Project Outline 04 - Anti-inflammatory drug discovery

Background to the project

Chronic inflammation is a fundamental driver of diverse human diseases, including rheumatoid arthritis, inflammatory bowel disease and psoriasis. At the centre of these conditions is the overproduction of Tumour Necrosis Factor-alpha (TNF-α), a potent pro-inflammatory cytokine primarily secreted by activated macrophages. While biological “TNF-blockers” have revolutionized treatment, their high cost and requirement for injection create a significant need for orally bioavailable small-molecule inhibitors of TNF-α production.

Many of the most successful and widely used drugs today, including examples targeting cancer, infection and heart disease, were discovered by the large-scale screening of natural products.

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 production of the key inflammatory cytokine - Tumour Necrosis Factor alpha (TNF-α).

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 cell culture, bioassay development, dose response assays, dataset management and statistical data analysis. Beyond gaining key industry-relevant skills, you will contribute to the search for new anti-inflammatory drugs, helping to address the problem of treating inflammatory diseases.

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 macrophage production of TNF-α
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 TNF-α production
4) Perform dose curve analysis of validated hits to test if their activity is dose-dependent
5) Analyse dose response curves to calculate IC_{50} values for your top hits
6) Compare the activity of top hit extracts with an established inhibitor of LPS signalling
7) Use these data and analyses to prepare your final report or dissertation

Please note:
⚠ This project is one of the more challenging options we offer, as it involves relatively complex techniques and requires good technical skills in cell culture and bioassay procedures. This option may be more appropriate to students with previous experience of cell culture experiments.

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 cell culture 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 IC50 values	✓
Bioassays for cytokine measurement	✓	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 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 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), actinomycin-D, polymyxin-B, crystal violet, acetic acid and methanol. 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, 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

- Tissue culture suite with a Class 2 biosafety cabinet and an incubator capable of maintaining a 5% CO_2 atmosphere at 37°C
- 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
- Squeezable wash bottle dispensing distilled water (H_2O)
- A large plastic tray to collect waste runoff from plate washing

- Waste stream for crystal violet dye
- Microplate reader capable of measuring absorbance of 96-well plates at 570 nm (or alternatively, any wavelength between 540 nm and 600 nm)

Reagents

- Growing culture of the J774, RAW or THP-1 macrophage cell-line (choose one)
- Growing culture of the L929 mouse fibroblast cell-line
- Cell culture medium (RPMI with L-glutamine and 10% FCS or 5% FCS)
- Dimethyl sulphoxide (~10 ml, as vehicle control)
- Actinomycin-D (~100 µl of 1 mg/ml in DMSO or ethanol, to sensitise the L929 cell-line)
- Crystal violet powder (~2 g, necessary to stain the L929 cell-line)
- Acetic acid (~200 ml, necessary to solubilise the crystal violet stain before measurement)
- Methanol (~200 ml, necessary to fix cells during staining)
- The antibiotic polymyxin-B (~100 µl of 10 mg/ml in water, binds LPS to block signalling)

Consumables

- Clear plastic sterile 96-well microplates (at least 60)
- 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
- Eye protection when performing washing and staining steps
- *Optional*: Benchkote laboratory surface protector for working with crystal violet dye

* If a filter is not available at 600 nm, comparable results can be obtained using a filter between 570 nm and 630 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: Plate macrophages for stimulation and screening

After receiving appropriate training in aseptic and cell culture techniques, prepare a bottle of complete medium (e.g. RPMI with L-glutamine, 10% FCS and penicillin / streptomycin supplement, or appropriate equivalent) for use in your experiments. Ask your supervisor to provide you with a flask of growing J774, RAW or THP-1 macrophages (choose one of these), or alternatively revive a vial of the cell-line of interest from the frozen stocks. Grow the cells in standard T75 tissue culture flasks using complete medium until they reach a confluence of 70-90%. Once sufficient cells have grown, passage the cells into a fresh flask and with the remainder, seed a suspension of cells at the correct concentration into every well of 96-well plates according to the advice given in **Method sheet 12**. See the note below on how many plates to prepare for the initial screen.

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 as some of the extracts may 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 guidance on how to defrost, open and reseal the kits given in **Method sheet 01**.

Step 8: Screen the *Phytotitre* library for inhibitors of TNF-α production

You should challenge the cell cultures in the 96-well plates with 1 µl of DMSO in each well of the first column of every plate (these wells will be your negative, or vehicle controls). 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 07**. Add nothing to any of the wells in the twelfth column of every plate (no TNF-α production controls).

