Project Outline 02 - Cancer drug discovery

Background to the project

Cancer is a leading cause of mortality worldwide. It is characterized by the uncontrolled proliferation of abnormal cells that evade the body's normal defence mechanisms. Despite significant advances in immunotherapy and targeted biologics, there remains a pressing need for the discovery of new classes of small-molecule drugs that can selectively induce apoptosis or arrest the cell cycle in cancer cells.

Historically, the plant kingdom has provided a rich repository of anti-cancer agents. Iconic chemotherapy drugs such as paclitaxel, vincristine, and etoposide, were all originally derived from natural sources. The large-scale screening of natural products has also given rise to successful and widely used drugs in many other categories.

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 tumour cell-line.

Project at a glance

- 400 extract library
- 8 weeks lab time
- BSc / MSc level
- Cancer 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 methods, dose response assays, dataset management and statistical data analysis. Beyond gaining key industry-relevant skills, you will contribute to the search for novel therapeutic leads to help develop new cancer-targeting drugs.

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 cancer cell-line
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 cell growth
4) Perform dose curve analysis of validated hits to calculate IC_{50} values for your top hits
5) Seek validation of your hits using a secondary assay of cell viability
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**	
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	✓
Cell viability 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 Cancer Drug 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 22 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 cancer biology.

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, and the solvent they are suspended in (dimethyl sulphoxide, DMSO). 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, mammalian cell-lines 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

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: Choose which cancer cell-line you will investigate

Discuss with your supervisor which cancer cell-line will be most appropriate for your research goals. We recommend selecting a cell-line that is **adherent**, and can be safely cultured in the Containment Level 2 (CL2) tissue culture laboratories that are typically available to students at UK universities. Please note that the methods we propose using will not work with suspension cell-lines, such as THP-1, since the viability assays are compatible only with adherent cell-lines.

The following cell-lines are commonly studied as *in vitro* models of various forms of human cancer, and should work well with these projects: MCF-7 (a breast cancer cell-line); PC3 (a prostate cancer cell-line), HepG2 (a liver cancer cell-line), A549 (a lung cancer cell-line). Please feel free to choose one of these, or any other appropriate tumour cell-line available in your laboratory, to focus on in your 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. Most of these are commonly available as stock items in university laboratories, but it is essential to check to ensure they are available 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

- A tissue culture facility with class II containment safety cabinets and CO_2 incubators
- 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 *
- *Optional*: Digital microscope capable of capturing images of cells

Reagents

- A growing culture of an adherent tumour cell-line of interest (e.g. MCF-7)
- Tissue culture medium (e.g. DMEM), foetal calf serum (FCS/FBS) and antibiotics
- Standard tissue culture reagents (sterile PBS, trypsin/EDTA)
- Dimethyl sulphoxide (~10 ml, as vehicle control)
- Crystal violet powder (~2 g, necessary to stain the cell-line)
- Acetic acid (~200 ml, necessary to solubilise the crystal violet stain before measurement)
- Methanol (~200 ml, necessary to fix cells during staining)
- MTT reagent ~100 mg (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
- Sodium dodecyl sulphate (SDS, 10% solution in H_2O)
- *Optional*: A positive control chemotherapy drug

Consumables

- Clear plastic sterile 96-well microplates (at least 30)
- Autoclaved plastic tips compatible with available pipettes
- 13 ml or 30 ml pipettes for use with a motorized pipette filler
- Plastic reagent reservoirs (sterile, for loading plates)
- 15 ml or 50 ml sterile plastic tubes (for preparation of cell suspensions)
- Nitrile gloves
- *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 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: Initiate a stock culture of the cancer cell-line of interest

After receiving appropriate training in aseptic and cell culture techniques, prepare a bottle of complete medium (e.g. DMEM with 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 cells, or revive a vial of the cancer 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%.

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 8: Seed 96-well plates for screening with the *Phytotitre* kit

You will have to seed 5 separate 96-well microplates with cultures of your chosen cell-line for each complete screen of the library in this project. For instructions on how to do this, please follow **Method sheet 06**.

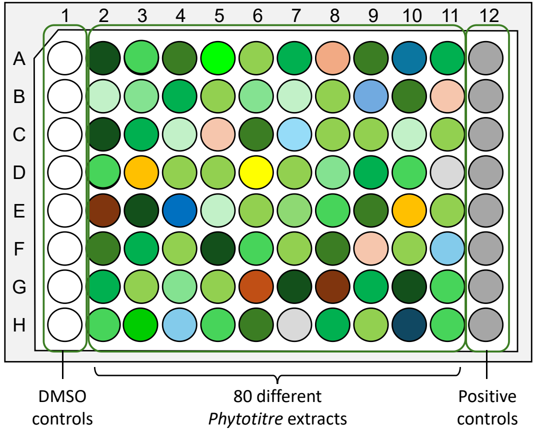
Step 9: Defrosting, opening and re-sealing the *Phytotitre* or *Puretitre* kits

⚠ 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 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 guide on how to defrost, open and reseal the kits given in **Method sheet 01**.

