Designing Drug-Response Experiments and Quantifying their Results

Marc Hafner,[1,2] Mario Niepel,[1,2] Kartik Subramanian,[1,2] and Peter K. Sorger[1]

[1]HMS LINCS Center, Laboratory of Systems Pharmacology, Department of Systems Biology, Harvard Medical School, Boston, Massachusetts
[2]These authors contributed equally to this publication

We developed a Python package to help in performing drug-response experiments at medium and high throughput and evaluating sensitivity metrics from the resulting data. In this article, we describe the steps involved in (1) generating files necessary for treating cells with the HP D300 drug dispenser, by pin transfer or by manual pipetting; (2) merging the data generated by high-throughput slide scanners, such as the Perkin Elmer Operetta, with treatment annotations; and (3) analyzing the results to obtain data normalized to untreated controls and sensitivity metrics such as IC_{50} or GR_{50}. These modules are available on GitHub and provide an automated pipeline for the design and analysis of high-throughput drug response experiments, that helps to prevent errors that can arise from manually processing large data files. © 2017 by John Wiley & Sons, Inc.

Keywords: experimental design • drug response • data processing • computational pipeline

How to cite this article:
Hafner, M., Niepel, M., Subramanian, K., & Sorger, P. K. (2017). Designing drug-response experiments and quantifying their results. *Current Protocols in Chemical Biology*, *9*, 96–116. doi: 10.1002/cpch.19

INTRODUCTION

Biological experiments are increasingly performed in high throughput and are becoming more reliant on automation. The emphasis in many high-throughput studies is on using the latest reagents and methods (e.g., different types of CRISPR genome editing) and ensuring the availability of state-of-the-art equipment (e.g., robotic plate handlers and screening platforms), while challenges in experimental design and data processing are often underemphasized. Current approaches rely on manual procedures and extensive human intervention such as manual entry or cut-and-paste operations in spreadsheets. These operations are not trackable and are susceptible to fragmentation, loss of metadata, and errors arising from forced formatting (which change numerical values and gene names into dates, for example; Zeeberg et al., 2004; Ziemann et al., 2016). When design and data are stored in disconnected spreadsheets, non-obvious mistakes lead to rapid accumulation of errors (Powell, Baker, & Lawson, 2008). With increasing attention to data reproducibility and public release, it has become apparent that manual data handling introduces errors at multiple levels and makes suboptimal use of the capabilities of automation.

While standards and tools are increasingly available for the management of data, reproducible and transparent science requires that these data be associated with metadata and linked to a machine-readable experimental design and protocol. Few tools exist for systematic creation, management, and logging of experimental designs (Stocker et al.,

Current Protocols in Chemical Biology 9:96–116, June 2017
Published online June 2017 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpch.19
Copyright © 2017 John Wiley & Sons, Inc.

2009; Wu & Zhou, 2014). The present article describes such a tool, with a specific focus on automating the design of drug-response experiments performed in multi-well plates. Such assays are sufficiently complex that they warrant dedicated tools, and they are paradigmatic of high-throughput experiments in general.

We (Saez-Rodriguez et al., 2008) and others (Stocker et al., 2009) have previously published tools that use graphical user interfaces (UIs) to assist in the layout and analysis of multi-well experiments. Over the years, we have found that UIs are very limiting in this context because they do not easily accommodate different experimental designs or research goals. We have therefore switched from UI-driven software to scripting tools that are run on the command line. This requires a bit more familiarity with programming, but we have found that the closer users are to the actual code, the less likely unintended errors are to occur. Moreover, interactive documents with embedded executable code, such as Jupyter notebooks, facilitate the use of simple executable functions and, once a script is implemented for a particular type of experiment, can be used repeatedly by individuals with little programming skill.

The tools we describe here are part of a Python package meant to systematize the design of the experiment and construct digital containers for the resulting metadata and data. Users download the packages and exemplar Jupyter notebooks from our GitHub repository (*https://github.com/datarail/datarail*) and then modify the scripts as needed to accommodate specific designs, which only requires minimal coding experience. As more experimental designs become available, it will be increasingly easy to find a notebook that is nearly ready to be used. For example, for a drug dose-response experiment, the user only needs to specify the cell type, drugs to be tested, and drug dose range. Digital designs benefit experimental scientists because they simplify and accelerate plate layouts, as well as computational biologists by making data analysis more rapid, transparent and error-free. This article is related to the companion article in *Current Protocols in Chemical Biology* (Niepel, Hafner, Chung, and Sorger, 2017), which describes experimental considerations in running drug-response experiments. While this article can be used by itself, ideally, both articles should be used in conjunction.

OVERVIEW OF THE METHOD

The following operations are described below: (1) laying out samples and doses across one or more multi-well plates, such that the design document can be used to guide either a human or, preferably, a robot, (2) gathering results from high-throughput instruments and merging them with the underlying metadata in a single data container, and (3) extracting drug-response metrics from the experimental results (Fig. 1). The response of cells to drugs is processed using the normalized growth rate inhibition (GR) method, which corrects for the effects of cell division time on the estimation of drug sensitivity and allows an estimation of time-dependent drug sensitivity (Hafner, Niepel, Chung, & Sorger, 2016). In addition to avoiding errors in the design and processing of experiments, our pipeline can fetch references from on-line databases to assist with reagent annotation (including PubChem annotation, internal IDs, and batch numbers) and perform quality control of results. The whole process is written in a documented script that becomes *de facto* a "literate" (human and machine-readable) archive of the overall experiment, including the design, results, and quality controls.

