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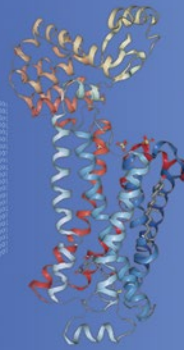

Richard S. Larson · Tudor I. Oprea *Editors*

Bioinformatics and Drug Discovery

Third Edition

Methods in Molecular Biology

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Bioinformatics and Drug Discovery

Third Edition

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Preface

A remarkable number of novel bioinformatics methods and techniques have become available in recent years, enabling us to more rapidly identify new molecular and cellular therapeutic targets. It is safe to say that bioinformatics has now taken its place as an essential tool in the process of rational drug discovery.

The first (2005), second (2012), and now third editions of *Bioinformatics and Drug Discovery* offer many examples that illustrate the dramatic improvement in our ability to understand the requirements for manipulating proteins and genes toward desired therapeutic and clinical effects.

This is partly due to our growing ability to modulate protein and gene functions, which has been facilitated by the emergence of novel technologies and their seamless digital integration. To address the rapidly changing landscape of bioinformatics methods and technologies, this edition has been updated to include four major topics: (1) Translational Bioinformatics in Drug Discovery; (2) Informatics in Drug Discovery; (3) Clinical Research Informatics in Drug Discovery; and (4) Clinical Informatics in Drug discovery. The topics covered range from new technologies in target identification, genomic analysis, cheminformatics and chemical mixture informatics, protein analysis, text mining and network or pathway analyses, as well as drug repurposing.

It is virtually impossible for an individual investigator to be familiar with all these techniques, so we have adopted a slightly different chapter format than other titles published by *Methods in Molecular Biology.* Each chapter introduces the theory and application of the technology, followed by practical procedures derived from these technologies and software. Meanwhile, the pipeline of methodologies and the biologic analysis that they perform has grown over time.

Bioinformatics and Drug Discovery is intended for those interested in the different aspects of drug design, including academicians (biologists, informaticists, chemists, and biochemists), clinicians, and scientists at pharmaceutical companies. This edition's chapters have been written by well-established investigators who regularly employ the methods they discuss. The editors hope this book will provide readers with insight into key topics, accompanied by reliable step-by-step directions for reproducing the techniques described.

Albuquerque, NM, USA

Richard S. Larson
Tudor I. Oprea

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Part I

Translational Bioinformatics in Drug Discovery

Chapter 1

Miniaturized Checkerboard Assays to Measure Antibiotic Interactions

Melike Cokol-Cakmak and Murat Cokol

Abstract

Drugs may have synergistic or antagonistic interactions when combined. Checkerboard assays, where two drugs are combined in many doses, allow sensitive measurement of drug interactions. Here, we describe a protocol to measure the pairwise interactions among three antibiotics, in duplicate, in 5 days, using only two 96-well microplates and standard laboratory equipment.

Key words Drug interactions, Checkerboard assay, Drug synergy

1 Introduction

Drug combinations may exhibit surprisingly high or low effect on a phenotype given the effects of constituent drugs, corresponding to synergistic or antagonistic drug interactions, respectively [1–4]. Experimental measurement of a drug interaction involves the preparation of combinations of constituent drugs in various concentrations [5]. A commonly used experimental setup for pairwise drug interaction measurement is the checkerboard assay, where two drugs are combined in a 2D matrix where the dose of each drug is linearly increased in one axis [6]. In such a setting, synergistic drug pairs will be more efficacious in many of the combinations, while high growth will be observed in antagonistic pairs.

Although in use for many decades, the preparation of a checkerboard assay is difficult, due to experimental variation of single-drug effects. In addition, checkerboard assays are often conducted in an 8 × 8 matrix of concentration combinations, resulting in significant cost in time and resources [6]. Here, we describe a simple and reproducible protocol to determine the pairwise antibiotic interactions using miniaturized checkerboard assays.

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2 Materials

2.1 Preparation of Bacterial Culture

1. Aliquots of *Escherichia coli* in 25% glycerol (*see* **Note 1**).
2. LB Broth Powder.
3. 15 ml breathable cell culture tube.
4. Pipette pump.
5. 5 ml cell culture serological pipette.
6. Manual pipette.
7. 200 μl tips.
8. Incubator.
9. Tube rotator.
10. 1.5 ml semi-micro cuvette.
11. Spectrophotometer.

2.2 Dose-Response and Checkerboard Assays

1. Drugs X, Y, and Z (*see* **Note 2**).
2. DMSO.
3. *1.5 ml Eppendorf microcentrifuge tubes.*
4. Manual pipette.
5. 20 μl and 1000 μl tips.
6. Vortex mixer.
7. 96-well plates.
8. Reagent reservoir.
9. Breathable sealing film.
10. Microplate reader.