Step 10: Screen the *Phytotitre* library for inhibitors of cell growth

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). You should then add 1 µl of 10% SDS into every well of the twelfth column of every plate (the positive 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**.

Return the plate to a 37°C incubator with 5% CO_2 atmosphere for 24 hours.



Step 11: Use the MTT assay to measure cellular metabolic activity

The MTT assay is commonly used to estimate cell number and / or viability in mammalian cell toxicity studies. Supplement every well of your 96-well plates with 25 µl MTT reagent and return to the incubator for 4 hours, according to **Method sheet 08**. Then supplement every well with 100 µl of 10% SDS, and leave the plate in the dark at room temperature overnight to solubilise the formazan crystals. Read the absorbance of the plate at 570 nm, or alternatively at a wavelength anywhere between 550 and 600 nm if not available, using a microplate reader the next day. For advice on how to get the most from your plate reader, follow **Method sheet 16**.

Step 12: 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 (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 19**.

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 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 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 growth of the tumour cell-line.

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 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 17: Test for compound interference with MTT assay reagents

Some natural products have potential to non-specifically increase absorbance values measured during MTT assays, either because they are strongly coloured, or because they have inherent reducing potential.

To account for this, you should repeat the dose response experiments from step 16 in the same way, except lacking any cells in the plates. In other words, aliquot 100 µl of complete cell culture medium only, containing no cells, to each well, then proceed directly to step 15 to add the hit

extracts, and perform the MTT assay as normal. The results of these experiments will be helpful for the discussion section of your dissertation, particularly if any extracts trigger a paradoxical uptick in apparent cellular viability at the higher concentrations of extract.

Step 18: Repeat the dose response assays using the crystal violet method

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 results of the previous experiment are correct by testing the bioactivity of the sample using a different kind of assay. You should attempt to do this for your hit extract(s) using a crystal violet assay for inhibition of cell growth. For instructions on how to do this, please follow **Method sheet 9**. Remember to repeat this experiment at least 3 times to enable statistical analysis of the data.

Step 19: Perform statistical analysis of crystal violet assay results

Once you have obtained data from dose response experiments using your top hits using the crystal violet assay, you will have to repeat the calculations of IC50, and perform a two way ANOVA on the new data to further confirm if the hits have a significant impact on cell growth, as shown in **Method sheet 24** and **Method sheet 25**.

Step 20: Microscopy to explore effects of extracts on cell morphology

Optional: If a digital microscope is available in your laboratory, it may be helpful to capture digital images of cells treated with DMSO (as control), and each your hit extracts for the duration of time chosen for your main screening assay (i.e. 24 hours). These images may show changes in cell morphology, which could be a helpful addition to the results section of your dissertation.

Step 21: Examine the kinetics of cell killing by hit extracts

To further explore the properties of your hit extracts, you should measure the kinetics of cell-line killing by each hit. For these experiments, you should choose a time of day when you can visit the laboratory at the same time on four consecutive days. This will enable you to remove the plate from the incubator at the same time on each day to challenge the cells with extract for different lengths of time. For example, you could plate the cells at 10 am on Monday, give the first challenge at 2 pm on the same day, then give the second challenge at 2 pm on Tuesday, the third challenge at 2 pm on Wednesday, and then begin the MTT assay process at 2 pm on Thursday. Further instructions are available in **Method sheet 38**.

Step 22: Perform statistical analysis of cell killing timecourse data

Analyse the results of the cell killing timecourse experiments using a two way ANOVA using the advice given in **Method sheet 25**, using treatment (DMSO or extract) as one factor, and time (in hours) as the other factor.

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 34**.

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-cancer 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 MTT interference 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 using the crystal violet assay

Simply repeat the primary screen as shown above using the crystal violet assay instead of the MTT assay. Is there any correlation between the growth-inhibition values measured by the MTT assay in comparison to those of the crystal violet assay? Can you explain why some differences occur?

Project variation 3 - Repeat the screening project using a different cell-line

Simply repeat the primary screen as shown above with a different cell-line. Is there any correlation between the extent to which the extracts inhibit growth of the two cell-lines, across the entire collection? Are any of the hits the same?

Project variation 4 - Compare your results with existing *Phytotitre* screen datasets

Complete the first part of the data analysis (dry) cancer biology project 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 cell-lines 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.

Project variation 5 - Explore potential mechanisms of cell-killing

If the reagents are available in your laboratory, it may be helpful to explore if your hit extracts affect rates of apoptosis or necrosis (two major forms of cell death) in your target cell-line. Apoptosis can be measured by flow cytometry using annexin-V reagent, and necrosis can be measured by a simple plate-based lactate dehydrogenase (LDH) release assay (not supplied).