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	01	09	17	25	33	41	49	57	65	73	RPMI
B	DMSO	02	10	18	26	34	42	50	58	66	74	RPMI
C	DMSO	03	11	19	27	35	43	51	59	67	75	RPMI
D	DMSO	04	12	20	28	36	44	52	60	68	76	RPMI
E	DMSO	05	13	21	29	37	45	53	61	69	77	RPMI
F	DMSO	06	14	22	30	38	46	54	62	70	78	RPMI
G	DMSO	07	15	23	31	39	47	55	63	71	79	RPMI
H	DMSO	08	16	24	32	40	48	56	64	72	80	RPMI

1 µl DMSO + LPS

1 µl each plant extract or stock compound, + LPS

No DMSO No LPS

Step 9: Challenge macrophage cultures with LPS and freeze supernatants

Unstimulated macrophages do not produce significant quantities of TNF-α. A suitable stimulus must therefore be provided to trigger secretion of TNF-α. In this experiment, you will challenge the cells with bacterial endotoxin (lipopolysaccharide, LPS), a highly pro-inflammatory molecule that is an agonist of Toll-like receptor 4. To do this, follow the advice given in **Method sheet 13**. Remember to not add any LPS to the wells in column 12, instead add 100 µl of RPMI / 10% FCS without LPS into every well of this column.

3 hours after challenging the plates with LPS, gently transfer 150 µl of supernatant from the challenged plates into empty 96-well plates and freeze the whole plates at -80ºC. Be sure to label each lid and base clearly to identify which experiment each plate belongs to. As you must be present 3 hours after challenge to collect and store the supernatants, it is best to perform the challenge part of the experiment in the morning.

As the supernatants are frozen, there is flexibility as to when you defrost them for analysis. However, the other steps must be performed with quite specific timing intervals. An example timetable to help plan your plating, challenge and harvesting steps is shown below:

Day	Task (J774 or RAW cells)	Task (THP-1 cells)
Mon	Split / passage stock culture and seed 96-well plate(s)	Split / passage stock culture and seed 96-well plate(s) with PMA
Tue	Challenge cells in 96-well plate(s) with extracts and LPS, collect and freeze supernatants 3 hours later	-
Wed	-	-
Thu	-	Challenge cells in 96-well plate(s) with extracts and LPS, collect and freeze supernatants 3 hours later
Fri	Split / passage stock culture	Split / passage stock culture

Step 10: Use the L929 cell bioassay to estimate relative TNF-α production

As the frozen supernatants are stable in the -80ºC freezer for several weeks, you have some flexibility as to when you measure the TNF-α content of the supernatants. Before dong so, you should establish a growing culture of the L929 mouse fibroblast cell-line. When these cells are growing well, you should seed one 96-well plate with these cells for every supernatant plate you have ready to measure. Follow **Method sheet 14** for advice on how to plate the L929 cells and supplement them with defrosted supernatants from the earlier challenge step. After adding supernatants to the L929 cell plates, return the supernatant plates to the freezer (in case you have

to repeat the assay), and the L929 plates to the incubator for overnight growth. During this time, the L929 cells will die at a rate proportional to the concentration of TNF-α in the medium.

The next day, you should stain the L929 cell plates with crystal violet according to the second part of **Method sheet 14**. This will reveal which treatments have produced large quantities of TNF-α, and those which have produced less. Solubilise the stain in acetic acid and measure the absorbance of the plate at ~570 nm using a microplate reader. Note here that the darker the stain, the less TNF-α was present in the supernatant.

Step 11: Collate and normalise 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 begin by **normalising** the data from every plate to transform the raw absorbance values into a percentage of the negative control values (in this case, they are the no-challenge controls in column 12 of each 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 21**.

Step 12: 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 13: 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 production of TNF-α.

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 14: 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 41**. Repeat this dose response experiment at least 3 times to enable statistical analysis of the data.

Step 15: 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 16: Test for compound interference with the L929 cell bioassay

Some natural products have the potential to non-specifically alter the rate of growth or viability of L929 cells, independent of any TNF-α signalling. To check for this interference, you should perform a Parallel Toxicity Screen. In this experiment, L929 cells are challenged directly with the hit extracts alone, in the absence of LPS, to test if they modify L929 cell growth. Please follow the advice given in **Method sheet 42**.