3 Methods

Carry out all protocols at room temperature. Thaw new aliquots of bacteria and drugs each day. Prior to experiments, prepare LB Broth with adding 25 g of powder to 1 l distilled water, autoclave it at 121 °C for 15 min, and store the autoclaved media at room temperature. Dissolve drugs X, Y, and Z in DMSO at a concentration of 2 mM, and freeze aliquots in 1.5 ml Eppendorf tubes at −20 °C.

3.1 Day 1: Start Bacterial Culture

1. Take one aliquot of *Escherichia coli* from −80 °C.
2. Add 100 μl of bacterial culture in 5 ml of growth media in a culture tube.
3. Leave to grow overnight on a tube rotator in a 37 °C incubator.

3.2 Day 2: Serial Dilution Dose Response

1. Take one aliquot of drugs X, Y, and Z from −20 °C, leave them in room temperature for 10 min, and prepare for serial dilution of these drugs.
2. Prepare LB-10% sol by mixing LB media and solvent (DMSO) in a 9:1 ratio.
3. Prepare LB-10% drug X by mixing LB media and drug X in a 9:1 ratio.
4. Vortex and add 20 μl of the 1:10 diluted solvent (LB-10% sol) to 10 wells in a 96-well plate.
5. Vortex and add 20 μl of the 1:10 diluted drug X (LB-10% drug X) into the first well.
6. Take 20 μl of content from first well and add to second well. Dilute the drug concentration serially in each well by adding 20 μl of content to its bottom adjacent well until ninth well (*see* Fig. 1a).
7. Discard the last 20 μl of content from ninth well (Last well of the column is used as a no drug control).
8. Repeat **steps 3**–**7** for the drugs Y and Z (*see* **Note 3**).
9. Measure the OD_{600} of the 1:10 dilution of the culture started in Day 1.
10. Dilute the cells in growth media to an OD of 0.01 (*see* **Note 4**).
11. Add 80 μl cells on drug serial dilutions prepared in **step 8.** The final drug concentration in each well is shown in Fig. 1a.
12. Seal plate to avoid evaporation.
13. Leave plate for 12 h at 37 °C in a shaker with 150 rpm.
14. Start new bacterial culture to use in Day 3 (*repeat* Subheading 3.1).

3.3 Day 3: Linear Dilution Dose Response

1. Measure OD_{600} absorbance for serial dilution dose-response plate from Day 2 (*see* Fig. 1b).
2. Normalize growth by dividing growth in each well with the growth in no drug control. For each drug, choose 1× as the dose which is twice the minimum concentration that results in no growth.
3. For each drug, prepare LB-10% drug by mixing LB media and drug in a 9:1 ratio, where drug's concentration is 50× of what is chosen at **step 2**. Similarly, prepare LB-10% sol by mixing LB media and solvent (DMSO) in a 9:1 ratio.
4. Prepare linearly increasing doses of drugs X, Y, and Z in ten concentrations, by mixing LB-10% drug and LB-10% sol in volumes shown in Fig. 2a (*see* **Note 3**).
5. Measure the OD_{600} of the 1:10 dilution of the culture started in Day 2.

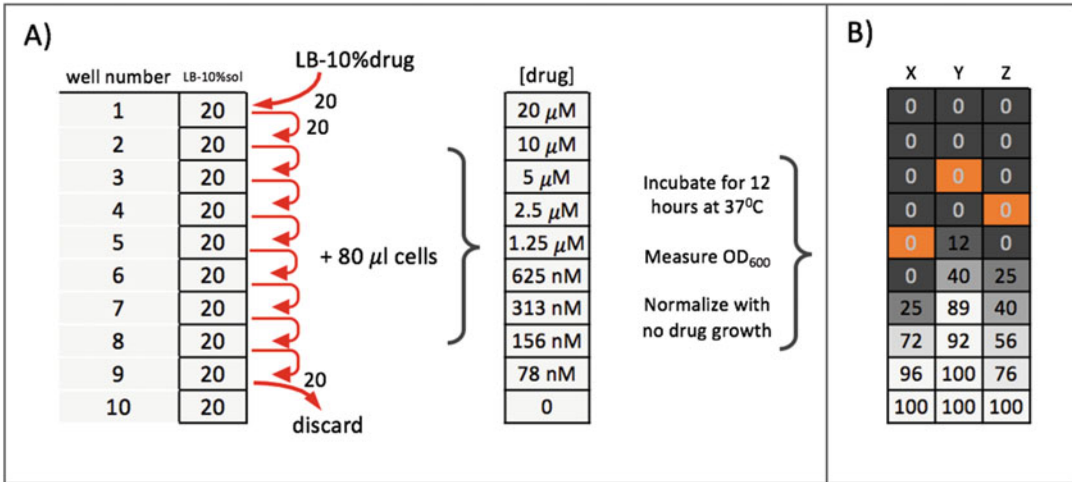

Fig. 1 Serial dilution dose-response experiment. (**a**) Preparation of serial dilution dose response for one drug and corresponding final concentrations of the drug. (**b**) Normalized growth in serial dilution of drugs X, Y, and Z. Each rectangle here represents a well of 96-well plate. Concentrations of each drug chosen for the next experiment are shown in orange