The pipeline comprises two flexible and modular open-source Python packages (*https://github.com/datarail/datarail*; *https://github.com/datarail/gr_metrics*) that can be used for end-to-end processing of drug-response experiments or for automating individual steps of such experiments (such as designing the plate layout). The approach assumes a standardized file format and set of keywords (such as `treatment_duration`,

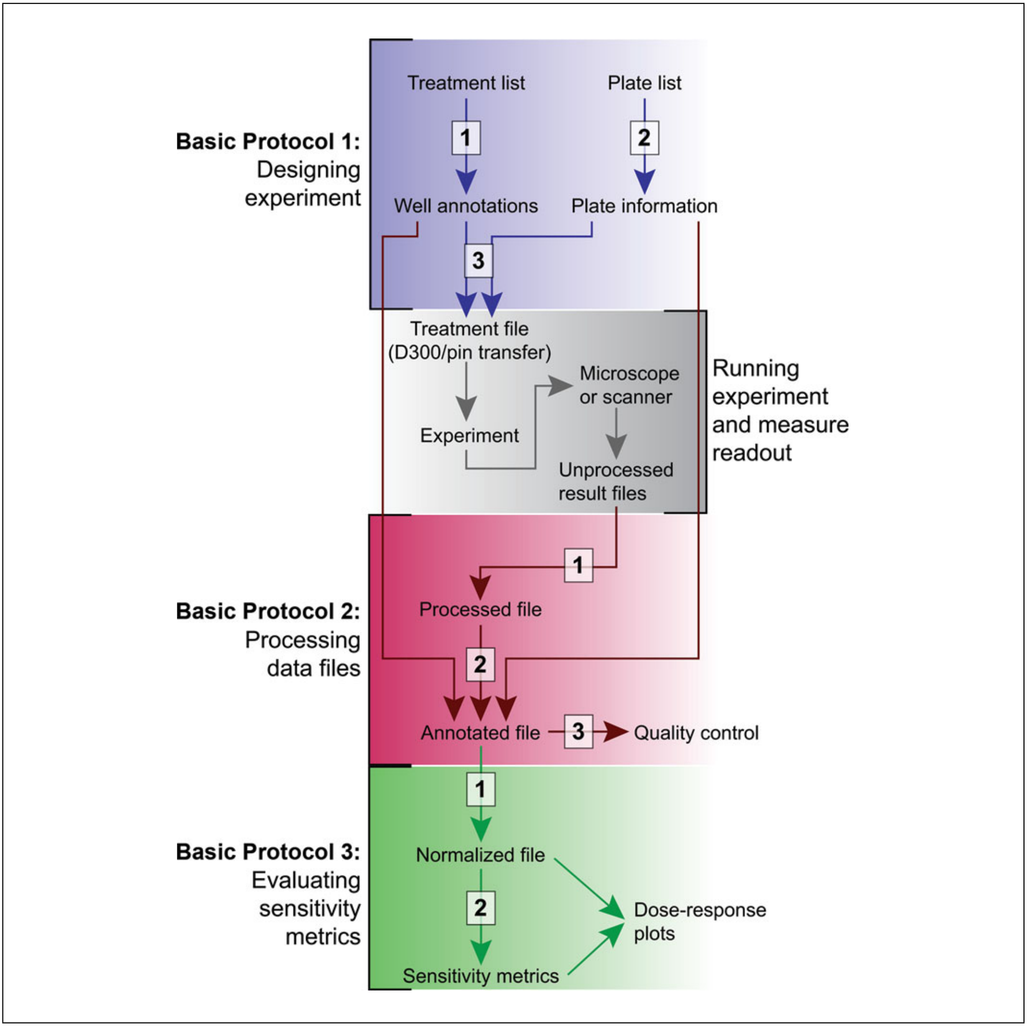


Figure 1 Pipeline for experimental design and analysis. The pipeline described in the manuscript comprises three protocols (colored sections), each with 2 to 3 steps (white boxes). The `datarail` package is used in Basic Protocols 1 and 2. Basic Protocol 3 relies on the `gr_metrics` package.

`cell_count`, `plate_identifier`, `well`, or `concentration`) that are used by the system to automate data processing. Data can be stored in long tabular representations as tab-separated values (tsv) files, or alternatively as more powerful hierarchical data format (HDF5) files. The main application is systematic high-throughput experiments, but the modular implementation makes the pipeline useful for simpler, low-throughput experiments that do not require interfacing with robotic arms or more complex multifactorial experiments for which the low-level functions can be used to generate and store the designs. The modules will continue to evolve as we include new functionality and add contributions from others.

Basic Protocol 1 deals with all steps necessary to set up a multi-well drug-response experiment. Basic Protocol 2 describes steps for processing data once an experiment is concluded. Basic Protocol 3 handles the analysis and parameterization of drug-response data. We typically use all three protocols in sequence, but individual protocols can be used in combination with personalized scripts. All three protocols can be modified to accommodate other types of studies in which cells are treated with exogenous ligands or small molecules, and their state is then measured in plate-based assays. We envision that future iterations of this pipeline will be able to support additional experimental assays and data structures.

STRATEGIC PLANNING

Types of Variables

The protocols in this article distinguish among three classes of variables: *model variables*, *confounder variables*, and *readout variables.*

Model variables describe aspects of the experiment that are explicitly changed as part of the experimental design and are expected to affect the values of the readout variables. In the context of drug-response assays, we separate *model variables* into (i) *treatment variables* that generally refer to agents that perturb cell state, such as drugs or biological ligands and their concentrations; and (ii) *condition variables* that refer to the states of the system at the start of the experiment such as seeding density, serum concentration, or oxygen level, as well as the experiment duration (Fig. 2). The distinction between *treatment* and *condition variables* is exploited for normalizing readout values. For example, in a multi-factorial design in which the effect of seeding density on drug response

