OCT 07, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.14egn7my6v5d/v1

Protocol Citation: Dennis Juarez, dfruman 2023. Annexin V/PI- Assay: Flow Based Medium Throughput Assessment of Apoptotic Response to Two and Three Drug Combinations . **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn7my6v5d/v1

Protocol status: Working
We use this protocol and it's working

Annexin V/PI- Assay: Flow Based Medium Throughput Assessment of Apoptotic Response to Two and Three Drug Combinations

Dennis Juarez[1], dfruman[1]

[1]University of California, Irvine

Fruman Lab

Dennis Juarez

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

ABSTRACT

Basic principals and protocol for medium throughput cell viability screening in suspension cells using Annexin V/PI staining on the Attune NXT used in the Fruman lab.

Created: May 04, 2022

Last Modified: Oct 07, 2023

PROTOCOL integer ID: 61999

MATERIALS

Composition of 1x Annexin V Binding Buffer

Components	[Final]	Molar Mass
HEPES, pH 7.5	10mM	238.3 g/mol
NaCl	140mM	58.44 g/mol
CaCl-2H2O	2.5mM	147.02 g/mol

Annexin V-Alexa Fluor 647: Invitrogen A23204

Propidium Iodide - 1.0 mg/mL Solution in Water Invitrogen P3566

Basic Principals and Planning Before Proceeding

1 In vitro viability experiments should not be underestimated; especially, when carried out in a higher throughput fashion.

With a structured an organized plan, a 96 well two drug combination response surface experiment can be plated in 30 minutes; whereas, a poorly thought out and planned experiment can eat away several hours of your day and can lead to non-meaningful results as prolonged experimental setup can affect your media quality and cell quality.

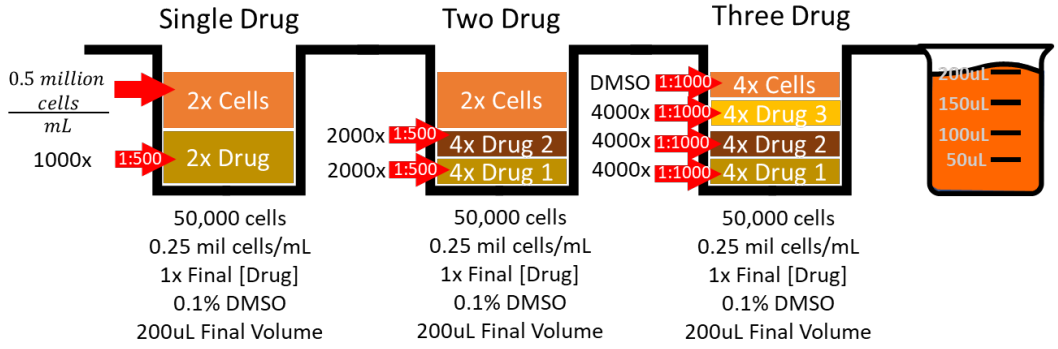
2 Several dilutions are required. To standardize the discussion, it's crucial to understand these key terms:

Stock Concentration: Refers to the concentration of the aliquots in long-term storage. Often stock concentrations are based on solubility of a compound or a practical concentration for aliquoting. As such, stock concentrations can be many times more concentrated than the final concentration.

Working Concentration: Refers to the concentration of any intermediate dilution of a drug in vehicle or media. As such, they are any concentration besides 1x and the stock concentration.

Final Concentration: Refers to the concentration of a drug which a cell is exposed to for the duration of the experiment. This is also known as 1x.

