” that completely stop bacterial growth - why is this?

**Probable Cause:** Bacteria resist or breakdown antimicrobial plant compounds.

**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.

## 10) 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 microbial growth 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 bacteria can survive.

### 11) 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.

### 12) 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° such that well H12, not A1, was in the top left position).

### 13) My disk diffusion assay results do not confirm the broth microdilution assays - why is this?

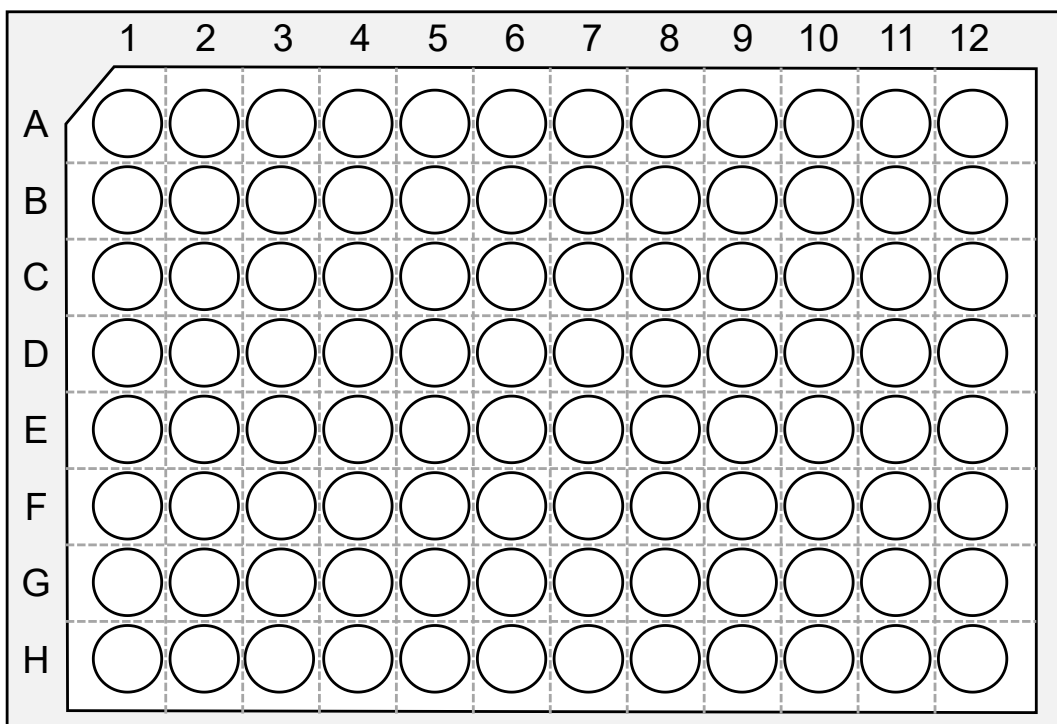
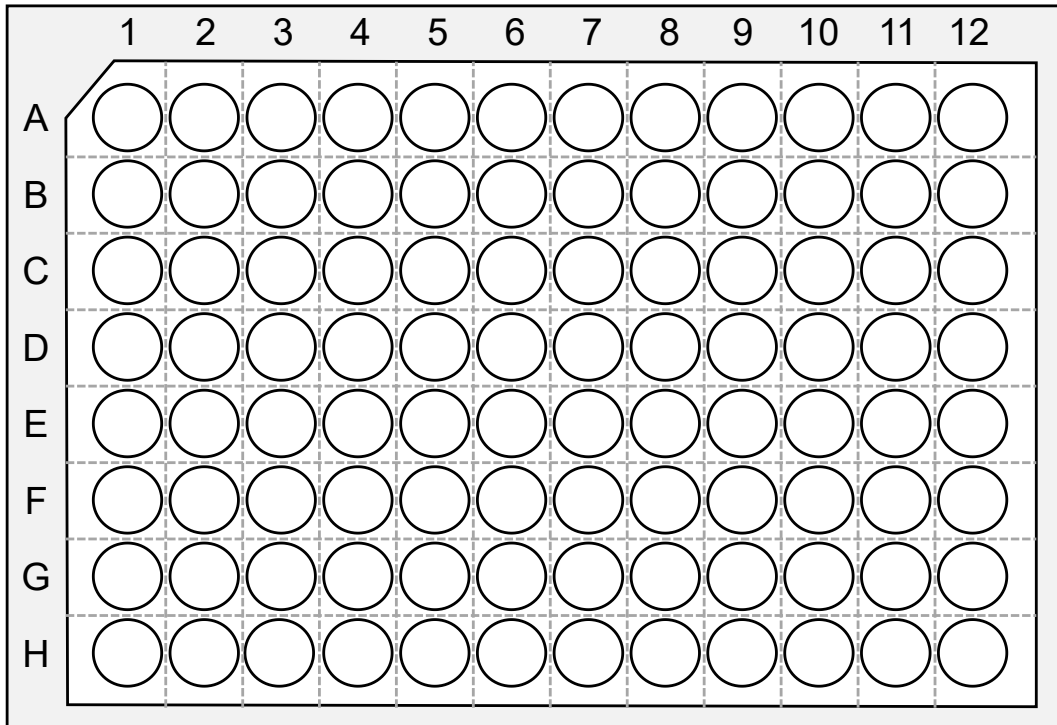
**Probable Cause:** Relatively low anti-bacterial activity of extract or compound.