Project variation 6 - Explore high content screening for cell morphology

High content screening involves the imaging of cell cultures exposed to hundreds of different compounds by microscopy. If you have access to a digital microscope, it may be possible to screen one or more of the *Phytotitre* extract plates and collect digital images of the cells treated with negative control (DMSO) and each of the extracts. Machine learning or AI tools could then be used to seek evidence of changes to cell morphology by certain extracts.

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 cell-line did not grow in the negative control (DMSO) wells - why is this?

Probable Cause: Improper cell seeding density or media preparation.

Suggested Action: Re-check the method of cell-counting, and the calculations used to prepare the diluted cell suspension before plating. Ensure the media contains serum.

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

Probable Cause: Interference from coloured or reducing compounds in the MTT assay.

Suggested Action: It is not uncommon to see an increase in apparent cell growth in MTT assays when challenging cells with natural extracts. This is because the compounds present in some extracts may be coloured, so adding to the absorbance value directly, or they may have reducing potential, so converting yellow MTT into purple formazan, even in the absence of any cells. Attempt replication of the key findings using an alternative method for cell viability assessment, such as the crystal violet assay.

4) 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, 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.

5) 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.

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 kill the tumour cell-line - 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.

8) Why is there a kick upwards in growth at the highest concentration of extract in my cell viability dose response assays?

Probable Cause: Interference from coloured or reducing compounds in the MTT assay.

Suggested Action: It is not uncommon to see a paradoxical uptick in absorbance in MTT assays when challenging cells with high concentrations of natural extracts. This is because the compounds present in some extracts may be coloured, so adding to the absorbance value directly, or they may have reducing potential, so converting yellow MTT into purple formazan even in the absence of any cells.

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

11) 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 out with 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. 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. Only then should you move on to adding the solubiliser to read the plate. By way of comparison, the MTT assay generally has higher reproducibility because it does not include a washing step.

12) The results of my crystal violet assays do not match exactly the results of the MTT assays - why is this?

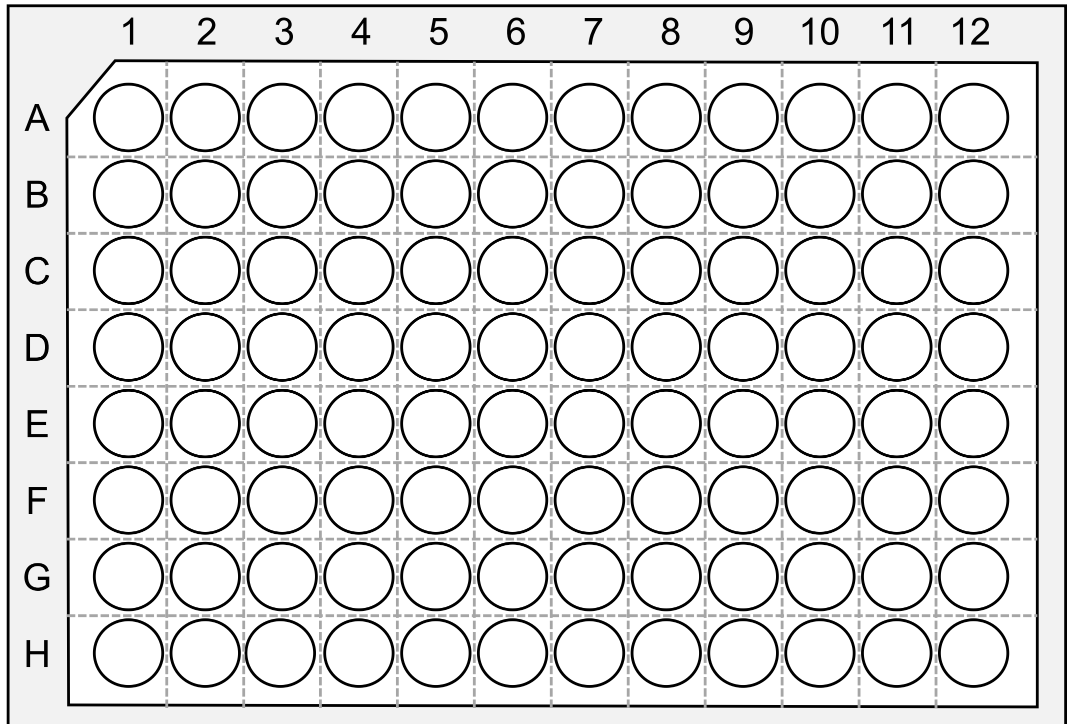
Probable Cause: This is normal - the MTT assay measures metabolic activity, and the crystal violet assay measures total biomass.