Step 17: Compare hit extract activity with an established LPS inhibitor

Repeat the dose response experiment performed in step 14, but instead of using your hit extract(s) as the inhibitor, use an established inhibitor of LPS signalling. We recommend using the antibiotic polymyxin B, at final concentrations of 256, 128, 64, 32, 16, 8, 4 and 2 µg/ml in the macrophage cultures, by modifying slightly the approach given in **Method sheet 41**. Use the data from these experiments to calculate the IC50 of polymyxin B, as shown in **Method sheet 24**.

Step 18: Attempt quantification of TNF-α using the L929 cell bioassay

Optional: The previous experiments give qualitative data regarding relative TNF-α secretion, but they do not give a quantitative value of how much TNF-α is present in the supernatants of macrophages after challenge with the various treatments. To address this limitation, if time and funds permit, purchase some recombinant TNF-α protein and perform a quantitative assay for TNF-α using the L929 cell bioassay. To do this, you should challenge macrophages with LPS alone, LPS with DMSO, and LPS with each of your hit extracts, in quadruplicate wells (i.e. four technical replicates per plate). Then transfer the supernatants from these wells onto a plate with sensitised L929 cells. On a separate row of wells within the same plate, prepare a standard curve of recombinant TNF-α protein, as shown in **Method sheet 43**. Incubate overnight, stain and read the plates as previously. Finally, analyse the results following the advice given in **Method sheet 43**.

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 36**.

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

It should be possible to complete the experiments suggested above for the basic Anti-inflammatory Drug 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 - Measure TNF-α production by ELISA

The key advantage of the crystal violet assay for TNF-α production is that the costs involved are a tiny fraction of the cost of performing the gold-standard technique for cytokine quantification, which is ELISA (Enzyme-Linked Immunosorbent Assay). However, the main disadvantage of the crystal violet assay is that the variability from this assay is high, and it is difficult to quantify absolute TNF-α concentrations with accuracy. If sufficient funds are available, you could attempt replication of Step 18 using ELISA instead of crystal violet assays to measure TNF-α concentrations in the macrophage supernatants. These values should then be compared with the values you obtained from the crystal violet method. Comment in your discussion section on whether there are any discrepancies between the two, and if so, why you think this may be.

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

Complete the first part of the data analysis (dry) project available on our website exploring existing datasets from screens for tumour cell-line killing. 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 the same as those for the killing of these cell-lines? If so, why do you think this may be? Collate information from the literature to support your hypothesis to explain for any similarities or differences.

Project variation 4 - Repeat the screening project with a different stimulus

If time and funds are available, you could repeat the entire screen and associated experiments as shown above using a different stimulus. For example, you could use the TLR2 stimulant Pam_3CSK_4 (at a final concentration of 1 µg/ml), or heat-killed Gram-positive bacteria (such as *Staphylococcus aureus* or *Micrococcus luteus* at a final concentration of 10^7 cells/ml). Is there any correlation between the extent to which the extracts inhibit TNF-α production by the two different types of stimulant across the entire collection (i.e. LPS vs the TLR2-stimulant)? Are any of the hits the same? If so, why do you think this might 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) I see high variability in my crystal violet assays - why is this?

Probable Cause: The crystal violet assay is very sensitive to the washing step - if washing too vigorously, the adherent cells can be washed away, or if washed too little, excess dye outwith cells confounds the reading.

Suggested Action: Be very careful with how vigorously you wash the cells when adding water to remove excess crystal violet dye from the plate after staining, as this is the number one cause of poor reproducibility in crystal violet assays. Do not point the nozzle of your water bottle directly at the cell monolayers - direct it instead gently against the side walls of the wells. Make sure every well in the plate is filled completely with water during each wash step, slowly and carefully. After inverting the plate to expel the water, remove the excess by patting gently on a paper towel. Keep repeating this process until the blue dye no longer appears on the paper towel. Hold your plate up to the light before adding the solubiliser. If you see 'clear spots' in the negative control wells (which should be solid dark blue), it indicates that cells were physically stripped away during washing. Only after completing the steps in this way should you move on to adding the solubiliser to read the plate. See point 3 for further advice on how to improve reproducibility in this assay.

3) 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, which can cause an increase in variability in the L929 cell bioassay.

Suggested Action: When performing screening assays where we must use all the wells of the plate, ensure the incubator has water in the lower tray (to increase humidity), and avoid placing the plates near the fan of the incubator (which dries them out more quickly). If that does not solve the problem, place the plates inside a secondary sealable container (e.g. a Tupperware box) with a damp stack of paper towels below them to maintain humidity, and open the lid of the Tupperware box just a crack to allow gas exchange. If crystal violet assays show the cells clustering around the edge of the outer wells, try incubating the cells at room temperature for 20 minutes straight after seeding the plates, before moving them into the 37°C incubator. This allows the cells to settle at the bottom of the wells by gravity in a thermally stable environment, preventing the “clumping” effect of rapid temperature change. When performing dose response assays, avoid using the outer wells of the plate.