**Suggested Action:** It is quite common for antibacterial activity seen in broth microdilution assays to not be seen in disk diffusion assays. This is because the microdilution assay is much more sensitive to antibiotic activity than the disk diffusion assay. Report the results as you find them and explain this limitation in the discussion section.

## 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	<input checked="" type="checkbox"/>
1	Read the introduction to the <i>Phytotitre</i> library (Method sheet 100)	<input type="checkbox"/>
2	Familiarise yourself with relevant <a href="#">Health and Safety information</a>	<input type="checkbox"/>
3	If necessary, complete a Research Ethics application	<input type="checkbox"/>
4	Choose which bacterial strain you will investigate	<input type="checkbox"/>
5	Make sure all the reagents and consumables you require are in stock	<input type="checkbox"/>
6	Plan strategies for Good Laboratory Practice, Data Management and Integrity	<input type="checkbox"/>
7	Prepare a bacterial culture for screening in 96-well format (Method sheet 02)	<input type="checkbox"/>
8	Plate bacterial cell cultures for screening with the <i>Phytotitre</i> kit (Method sheet 03)	<input type="checkbox"/>
9	Learn how to defrost, open and re-seal the <i>Phytotitre</i> kit (Method sheet 01)	<input type="checkbox"/>
10	Screen the <i>Phytotitre</i> library for inhibitors of bacterial growth (Method sheet 04) *	<input type="checkbox"/>
11	Measure bacterial growth by absorbance (Method sheets 05 & 16)	<input type="checkbox"/>
12	Perform background correction on the primary screen data (Method sheet 18)	<input type="checkbox"/>
13	Calculate Z' factor values for each of your plates (Method sheet 22)	<input type="checkbox"/>
14	Identify hits from the primary screen (Method sheet 23)	<input type="checkbox"/>
15	Perform dose response assays using individual hit extracts (Method sheet 11) *	<input type="checkbox"/>
16	Perform statistical analysis of dose curve data (Method sheets 24 & 25)	<input type="checkbox"/>
17	Perform disk diffusion assays of individual hit extracts (Method sheet 26) *	<input type="checkbox"/>
18	Perform statistical analysis of disk diffusion assay results (Method sheet 27)	<input type="checkbox"/>
19	Examine effects of hit extracts on kinetics of bacterial growth (Method sheet 29) *	<input type="checkbox"/>
20	Perform statistical analysis of bacterial growth kinetics data (Method sheet 30)	<input type="checkbox"/>
21	Writing the dissertation - hints, tips and advice available in Method sheet 33	<input type="checkbox"/>

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

### Found a Bug?

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