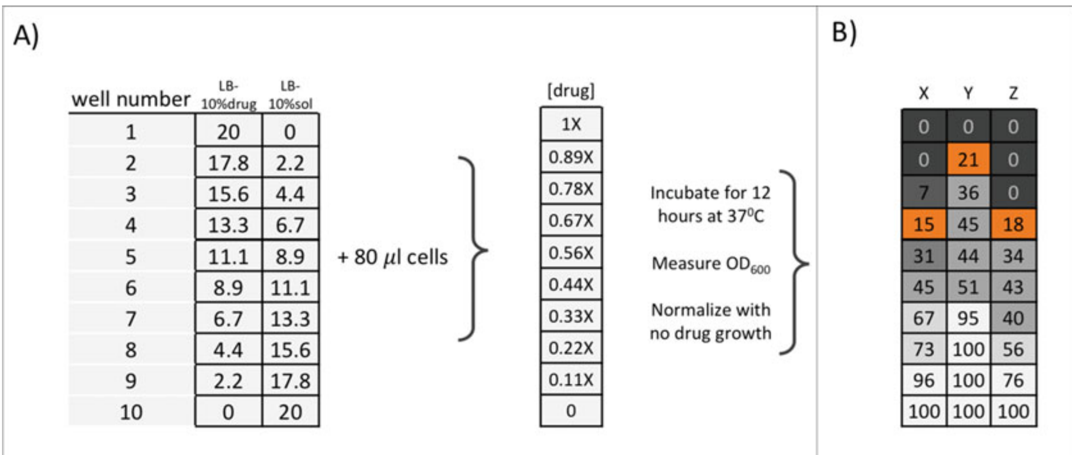

Fig. 2 Linear dilution dose-response experiment. (**a**) Preparation of linear dilution dose response for each drug and corresponding final concentrations of the drug. (**b**) Normalized growth in linear dilution of drugs X, Y, and Z. Each rectangle here represents a well of 96-well plate. Concentrations of each drug chosen for the next experiment are shown in orange

6. Dilute the cells in growth media to an OD of 0.01 (*see* **Note 4**).
7. Add 80 μl cells on drug linear dilutions prepared in **step 4**. The final drug concentration in each well is shown in Fig. 2a as ratios of 1×.
8. Seal plate to avoid evaporation.
9. Leave plate for 12 h at 37 °C in a shaker with 150 rpm.
10. Start new bacterial culture to use in Day 4 (*repeat* Subheading 3.1).

3.4 Day 4: Checkerboard Assay Experiment

1. Measure OD_{600} absorbance for linear dilution dose-response plate from Day 3 (*see* Fig. 2b).
2. For each drug, choose the concentration that resulted in 80% growth inhibition (IC80) as 1× (*see* **Note 5**).
3. For drug X, label four tubes as LB-drugX0, LB-drugX1, LB-drugX2, and LB-drugX3, and add 189 μl of LB media to these tubes.
4. In each tube, add 0, 7, 14, or 21 μl of 100× drug X, and add 21, 14, 7, or 0 μl of solvent (DMSO), as shown in Fig. 3a.
5. Repeat **steps 3** and **4** for the drugs Y and Z (*see* **Note 6**).
6. Preparation of a 4x4 checkerboard assay for drug X + drug Y is shown in Fig. 3a.