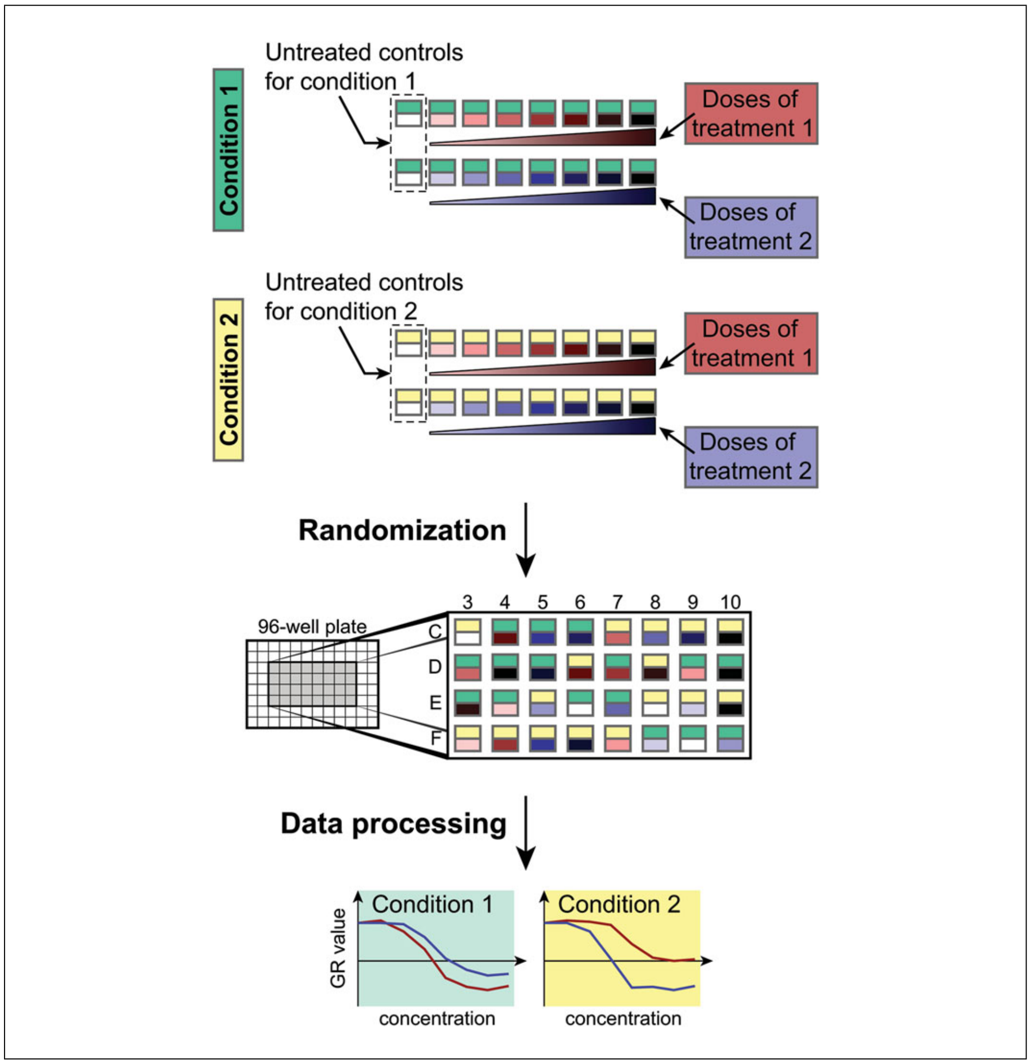


Figure 2 Illustration of an experimental design including different conditions and treatments. Serial dilutions are measured for two treatments in two different conditions. The provided scripts help to design the treatments and randomize the conditions for avoiding bias during the experiment. Data processing scripts normalize the different treatments to condition-specific untreated controls and yield the dose-response curves. In the "Randomization" panel, for clarity, only the center wells of a 96-well plate are depicted to illustrate the random distribution of experimental conditions across an assay plate.

is being examined, dose-response curves for different drugs (*treatment variable*) can be compared at the multiple seeding densities (*condition variable*).

Confounder variables are implicit variables that are not intended to affect readouts, but that are recorded to fully document the experiment. These can be media batch number, drug supplier, or assay date. It is frequently observed *post facto* that assays depend on the value of a particular *confounder variable*; in this case, forensic analysis might involve a new experiment in which the confounder becomes an explicitly controlled *model variable*. Among *confounder variables*, the plate identifier (ID number or barcode) and well number are of particular importance in performing quality-control and matching *readout* and *model* variables.

Readout variables are values measured while the experiment is underway or when it ends. They constitute the data being collected by the experimentalist; cell number and fraction of viable or apoptotic cells are typical readout variables for an experiment with anti-cancer drugs. However, readout variables can also be more complex: multi-channel microscope images in the case of high-content experiments, for example. A set of readout variables is typically collected from each well in a multi-well plate. For simplicity, the protocols described below use viable cell number, or a surrogate such as ATP level measured by CellTiter-Glo (Promega), as the readout variable.