4) There was no reduction in L929 cell viability in response to any of the supernatants - why is this?

Probable Cause: If all wells of the L929 cell plate remain blue after washing, there are a number of possible causes, including: lack of actinomycin-D sensitisation, improper macrophage cell seeding density, poor crystal violet washing step or incorrect LPS concentration.

Suggested Action: Ensure that actinomycin-D is added to the L929 cell cultures before challenge, as without it they are not sensitive to TNF-α. Re-check the method of cell-counting, and the calculations used to prepare the diluted cell suspension before plating. Check also that the final LPS concentration is correct. Ensure the media contains serum. See the advice given in point 2 on how to improve reproducibility of the crystal violet assay during the washing step.

5) Why do some of the extracts seem to increase L929 cell growth?

Probable Cause: Interference from plant compounds with rate of growth of L929 cells.

Suggested Action: It is not uncommon to see an increase in apparent cell growth in crystal violet assays when challenging cells with natural extracts. This is because some natural compounds enhance the growth of certain cell-lines. Some may even inhibit the action of actinomycin-D. Exposure to such compounds may result in increased absorbance values measured during the crystal violet assay.

6) Why are some of my absorbance results negative numbers?

Probable Cause: Expected variation in pipetting accuracy.

Suggested Action: When subtracting the mean value of the positive control for cell killing, some of the values from the treated samples may be also close to zero. As there is always some variability in the accuracy of pipetting from well to well, and also in the accuracy of the plate reader, it is possible that some values slip just below zero as a result of random variation in the experiment.

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

Probable Cause: Pipetting errors, or issues in crystal violet staining or washing steps.

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. Check also that the plates are washed correctly after crystal violet staining, as suggested in point 2.

8) I did not find any "hits" that completely block TNF-α release - why is this?

Probable Cause: The cell-line is resistant to all tested 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 cell killing, 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.

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?

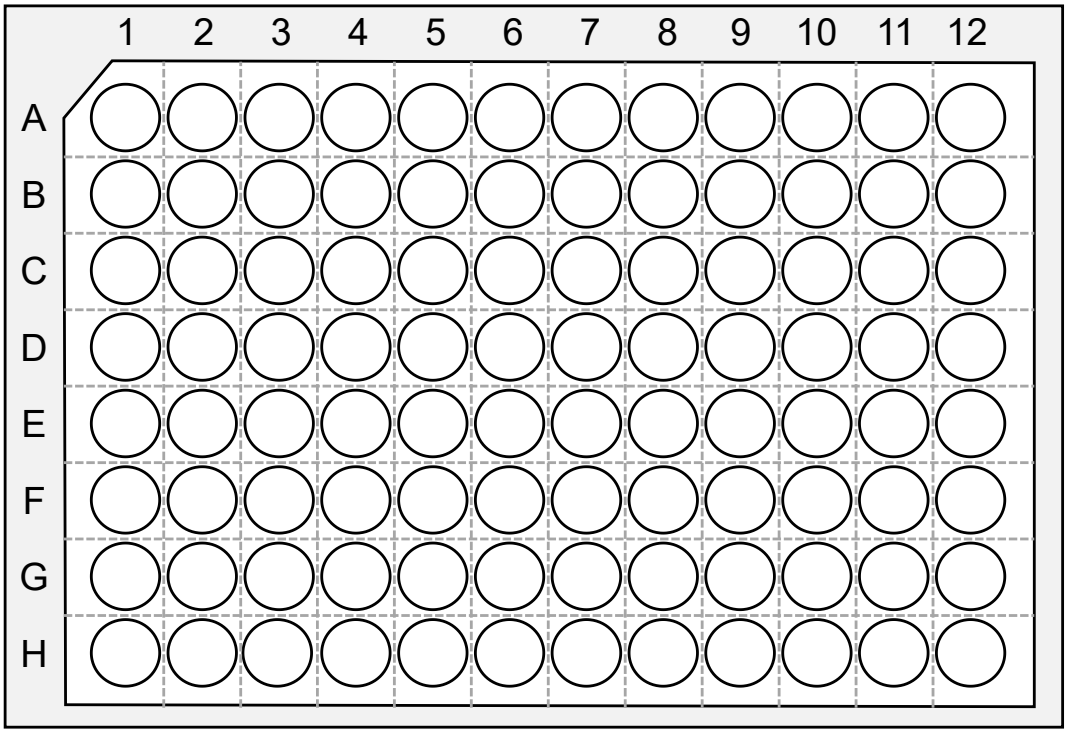
Probable Cause: Microsoft Excel data handling error.