3



A schematic for three different experimental designs that accommodate 1, 2 and 3 drugs respectively, left to right. Depicted are 4 wells of a 96 well plate that are filled with a total volume of 200uL (right). Stock concentrations of drugs are not pictured here in this generalizable model. Working dilutions from the stock include 1000x (in DMSO) and 2x (in media) for a single drug setup, 2000x (in DMSO) and 4x (in media) for a two drug setup, and 4000x (in DMSO) and 4x (in media) for a three drug setup. In all cases, 50,000 cells are delivered to each well, but in different volumes, thus requiring different starting cell concentrations. 100uL of 0.5 million cells per mL (2x the final cell concentration in a well) are required for single and two drug setups while the three drug setup requires 50uL of 1 million per mL cells (4x the final concentration in a well). Unique to the three drug setup is an addition of 1uL to every 1000uLs of cells used. In the single drug setup, 100uL of 2x drug diluted in media from a 1000x DMSO-based working dilution is added to a well. In the two and three drug set up, 50uL of 4x drug is added. The dmso-based working concentration for the two drug setup is 2000x while for the three drug setup is 4000x. The final outcome of each setup is a cell concentration of 0.25 million per mL, 1x final concentration of the utilized drugs, 0.1% (vol/vol) DMSO in media, and a final volume of 200uL. Delivery of at least 50uL ensures precise volumes and simple mixing by diffusion.

4 Cells in culture can only tolerate small amounts of the vehicle in which a drug is dissolved in.

As such, you should minimize and standardize the amount of vehicle used in an experiment across all treatments.

4.1 I cap all experiments at 0.1% DMSO in the final experimental condition (1:1000 dilution of DMSO in media).

Note

You may encounter other vehicles based on compound solubility.

For example:
PBS or basal media; however, these vehicles are not commonly used for long term storage.
Ethanol/methanol
Water

Whatever it is, you must make sure all your cells are exposed to the same amount of vehicle as all other cells.

4.2 Single drug treatments are thus prepared at working dilutions that are 1000x their final concentration with DMSO.

4.3 Each agent of two drug treatments are prepared at working dilutions that are 2000x their final concentration.

4.4 Each agent of three drug combination treatments are prepared at working dilutions that are 4000x their final concentration.

5 Precision is key when carrying out these assays. Minimize variation by stepwise dilutions and using volumes that are easy to pipet and verify.

5.1 In regards to preparing your working dilutions, stepwise dilution results in greater precision than direct dilution.

Note

Half log dilutions in drug concentrations are often used to cover a large drug titration range.

	A	B	C	D	E	F	G	H
	[Drug] in nM	10.0	31.6	100	316.2	1000	3162.3	10000
	LogTransformed	1	1.5	2	2.5	3	3.5	4

An example of exact half log doses.

Approximation of a half log are accepted in the field, rather than a exact half log, because they're easier to use.

	A	B	C	D	E	F	G	H
	[Drug] in nM	10	30	100	300	1000	3000	10000
	LogTransformed	1.00	1.48	2.00	2.48	3.00	3.48	4.00

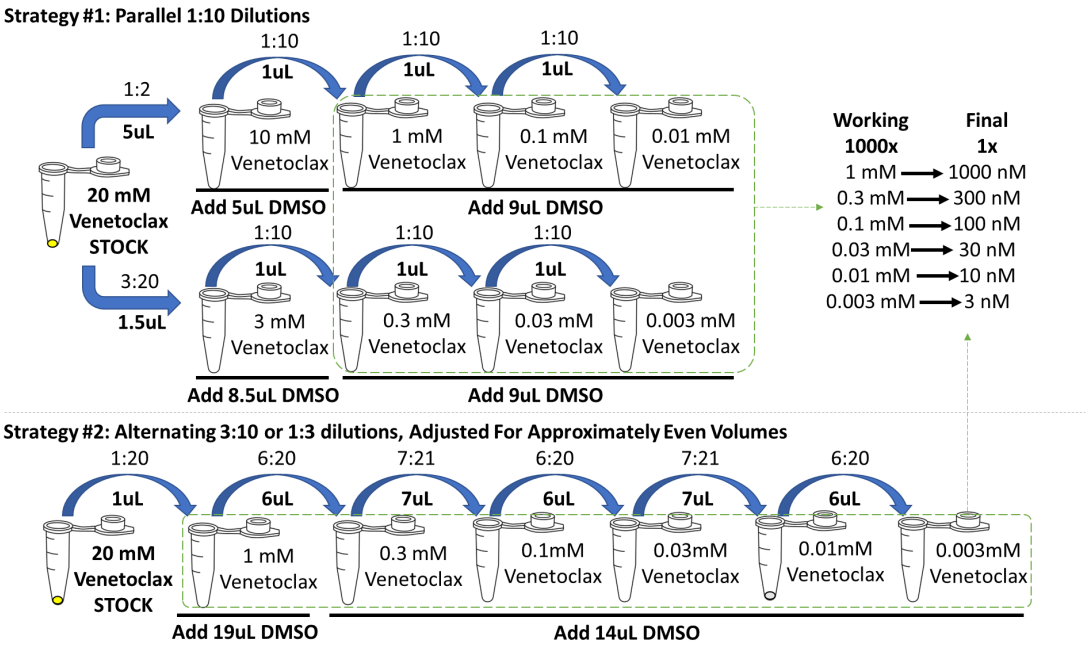
An example of approximated half log doses.