Jupyter Notebooks and File Structure

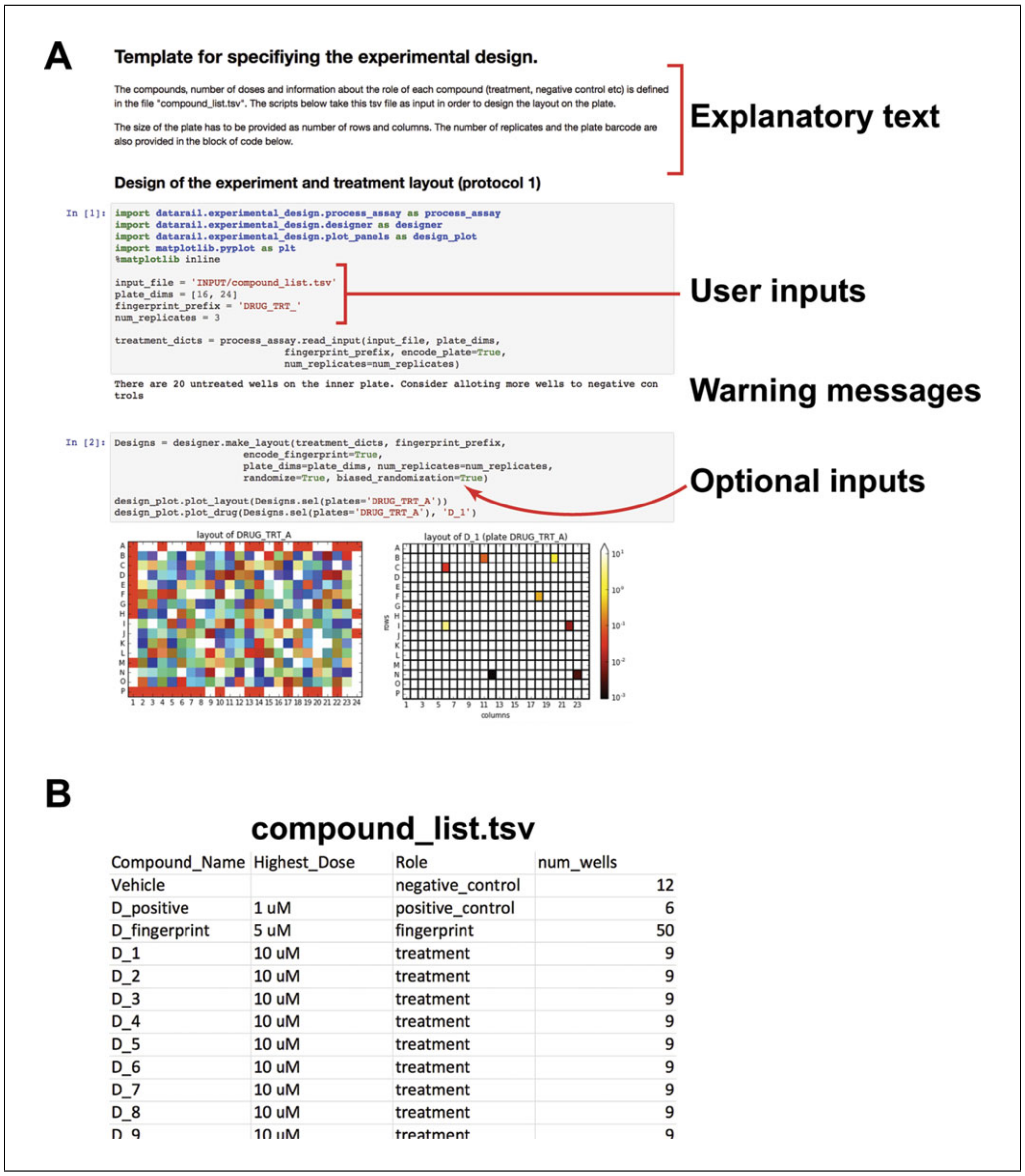
Proper documentation of the details and rationale behind the design of an experiment is a necessity for reproducibility and transparency. We use Jupyter, which is a Web application that allows snippets of code, explanatory text, and figures to be contained within a single page, called a notebook. We provide templates for commonly used experimental designs to assist in notebook creation. *Experimental design notebooks* define all *model* and *confounder* variables of an experiment such as the layout of drugs, doses, and cell lines on 384-well plates (Fig. 3), whereas *processing notebooks* comprise the commands used to process and analyze the data of an experiment (Fig. 4). Recording these commands in a notebook makes it possible to establish the full provenance of each piece of data.

For large-scale experiments involving multiple collaborators, it is important to establish a clear file and folder structure for the project so that all investigators can work together efficiently with a minimum of confusion. We suggest creating for each project a set of folders entitled:

1. `SRC` (conventional name for folders containing source code) that contains all Juypter notebooks or scripts.
2. `INPUT` that stores data and metadata files read by Juypter notebooks or scripts. These files can be created manually by recording measurements or, preferably, generated by assay instruments. In either case, they should never be modified, to ensure data integrity.
3. `OUTPUT` that contains all files generated by the Jupyter *processing notebook* or scripts; like the files in the INPUT folder, these should not be manually modified.

Experimental Design

A major consideration in designing a multi-plate experiment is the number of 96- or 384-well plates that will be used, as this determines the maximum number of samples. The experimental design constitutes the way in which treatments and controls are split among the available number of samples and mapped to each plate. Obtaining reliable results depends on using replicates, including sufficient untreated controls and, in the case of dose-response experiments, assaying an adequate dose range at an effective step size. The following considerations impact the final design:



Template for specifiying the experimental design.

The compounds, number of doses and information about the role of each compound (treatment, negative control etc) is defined in the file "compound_list.tsv". The scripts below take this tsv file as input in order to design the layout on the plate.

The size of the plate has to be provided as number of rows and columns. The number of replicates and the plate barcode are also provided in the block of code below.

Design of the experiment and treatment layout (protocol 1)