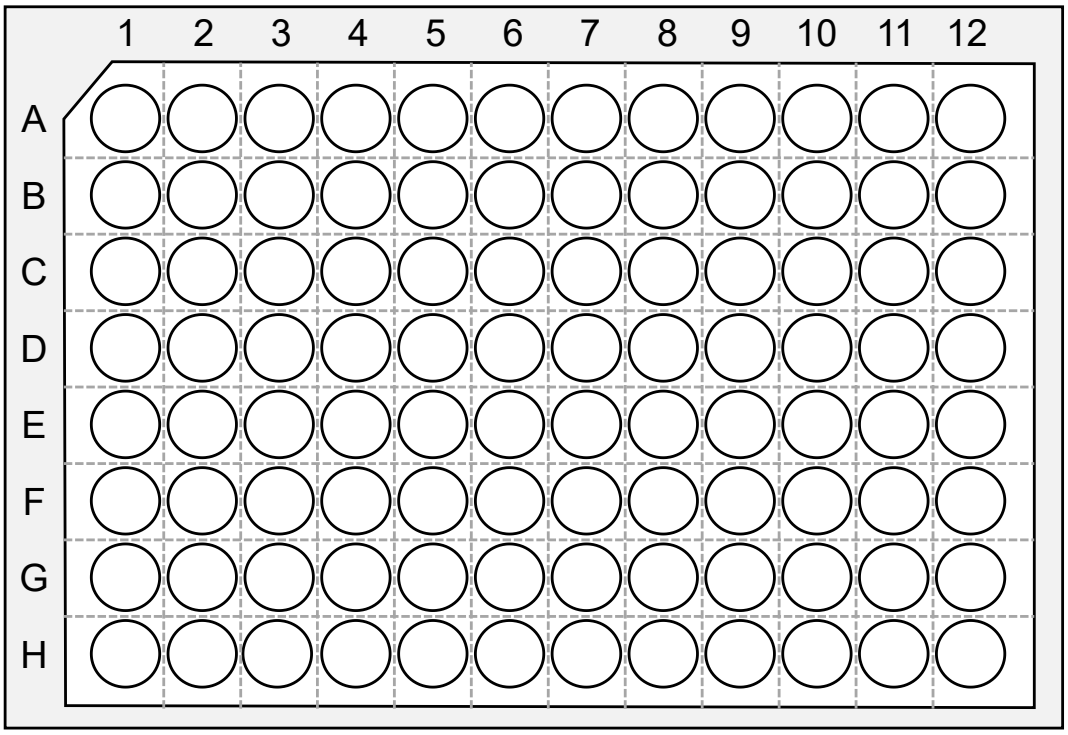
Suggested Action: If your assay plate had the highest concentration at the left-hand side of the plate, and the data are copied into your spreadsheet in the same orientation as the plate, make sure you type the concentrations in the correct (descending) order from left to right, so that the scatter plot 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). Remember also to plot your doses on the x-axis using a log scale, not a linear scale.

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	Plate macrophages for stimulation and screening	☐
7	Learn how to defrost, open and re-seal the *Phytotitre* kit (Method sheet 01)	☐
8	Screen the *Phytotitre* library for inhibitors of TNF-α production (Method sheet 07) *	☐
9	Challenge macrophages with LPS and freeze supernatants (Method sheet 13)	☐
10	Use L929 cell bioassay to estimate relative TNF-α production (Method sheet 14)	☐
11	Collate and normalise the primary screen data (Method sheet 21)	☐
12	Calculate Z' factor values for each of your plates (Method sheet 22)	☐
13	Identify hits from the primary screen (Method sheet 23)	☐
14	Perform dose response assays using individual hit extracts (Method sheet 41) *	☐
15	Perform statistical analysis of dose curve data (Method sheets 24 & 25)	☐
16	Test for compound interference with the L929 cell bioassay (Method sheet 42) *	☐
17	Compare hit extract activity with an established LPS inhibitor (Method sheet 41) *	☐
18	Attempt quantification of TNF-α using the L929 cell bioassay (Method sheet 43) *	☐
19	Writing the dissertation - hints, tips and advice available in Method sheet 36	☐

* 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.