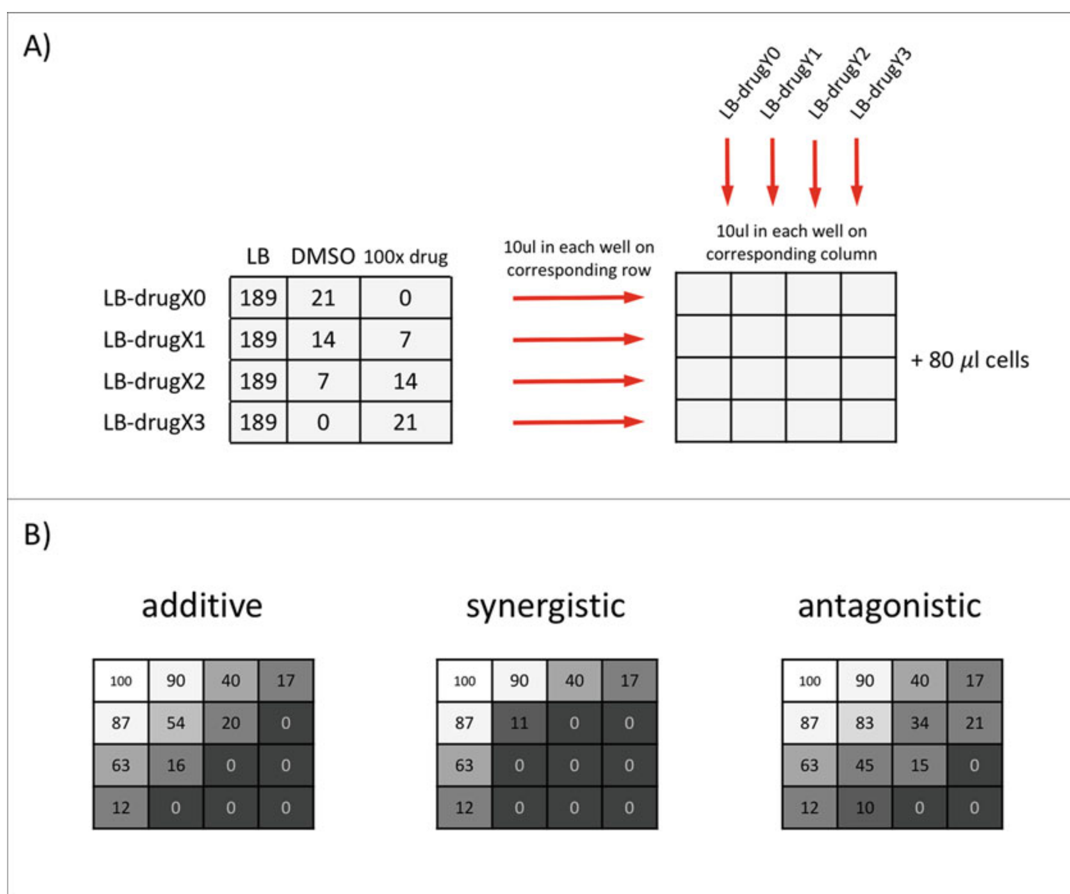

Fig. 3 Miniaturized checkerboard assay. (**a**) Preparation of drug mixes and placement of each drug in 96-well plate for 4 × 4 checkerboard. Each rectangle here represents a well of 96-well plate. Drug X and drug Y pairs are used as an example for preparation. (**b**) Interpretation of drug pairs results in 4 × 4 checkerboard assay as additive, synergistic, or antagonistic

7. Add 10 μl of LB-drugX0, LB-drugX1, LB-drugX2, and LB-drugX3 in each well on first, second, third, and fourth rows, respectively.
8. Add 10 μl of LB-drugY0, LB-drugY1, LB-drugY2, and LB-drugY3 in each well on first, second, third, and fourth columns, respectively.
9. Repeat the **steps 6–8** for X + Z and Y + Z, in duplicate, which corresponds to one 96-well plate ($4 \times 4 \times 6 = 96$) (*see* **Note 3**).
10. Measure the OD_{600} of the 1:10 dilution of the culture started in Day 3.
11. Dilute the cells in growth media to an OD of 0.01 (*see* **Note 4**).
12. Add 80 μl cells on 4×4 checkerboards assay.
13. Seal plate to avoid evaporation.
14. Leave plate for 12 h at 37 °C on a shaker with 150 rpm.

3.5 Day 5: Checkerboard Assay Result

1. Measure OD_{600} absorbance for checkerboard assay experiment plate from Day 4.
2. Example results for additive, synergistic, or antagonistic drug pairs are shown in Fig. 3b.
3. For each experiment, count the number of wells where there is no growth. This count will be high for synergistic drug pairs, medium in additive drug pairs, and low in antagonistic drug pairs. Compare results from replicates.
4. For further exploration on how to score checkerboard assays, the reader is suggested to consult refs. 2, 4, 6–8.

We have previously used this miniaturized checkerboard assay protocol in two antibiotic interaction screens, where all pairwise interaction scores for 24 compounds (276 pairs) were determined in replicate. For these screens, we developed a scoring method based on Loewe additivity model, where negative, zero, or positive values correspond to synergy, additivity, or antagonism. MATLAB functions that use 4×4 growth metrics and compute a drug interaction score are shared as the supplementary material of ref. 8, as well as all the raw growth measurements recorded in this screen.

In this screen, we have found that the pairwise interactions among fusidic acid, oxacillin, and amikacin cover all possible three drug interaction types: Fusidic acid and oxacillin are synergistic; fusidic acid and amikacin are additive; and oxacillin and amikacin are antagonistic. We suggest that the reader use these three drugs for trying this protocol, in order to observe the full extent of the drug interaction phenotypes. The reader may use the simple scoring method described in the protocol's Day 5 **step 3** or the more involved synergy metric described in ref. 8. With materials that can

be found in an undergraduate laboratory class, our protocol describes an efficient and reproducible method to measure antibiotic interactions.

4 Notes

1. While antibiotic interaction in *E. coli* is the example here, any species can be substituted here, with their respective growth media and growth conditions supplanted.
2. Any small molecule that inhibits growth and corresponding solvent can be used.
3. In our protocol, there is 2% solvent in all microplate growth experiments, ensuring the effects we observe are not due to the solvent.
4. Since the cell density influences the inhibitory concentration of a drug, it is important that cells used are at an OD = 0.01.
5. In our experience, we have found IC80 is the most informative top concentration in a miniaturized checkerboard assay.
6. Although we need 160 μl for each concentration (10 μl × 4 per interaction assay × 4 interaction assays), we prepare 210 μl because of ease of calculation and pipetting.