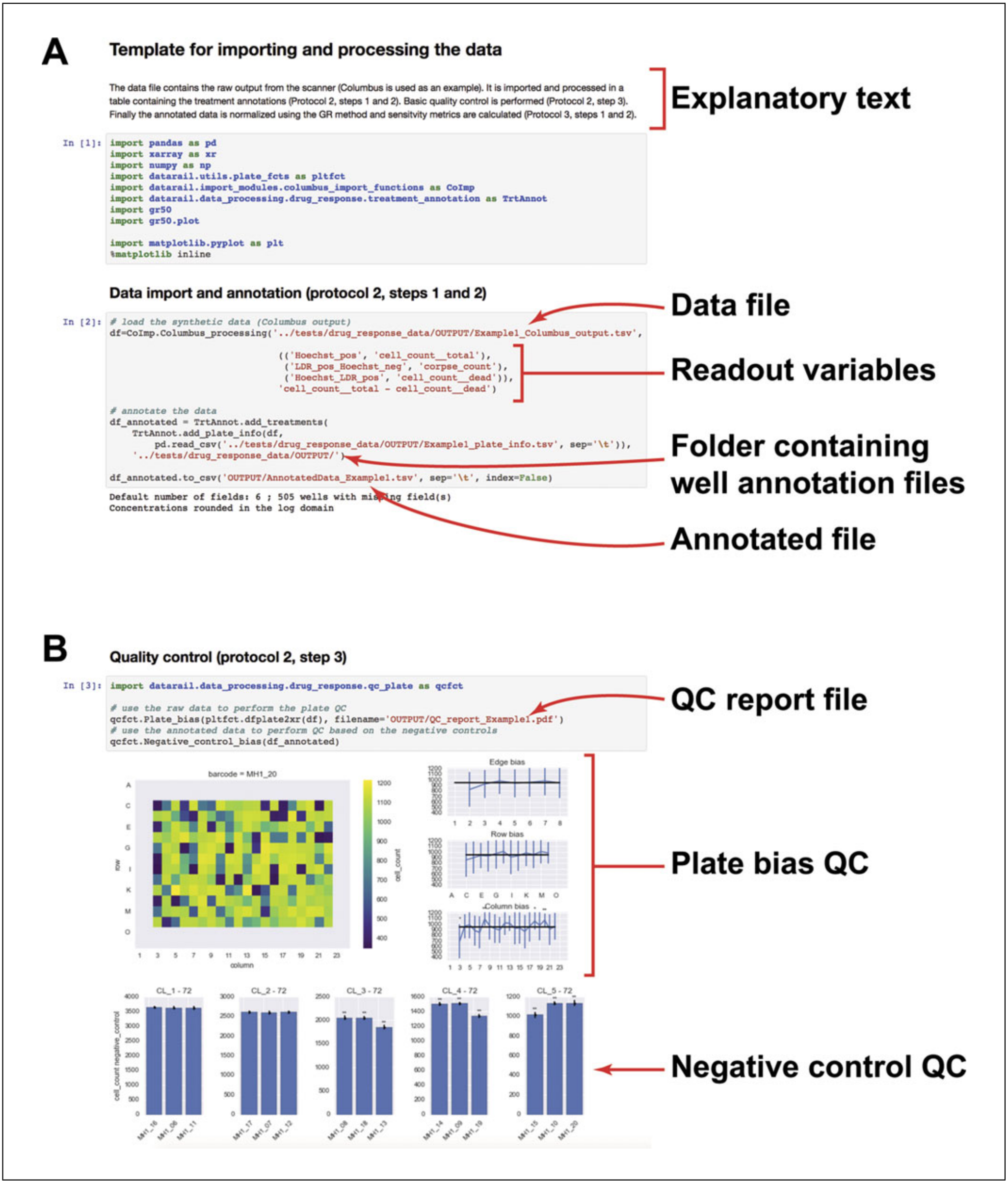
```
In [1]: import datarail.experimental_design.process_assay as process_assay
        import datarail.experimental_design.designer as designer
        import datarail.experimental_design.plot_panels as design_plot
        import matplotlib.pyplot as plt
        %matplotlib inline

        input_file = 'INPUT/compound_list.tsv'
        plate_dims = [16, 24]
        fingerprint_prefix = 'DRUG_TRT_'
        num_replicates = 3

        treatment_dicts = process_assay.read_input(input_file, plate_dims,
                                                   fingerprint_prefix, encode_plate=True,
                                                   num_replicates=num_replicates)

        There are 20 untreated wells on the inner plate. Consider alloting more wells to negative con
        trols

In [2]: Designs = designer.make_layout(treatment_dicts, fingerprint_prefix,
                                       encode_fingerprint=True,
                                       plate_dims=plate_dims, num_replicates=num_replicates,
                                       randomize=True, biased_randomization=True)

        design_plot.plot_layout(Designs.sel(plates='DRUG_TRT_A'))
        design_plot.plot_drug(Designs.sel(plates='DRUG_TRT_A'), 'D_1')
```

compound_list.tsv

Compound_Name	Highest_Dose	Role	num_wells
Vehicle		negative_control	12
D_positive	1 uM	positive_control	6
D_fingerprint	5 uM	fingerprint	50
D_1	10 uM	treatment	9
D_2	10 uM	treatment	9
D_3	10 uM	treatment	9
D_4	10 uM	treatment	9
D_5	10 uM	treatment	9
D_6	10 uM	treatment	9
D_7	10 uM	treatment	9
D_8	10 uM	treatment	9
D_9	10 uM	treatment	9

Figure 3 Exemplar Jupyter notebook for the experimental design (Basic Protocol 1). (**A**) Exemplar *experimental design notebook*: The Jupyter notebook allows computer code (gray-shaded block) and text to be contained in the same document. The user can add or modify the explanatory text portions to describe the rationale of the experiment. In order to generate the experimental design, the user has only to modify parts of the code that specify the input filename, the dimensions and fingerprint of the plate, and the number of replicates in the experiment. The *experimental design notebook* also provides warning messages if the number of wells for treatments and controls is erroneous or suboptimal. (**B**) User-created tsv file that lists the name and role of compounds to be used in the experiment. The `num_wells` column defines the number of doses for compounds that are treatments, or the number of wells reserved for compounds that serve as controls.

- For cell lines and experimental setup in which strong edge effects have been observed, we recommend avoiding using the outermost wells. Potential causes and solutions for edge effects have been discussed elsewhere (Lundholt, Scudder, & Pagliaro, 2003; also see Internet Resources for helpful hints on how to manage edge effects), and are also covered in the accompanying article to this one in *Current Protocols in Chemical Biology* (Niepel et al., 2017). If enough wells are available on each plate to accommodate the experimental design, the scripts we provide automatically avoid using wells at the edge of the plate.