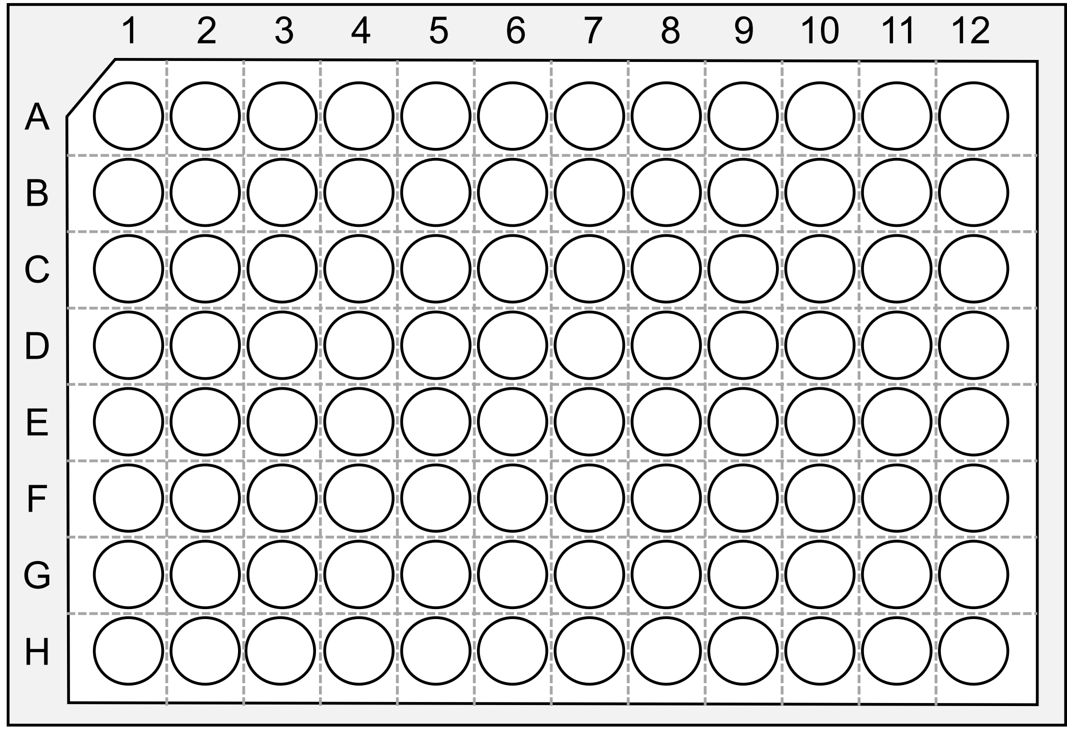
Suggested Action: You can explain in your discussion that there are advantages and disadvantages to the various methods of measuring cell growth and viability. The MTT assay is easy to perform and highly reproducible, but it can be confounded by coloured compounds and compounds with reducing potential. The crystal violet assay is not confounded by either of these issues as any compounds that may interfere with the assay are washed away before the staining steps. However, this assay is more difficult to perform and has lower reproducibility, because of the risk of washing away healthy cells if performing the washing steps too vigorously.

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	Choose which cancer cell-line you will investigate	☐
5	Make sure all the reagents and consumables you require are in stock	☐
6	Plan strategies for Good Laboratory Practice, Data Management and Integrity	☐
7	Initiate a stock culture of the cancer cell-line of interest	☐
8	Seed 96-well plates for screening with the *Phytotitre* kit (Method sheet 06)	☐
9	Learn how to defrost, open and re-seal the *Phytotitre* kit (Method sheet 01)	☐
10	Screen the *Phytotitre* library for inhibitors of cell growth (Method sheet 07) *	☐
11	Use the MTT assay to measure cellular metabolic activity (Method sheet 08)	☐
12	Collate and normalise the primary screen data (Method sheet 19)	☐
13	Calculate Z' factor values for each of your plates (Method sheet 22)	☐
14	Identify hits from the primary screen (Method sheet 23)	☐
15	Perform dose response assays using individual hit extracts (Method sheet 11) *	☐
16	Perform statistical analysis of dose curve data (Method sheets 24 & 25)	☐
17	Test for compound interference with MTT assay reagents (Method sheet 08) *	☐
18	Repeat the dose response assays using crystal violet assay (Method sheet 09) *	☐
19	Perform statistical analysis of crystal violet assay results (Method sheets 24 & 25)	☐
20	*Optional*: Microscopy to explore effects of extracts on cell morphology	☐
21	Examine the kinetics of cell killing by hit extracts (Method sheet 38) *	☐
22	Perform statistical analysis of cell killing timecourse data (Method sheet 25)	☐
23	Writing the dissertation - hints, tips and advice available in Method sheet 34	☐

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

🐞 **Found a Bug?**
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