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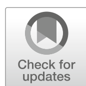

Chapter 2

High-Throughput Screening for Drug Combinations

Paul Shinn, Lu Chen, Marc Ferrer, Zina Itkin, Carleen Klumpp-Thomas, Crystal McKnight, Sam Michael, Tim Mierzwa, Craig Thomas, Kelli Wilson, and Rajarshi Guha

Abstract

The identification of drug combinations as alternatives to single-agent therapeutics has traditionally been a slow, largely manual process. In the last 10 years, high-throughput screening platforms have been developed that enable routine screening of thousands of drug pairs in an in vitro setting. In this chapter, we describe the workflow involved in screening a single agent versus a library of mechanistically annotated, investigation, and approved drugs using a full dose-response matrix scheme using viability as the readout. We provide details of the automation required to run the screen and the informatics required to process data from screening robot and subsequent analysis and visualization of the datasets.

Key words Drug combination screening, Acoustic dispensing, Automation, Compound management, Synergy

1 Introduction

High-throughput screening for compounds that affect cell viability has been utilized as a method for discovery of novel treatments for various human diseases. For patients with cancer and certain infectious diseases, combinations of drugs are given to achieve maximal clinical benefit. An additional benefit of a clinically synergistic drug combination is that both drugs may be synergistic at a low dose, which can reduce off target toxicities. For infectious diseases such as HIV, drug combinations are critical to prevent infectious agents from acquiring mutations to evade the action of a single drug. The search for novel synergistic drug pairs requires the development of a systematic, large-scale screening platform. CombinatoRX, a biotech company acquired in 2014 by Horizon Discovery, was the first to publish a series of papers utilizing drug combination screening to explore synergistic drug responses in various disease models such as cancer and drug-resistant bacteria [1–3]. A recent study spearheaded by AstraZeneca and NCI-DREAM utilized a

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crowdsourcing approach to predict synergistic drug combinations for treatment of B-cell lymphoma [4].

The development of a methodology for large-scale testing of drug combinations in vitro was advanced by the incorporation of acoustic dispensing technology, which allows for the flexibility of an anywhere-to-anywhere compound transfer. Given that drug combination screening requires two or more compounds present in a single well, contact-based transfer methods would be costly in time and resources to reduce the possibility of contamination between transfer steps. Using a noncontact dispenser greatly reduces the amount of sample and consumables used as well as the complexity that would be involved if traditional contact-based pipetting had been applied.

Here we report the methods and workflow specifically for drug combination screening that has been implemented and optimized at the National Institutes of Health's National Center for Advancing Translational Sciences (NCATS). This drug combination screening platform has been applied to multiple areas of drug discovery including cancer, malaria, Ebola, and various other disease models [5–8] and as of 2016 has tested over 200,000 discrete drug combinations. This automated screening platform has required the use of in-house software development as well as integration of various instrumentations in order to achieve an almost fully automated workflow. We typically refer to this as Matrix screening, due to the layout of the drug combinations in a grid format on the final plate. The workflow presented here was utilized for screening of the Ewing's sarcoma cell line and has been published [8].

2 Materials

The DMSO stock solutions are stored at −20 °C, but all other operations occur at room temperature.

2.1 Consumables

1. Dimethyl sulfoxide (DMSO): 100% DMSO, ACS grade.
2. 1.4 mL Matrix 2D barcode tube (sample tube): Thermo Scientific, #3711.
3. 96-well Society for Biomolecular Screening (SBS) footprint rack that holds sample tubes (compound source rack).
4. SepraSeal cap (cap): Thermo Scientific, # 4463.
5. 96-well polypropylene compound plate (intermediate plate): VWR, #82006-704.
6. 384-well polypropylene compound plate (mother plate): Greiner, #784201.
7. SBS footprint reservoir (DMSO reservoir).

8. 384-well cyclic olefin copolymer (COC) plate (acoustic source plate): Greiner, #788876.
9. P25 JANUS tips (P25 tips): Perkin-Elmer, #6000689.
10. Biomek FX P30XL tips (P30XL tips): Beckman Coulter, #A22288.
11. Biomek FX P30 tips (P30 tips): Axygen, FX-1536-30FP-R-S.
12. DMSO-resistant adhesive foil seal (foil seal): 4titude, 4Ti-0512.
13. Deionized water.
14. 70% ethanol.
15. White 1536, tissue culture treated, high base plates (assay plate): Aurora, EWB04100A.
16. T175 tissue culture flasks.
17. TC71, Ewing's sarcoma cancer cell line, DSMZ repository #ACC 516.
18. RPMI-1640 cell culture media, Thermo Fisher Scientific #11875093.
19. Fetal Bovine Serum, GE Healthcare Life Sciences, #SH30071.03.
20. Penicillin-streptomycin, Thermo Fisher Scientific #15140122.
21. 0.25% Trypsin-EDTA, Thermo Fisher Scientific #25200056.
22. CellTiter-Glo® One Solution (CellTiter-Glo): Promega G7573.