A

Template for importing and processing the data

The data file contains the raw output from the scanner (Columbus is used as an example). It is imported and processed in a table containing the treatment annotations (Protocol 2, steps 1 and 2). Basic quality control is performed (Protocol 2, step 3). Finally the annotated data is normalized using the GR method and sensitvity metrics are calculated (Protocol 3, steps 1 and 2).

```
In [1]: import pandas as pd
        import xarray as xr
        import numpy as np
        import datarail.utils.plate_fcts as pltfct
        import datarail.import_modules.columbus_import_functions as CoImp
        import datarail.data_processing.drug_response.treatment_annotation as TrtAnnot
        import gr50
        import gr50.plot

        import matplotlib.pyplot as plt
        %matplotlib inline
```

Data import and annotation (protocol 2, steps 1 and 2)

```
In [2]: # load the synthetic data (Columbus output)
        df=CoImp.Columbus_processing('../tests/drug_response_data/OUTPUT/Example1_Columbus_output.tsv',

                                      (('Hoechst_pos', 'cell_count__total'),
                                       ('LDR_pos_Hoechst_neg', 'corpse_count'),
                                       ('Hoechst_LDR_pos', 'cell_count__dead')),
                                      'cell_count__total - cell_count__dead')

        # annotate the data
        df_annotated = TrtAnnot.add_treatments(
            TrtAnnot.add_plate_info(df,
                pd.read_csv('../tests/drug_response_data/OUTPUT/Example1_plate_info.tsv', sep='\t')),
            '../tests/drug_response_data/OUTPUT/')

        df_annotated.to_csv('OUTPUT/AnnotatedData_Example1.tsv', sep='\t', index=False)
        Default number of fields: 6 ; 505 wells with missing field(s)
        Concentrations rounded in the log domain
```

B

Quality control (protocol 2, step 3)

```
In [3]: import datarail.data_processing.drug_response.qc_plate as qcfct

        # use the raw data to perform the plate QC
        qcfct.Plate_bias(pltfct.dfplate2xr(df), filename='OUTPUT/QC_report_Example1.pdf')
        # use the annotated data to perform QC based on the negative controls
        qcfct.Negative_control_bias(df_annotated)
```

Figure 4 Exemplar Jupyter notebook for processing the data (Basic Protocol 2). (**A**) Data output by Columbus are imported (Basic Protocol 2, step 1) in a dataframe. Function input allows selection of the desired readouts and labels them with a standard name (e.g., `cell_count`). Data are then merged with the design (Basic Protocol 2, step 2). (**B**) Different quality controls can be performed on the plate (Basic Protocol 2, step 3): bias across the plate can be quantified by distance from the edge, row, or column; difference in the untreated controls can be quantified across plates to test for bias.

- Multiple untreated (or vehicle-treated) controls should be included on each plate. If outermost wells are used, we recommend at least six controls on the edges and six in the center of the plate to test if edge effects occurred. The scripts we provide will position controls in this manner by default and randomly assign any remaining untreated wells across the plate.
- For simple dose-response experiments, we recommend at least three technical replicates. For experiments that aim to identify small differences in GR value (less than 0.2) between treatments or conditions, more technical replicates are generally necessary to properly assess significance. More replicates are also necessary to precisely assess time-dependent GR values, especially for short time intervals or for cell lines growing slowly. We generally recommend six replicates if measurements are made

four times per division, which corresponds to a population growth of about 20% between measurements.

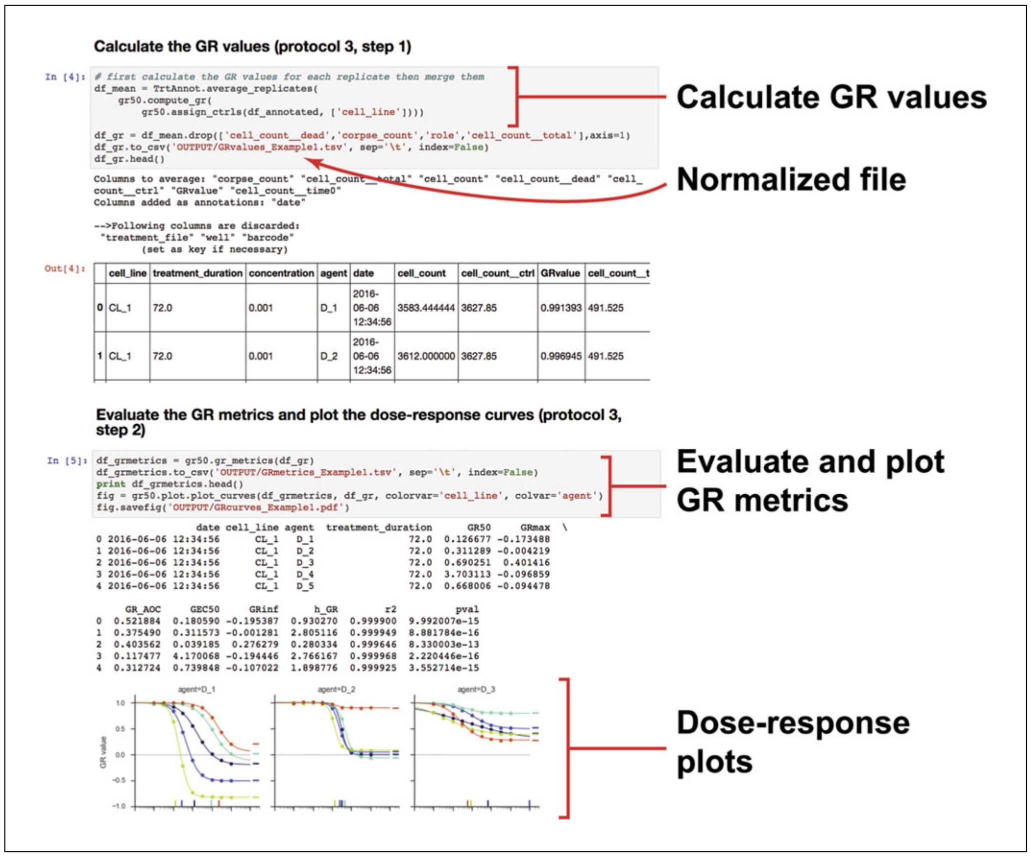
- Technical replicates should be spread across multiple plates using different randomization schemes. Default randomization schemes are arranged so that a particular condition (e.g., drug/concentration pair) is not assigned to the same region of the plate across replicates.
- For dose-response experiments in which the likely range of cellular sensitivity is unknown, or for drug master plates that will be used to treat multiple cell lines having differing sensitivities, we recommend using a wide range of drug concentrations. In experiments exploring the responses of cancer cells to small-molecule drugs, we typically use nine doses separated by 3.16-fold and spanning 1 nM to 10 μM (or from 0.1 nM to 1 μM for compounds that are known to be potent).
- For evaluating drug response using GR values and metrics, the number of cells present at the time of treatment must be measured. We recommend that an untreated plate, prepared in parallel to the plates to be treated, be fixed at the time the other plates are being exposed to drugs.
- For evaluating time-dependent GR values and metrics, the number of cells must be measured at multiple time points. This can be done either as a time course where samples are measured multiple times in a non-destructive manner, or with multiple plates fixed at different time points.
- Further experimental parameters such as cell plating, recovery time, and control treatments are discussed in an accompanying article (Niepel et al., 2017) and should be determined prior to generating treatment files.