Alternatively, if you are interested in covering a specific range more completely, you may cover the range of 1x, 2.5x, 5x, 7.5x, 10x and cover a smaller range. You may do this when you have a better idea of the effective range of a drug.

	A	B	C	D	E	F	G	H
		1x	2.5x	5x	7.5x	10x	25x	50x
	[Drug] in nM	10	25	50	75	100	250	500

An example to cover a dose range more thoroughly.

5.2

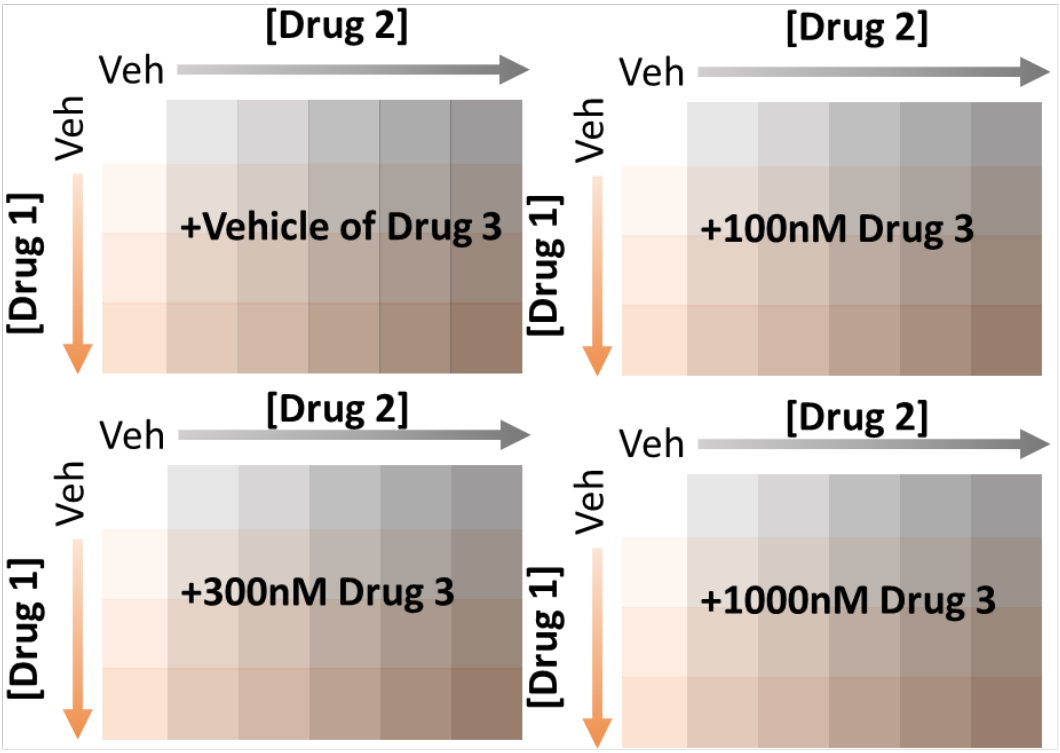


Two strategies for stepwise dilutions. Dilutions should always be simple math. Understand how to use $M_1V_1 = M_2V_2$ to make dilutions simple with easy to dilute volumes. For example: if we make V2=M1 then V1=M2, or in words: if we decide that our desired volume is the concentration of the drug we are diluting, then the volume we'll have to dilute from that drug will be equivalent to our desired concentration. This is exemplified by the 3:20 dilution-- by deciding my desired volume was 20, I knew I had to take 3 and bring it up to 20. The dilution ratio can also be reduced down to 1.5:10, which is what I do in strategy #1. Strategy #2 is excellent when the amount of drug is the limiting reagent.