2.2 Equipment and Instrumentation

1. Benchtop vortex mixer.
2. Sonicating water bath.
3. Automated compound store (ACS): Brooks Automation, A3+.
4. Automated decapper: Univo, #DC480.
5. TubeAuditor: automated volume measurement device from Brooks Automation.
6. JANUS liquid handler (JANUS): Perkin-Elmer.
7. Handheld barcode scanner.
8. Matrix WellMate bulk liquid dispenser (WellMate), Thermo Scientific.
9. Handheld 8-channel pipettor.
10. Biomek FX liquid handler (FX), Beckman Coulter.
11. Benchtop centrifuge.
12. Handheld pipettor.
13. Rubber roller.

14. ATS-100 acoustic dispenser (ATS-100), EDC Biosystems, Gen4+.
15. Multidrop Combi dispenser (Multidrop), Thermo Fisher Scientific, 5840300.
16. Multidrop Combi dispensing cassette (cassette), Thermo Fisher Scientific #24073290.
17. Metal, foam gasketed lid (compound plate lid).
18. Clear assay lid.
19. Stainless steel, rubber gasketed assay lid (assay lid).
20. ViewLux reader (ViewLux): Perkin-Elmer.
21. Polystyrene Universal Microplate Lid (plastic lid), Corning #3098.
22. Automated acoustic plate reformatter (HRB): HighRes Biosolutions, ACell.

2.3 Software Components

1. Microsoft Excel or equivalent spreadsheet program.
2. Matrix Script Plate Generator (MSPG).
3. R 3.3.1 and the ncgcmatrix package.

3 Methods

3.1 Preparation of Stock Compound Solution

1. Prepare compound stock solutions by weighing compound into sample tube to make 800 μL of 10 mM DMSO solution.
2. Cap and vortex the sample tube for 10 s at 3200 rpm. Visually inspect that the compound has completely dissolved; sonicate the sample tube for up to 10 s in a sonicating water bath to assist in dissolution, if necessary.
3. Register the sample tube barcode to sample ID association in the database, and load the sample tube to the ACS.

3.2 Compound Source Rack Plate Map Creation

1. Based on prior IC_{50} determination of the compounds of interest, prepare a Matrix screening request form following the template format as shown in Figs. 1a and 2.
2. Identify a list of available sample tubes from the chemical inventory system, and input the list of sample tube barcodes to the ACS to cherry-pick the compounds needed to prepare the acoustic source plate.
3. Remove the compound source racks from the ACS, and allow the samples to thaw at room temperature. Briefly centrifuge the compound source racks for 30 s at 234 × *g* (*see* **Note 1**). Export the cherry-pick plate map from the ACS database to Microsoft Excel, and save (Fig. 1b). This file is called the compound source rack plate map.

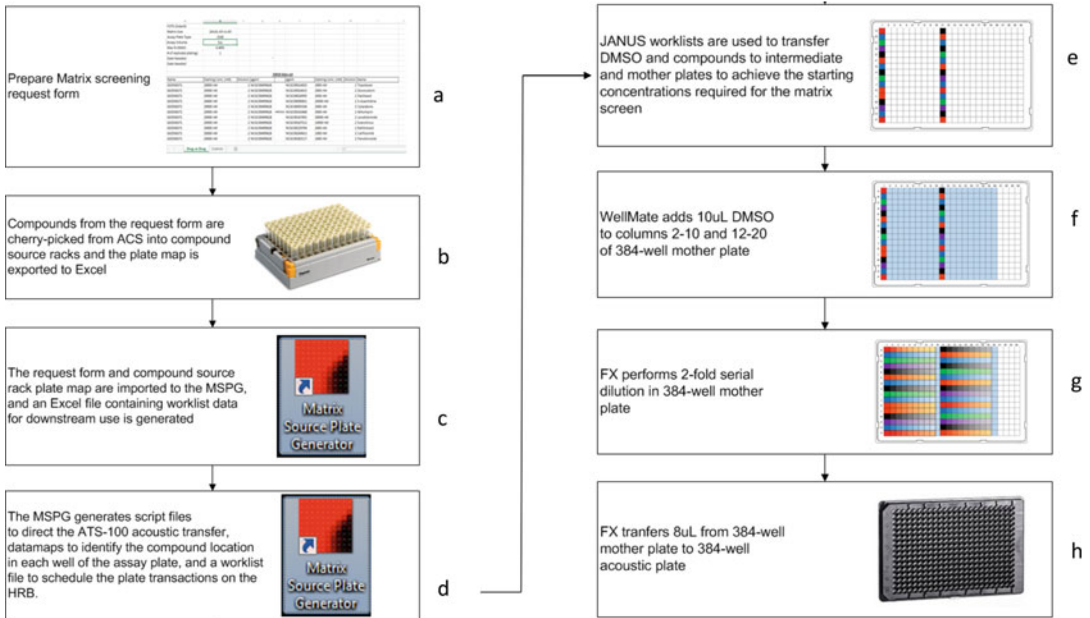

Fig. 1 An overview of the process to prepare an acoustic source plate and related files