The Value of Automation

Assays in multi-well plates can be performed using a wide variety of instruments, including multi-channel pipettors, but even experienced bench scientists are not particularly good at accurately dispensing dilutions across multiple plates. This is particularly true in the case of 384-well plates, because of tight well spacing and small volumes. We therefore strongly advocate the use of automated cell counters, plate fillers, compound dispensers, and plate washers. The Hewlett-Packard D300 Digital Dispenser is particularly valuable for dispensing small volumes of treatment agents and, when combined with the scripts described here, allows samples and controls to be arranged randomly across a plate so that successive doses are not always next to each other. This helps to reduce systematic error arising from edge effects or gradients in cell adherence or growth. All automation instruments that we suggest in this article fit on a benchtop, are simple to operate, and are highly recommended for any laboratory or organization wishing to assay the responses of mammalian cells to perturbation on a regular basis.

OVERVIEW OF THE SOFTWARE

Basic Protocols 1 to 3 describe software routines for specifying the experimental design, processing the readout, and analyzing the results (Fig. 1). The two packages used in this article are `datarail` and `gr_metrics`, which are both open source and can be downloaded directly from our GitHub repositories (*https://github.com/datarail/datarail*; *https://github.com/datarail/gr_metrics*). All functions in these packages are written in Python and require the following Python packages: `numpy`, `scipy`, `sklearn`, `pandas`, `matplotlib`, and `xarray`. Submodules in both packages can be used independently of the pipeline, and each step can be substituted by a customized function. All inputs and outputs are tsv or HDF5 files and handled internally as `pandas` tables and `xarray` datasets. This duality allows more flexibility in interfacing with other scripts or programmatic languages.



Calculate the GR values (protocol 3, step 1)

```
In [4]: # first calculate the GR values for each replicate then merge them
        df_mean = TrtAnnot.average_replicates(
            gr50.compute_gr(
                gr50.assign_ctrls(df_annotated, ['cell_line'])))

        df_gr = df_mean.drop(['cell_count__dead','corpse_count','role','cell_count__total'],axis=1)
        df_gr.to_csv('OUTPUT/GRvalues_Example1.tsv', sep='\t', index=False)
        df_gr.head()

        Columns to average: "corpse_count" "cell_count__total" "cell_count" "cell_count__dead" "cell_
        count__ctrl" "GRvalue" "cell_count__time0"
        Columns added as annotations: "date"

        -->Following columns are discarded:
         "treatment_file" "well" "barcode"
               (set as key if necessary)
```

Out[4]:

	cell_line	treatment_duration	concentration	agent	date	cell_count	cell_count__ctrl	GRvalue	cell_count__t
0	CL_1	72.0	0.001	D_1	2016-06-06 12:34:56	3583.444444	3627.85	0.991393	491.525
1	CL_1	72.0	0.001	D_2	2016-06-06 12:34:56	3612.000000	3627.85	0.996945	491.525

Evaluate the GR metrics and plot the dose-response curves (protocol 3, step 2)

```
In [5]: df_grmetrics = gr50.gr_metrics(df_gr)
        df_grmetrics.to_csv('OUTPUT/GRmetrics_Example1.tsv', sep='\t', index=False)
        print df_grmetrics.head()
        fig = gr50.plot.plot_curves(df_grmetrics, df_gr, colorvar='cell_line', colvar='agent')
        fig.savefig('OUTPUT/GRcurves_Example1.pdf')

                          date cell_line agent  treatment_duration      GR50     GRmax  \
        0 2016-06-06 12:34:56      CL_1   D_1                 72.0  0.126677 -0.173488
        1 2016-06-06 12:34:56      CL_1   D_2                 72.0  0.311289 -0.004219
        2 2016-06-06 12:34:56      CL_1   D_3                 72.0  0.690251  0.401416
        3 2016-06-06 12:34:56      CL_1   D_4                 72.0  3.703113 -0.096859
        4 2016-06-06 12:34:56      CL_1   D_5                 72.0  0.668006 -0.094478

             GR_AOC     GEC50     GRinf      h_GR        r2          pval
        0  0.521884  0.180590 -0.195387  0.930270  0.999900  9.992007e-15
        1  0.375490  0.311573 -0.001281  2.805116  0.999949  8.881784e-16
        2  0.403562  0.039185  0.276279  0.280334  0.999646  8.330003e-13
        3  0.117477  4.170068 -0.194446  2.766167  0.999968  2.220446e-16
        4  0.312724  0.739848 -0.107022  1.898776  0.999925  3.552714e-15
```

Figure 5 Exemplar Jupyter notebook for evaluating sensitivity metrics (Basic Protocol 3). GR values and metrics are calculated using the *gr_metrics* module (Basic Protocol 3, steps 1 and 2). Dose response curves can be plotted and exported as a pdf file.