Nevertheless, always plan and write down your dilutions to use during experimental setup and to reference later.

6

Plan your plate set up to include all single drug controls. To accomplish this, I recommend setting up a matrix.



A three drug combination study on a 96 well plate including every possible drug combination within a selected range. Every square represents either single treatment or a combination of treatments in a 96 well plate. Three doses of drug 1, five doses of drug 2 and 3 doses of drug 3 are used in this example. A single drug treatment can be thought of as a single axis of the upper left matrix. A two drug matrix is well represented as the upper left matrix. Adding a third drug vector vastly increases the dimensions and complexity of analysis.

Prepare Dilutions and Plate Drugs

7 Prewarm your media.

8 Prepare your DMSO-based working dilutions sterilely. From your stock solution, make your DMSO-based working dilutions per your experimental design in step 3.

Note

There are several things that can affect your dilutions that you as the technician need to be aware of.

1. Use trustworthy pipets to measure volumes and always look at your pipet tip before and after pipetting.
2. Forward pipet your dilutions.
3. When possible, be aware of what the markings on the pipet tip indicate and use these markings to mentally benchmark that you're delivering the correct volumes.
4. Know what 1uL, 2.5uL, 5uL, and 10uL look like.
5. Do not accidentally deliver unintended volumes that stick to the outside of the pipet tip. These can make a huge difference in your variation from experiment to experiment.
6. Always thoroughly mix your intermediary dilution before further diluting.
7. If you are in doubt about your mixing by pipet, vortex the dilution before further diluting.
8. Failure to properly mix dilutions will introduce massive variability.

9 Dilute your DMSO-based working dilutions into prewarmed media from step 7 as depicted in step 3. Only make as much as you need for each drug.

Calculate this by multiplying the volume you will use per well by the number of wells that will get the specific drug dose. Make extra.

10 Think of the vehicle column and rows of your matrix as a drug and prepare an appropriate dilution per step 3 of DMSO in media. Usually you'll need more of this than the other drug doses.

11 Using 8 channel reservoirs and a multichannel pipet, distribute your drugs into a 96 well flat bottom.

- To minimize tip usage, you can reuse tips for multiple wells receiving the same drug.
- In order to minimize well to well contamination with other drugs, deliver your first drug on the bottom of the plate. Your second drug should be delivered on the side wall of the plate. Your third drug will be delivered on the opposite side wall of the plate.

12 When plating of all the drugs is complete, place the plate in the incubator. Plan to minimize this amount of time without the cells to avoid unnecessary evaporation.

12.1 If you have a lot of cell lines that you will be plating, you can count your cells before starting your dilutions and drug plating. If you have a couple of cell lines, then preparing them next will

not be too time consuming.

Preparing and Plating Cells

13 Count your cells and either resuspend in fresh media or dilute cells to get them to either 0.5 million per mL or 1 million per mL if carrying out a triple combination.

14 If doing a triple combination, don't forget to add 1:1000 DMSO to the cells.

15 Transfer the appropriately concentrated cells to a reservoir and pull your plate with drugs from the incubator.

16 Deliver cells by multichannel to an unused side wall.

17 Incubate from 24-72 hours depending on your experiment.

Annexin V PI Staining and Readout

18 After completion of incubation, transfer 100 uL of cells to a V-Bottom well plate. Centrifuge at 500g x 5 min

Note

The cells have settled at the bottom of the plate. Pipet up and down throughout the entire bottom of the plate to resuspend the cells. Do not just pipet up and down in a single place. You must spray the entire bottom with your pipet to get cells dispersed into solution.

19 Resuspend in 100uL of 1:2000 Annexin V and 1:2000 propidium iodide diluted in annexin V binding buffer and transfer to a U-Bottom well plate.

20 After 5 minutes, the samples should be ready to read on the attune with the high throughput setting checked.