A	B	C	D	E	F	G	H	I
FOTS OrderID								
Matrix Size	10x10; All vs All							
Assay Plate Type	1536							
Assay Volume	5uL							
Max % DMSO	0.40%							
# of replicate platings	1							
Date Needed								
Date Needed								
				10X10 data set				
Name	Starting Conc. (nM)	Dilution	agent		agent	Starting Conc. (nM)	Dilution	Name
G02938371	20000 nM	2	NCGC00499628		NCGC00014925	2000 nM	2	Topotecan
G02938371	20000 nM	2	NCGC00499628		NCGC00024415	2000 nM	2	Doxorubicin
G02938371	20000 nM	2	NCGC00499628		NCGC00024995	2000 nM	2	Paclitaxel
G02938371	20000 nM	2	NCGC00499628		NCGC00090851	20000 nM	2	5-Azacitidine
G02938371	20000 nM	2	NCGC00499628		NCGC00093356	2000 nM	2	Cytarabine
G02938371	20000 nM	2	NCGC00499628	versus	NCGC00163468	5000 nM	2	Mitomycin
G02938371	20000 nM	2	NCGC00499628		NCGC00167491	20000 nM	2	Lenalidomide
G02938371	20000 nM	2	NCGC00499628		NCGC00167512	10000 nM	2	Everolimus
G02938371	20000 nM	2	NCGC00499628		NCGC00229704	2000 nM	2	Raltitrexed
G02938371	20000 nM	2	NCGC00499628		NCGC00249613	1000 nM	2	Carfilzomib
G02938371	20000 nM	2	NCGC00499628		NCGC00263117	1000 nM	2	Panobinostat

Drug vs Drug | Controls

Fig. 2 An example of the matrix screening request form in Microsoft Excel format with the required information filled in. Each drug combination should be listed by row. Drug A should be listed with the name, starting concentration in the assay in nanomolar (nM), dilution factor of the drug in the screen, and internal compound ID. Drug B in the combination should be listed next with the same information. The researcher should also specify the size of the matrix block as well as information regarding the number of replicates needed and assay types used

3.3 Preparation of All Files Needed for Creation of the Acoustic Source Plate

The MSPG application will create the appropriate files needed for each critical instrument used in creation of the acoustic source plate. MSPG will take the submitted requestor form and create the JANUS worklist file to prepare the mother plates which is then transferred to acoustic source plates. It will also generate transfer script files that are used on the ATS-100 for acoustic dispensing, a worklist file used to schedule the movement of plates on the HRB, and a plate map of the assay plate which is used for data analysis (*see* **Note 2**).

1. Open the MSPG application, and click on Matrix Order which will open the "Import Compound Combinations Wizard" (Figs. 1c and 3).
2. Click Next to begin the Wizard. On the second window, select the "Browse" button, and select the Matrix screening request form (Fig. 2) containing all the drug combinations you wish to process. After selecting the file, a preview of the combinations will appear in the window as seen in Fig. 4. Click "Next" to advance to the next screen.
3. Use the drop-down menu to select the Excel worksheet tab that contains the compound pairs. For each Compound A and

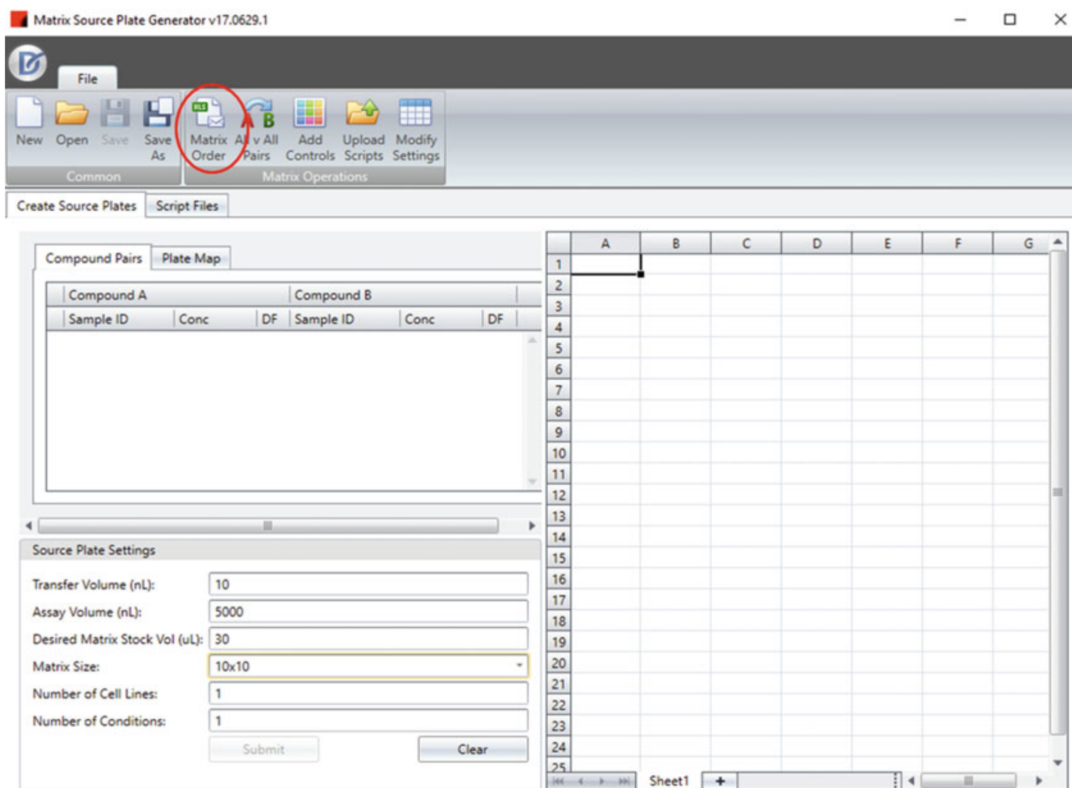

Fig. 3 Select the "Matrix Order" menu item to initiate the wizard that will walk you through the use of the tool, which will open the import compound combination wizards

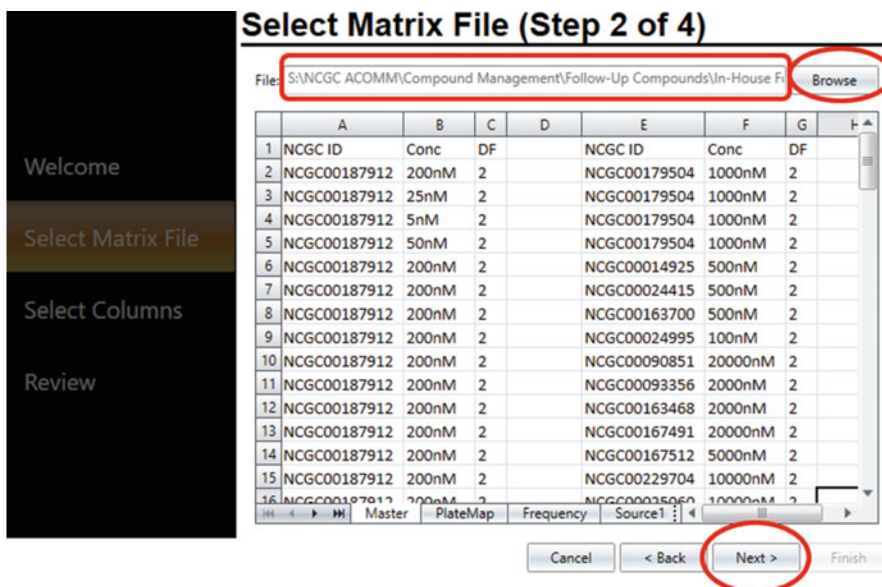

Fig. 4 Browse to and select the request form to display a preview of the desired matrix combinations

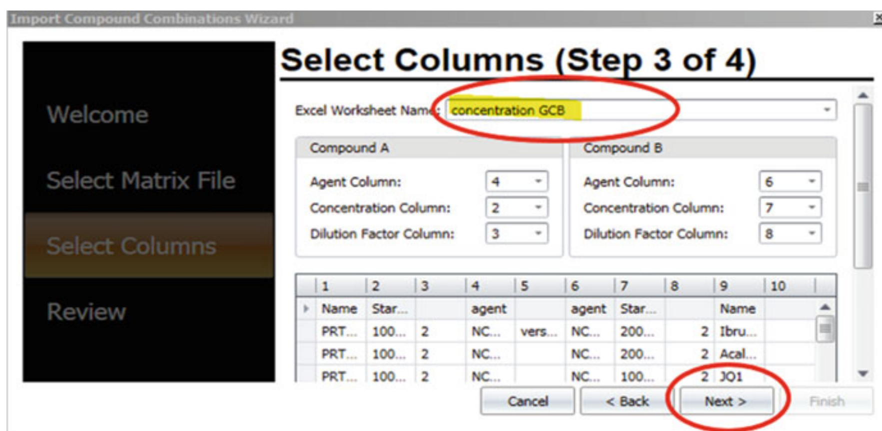

Fig. 5 Data fields from the matrix request form are associated with the variable fields in the MSPG

Compound B pair, use the drop-down menus to map the appropriate columns in the spreadsheet to the appropriate column headers named “Agent Column,” “Concentration Column,” and “Dilution Factor Column.”. Click “Next” (Fig. 5). Verify that the columns in the Review window have been mapped properly, and click Finish (Fig. 6). The software has now recorded the requested drug combinations to be made.

4. In the next window, input the assay parameters in the Source Plate Settings window (Fig. 7a). Copy the Compound source