As mentioned above, we recommend using Jupyter notebooks (*http://jupyter.org/*) to describe and generate the experiment design as well as to document and execute the data processing (Figs. 3 to 5). In addition to the blocks of code that execute the commands (gray blocks in Figs. 3 to 5), the *experimental design* notebook should include the background, rationale, and assumptions of the experiment as explanatory text (white blocks in Figs. 3 to 5). The *processing* notebook may include an amended version of the protocol to account for any deviation from the intended plan, as well as code implementing quality control or data filtering. For data fidelity, it is important to keep the raw data file untouched and filter or correct data only in a programmatic manner while recording all operations in the notebook.

BASIC PROTOCOL 1

DESIGNING THE EXPERIMENT

Basic Protocol 1 covers experimental design and the generation of files needed to control the robots that dispense treatments. Upon completion of Basic Protocol 1, the user will have:

- One *Jupyter* notebook that serves as an archive of the design
- Several *well annotation files* describing well-based treatments on a plate basis
- One *plate information file* listing all plates in the experiments and mapping them to treatments
- One *treatment file* describing the pin transfer procedure or specifying the actions of the drug dispenser

Table 1 Names and Descriptions for Typical Model Variables

Variable name	Description
`cell_line`	Cell line identity
`agent`	Name of agent used to treat cells; e.g., drug name, ligand name
`agent__concentration`	Concentration of the agent (μM)
`agent2`	Name of the second treatment used in a combination
`agent2__concentration`	Concentration of the second agent (μM)
`seeding_number`	Number of cells seeded in a well
`serum_pct`	Percent of serum in the medium
`media`	Type of medium

File Types

We split the description of the *model variables* for a specific experiment into two types of files: *well annotation files* and *plate information file*s. *Well annotation files* define the layout of treatments on a plate basis. These files are long tables with one row per treated well and as wide as necessary to describe the treatment, with columns such as `agent` or `agent_concentration` (see Table 1 for suggested column headers). The second type of file is the *plate information file*, which associates physical plates based on their plate identifier to a specific *well annotation file*. The *plate information file* also contains all the plate-related annotations, such as `treatment_time` or `treatment_duration`. Model variables such as `cell_line` or `seeding_density` are recorded either in *well annotation files*, if they differ from well to well, or in the *plate information file* if they are constant across a plate. Note that this distinction enhances the clarity of the experimental design, but will not change subsequent analysis. The only hard constraints in the structure of these files are as follows:

- A *well annotation file* needs to have a column whose header is `well` and that contains the names of each treated well (in conventional alphanumeric plus numerical core, e.g., B04, F12). *Well annotation* files can be created manually, but we strongly recommend scripting this process to avoid errors in parsing names; example scripts are provided in the datarail GitHub repository (*https://github.com/datarail/datarail*).
- A *plate information file* needs to have (1) a column `plate_identifier` containing plate identifiers (barcodes or ID numbers) as they will be reported in the data file (see Basic Protocol 2); (2) a column `well_file` with the name of a well annotation file (including the appropriate extension such as `.tsv`) or, for untreated plates, an empty field or a dash; and (3), for end-point experiments, a column `treatment_duration` with the duration of the treatment; a value 0 is used for untreated plates fixed at the time of treatment, or for time-course experiments, a column `treatment_time` with the date and time of treatment.

A *treatment file* is generated in the final step of Basic Protocol 1 by merging the two types of files described above into a single document that drives a drug dispenser such as the D300, maps drugs for generating a master plate(s) for pin transfer, or, less desirably, is used to guide manual pipetting.

The `datarail` GitHub repository includes exemplar *experimental design notebook*s that can be adapted to run specific experiments (Fig. 3A). These notebooks are executable and generate the necessary files but also serve as a record of the experimental design. Based on the functions provided in the `datarail` package, advanced users can write a Python script to generate more complicated designs. Users should explicitly set the seed for the random number generator such that the randomization can be reproduced.

Enforcing standardized names and unique identifiers in design files is necessary for merging datasets collected at different times or by different investigators and prevents mismatches between protocols and actual experiments. We provide a function to check the names of reagents in *experimental design notebook*s against a reference database and to add unique identifiers (see the function help for more details on the available reference databases).

Materials

The module `experimental_design` of the `datarail` Python package (*https://github.com/datarail/datarail*) containing:
- Functions to generate various experimental designs such as single-drug dose-response or pairwise drug combinations across multiple concentrations
- A function to randomize the positions of the treatments
- Functions to plot the experimental design for error checking, troubleshooting, and subsequent data interpretation
- Functions to export designs as either tsv files for manual pipetting and pin transfer or hpdd files (xml format) to drive the D300 drug dispenser

List of treatments including:
- Treatment reagent names and any associated identifiers
- Concentrations for each agent (μM by default)
- Stock concentration for each agent (mM by default)
- Vehicle for dissolution of treatment agent (typically DMSO for small molecules)

Information about plates
- List of plate identifiers (barcodes or ID numbers)
- Any plate-based *model variable* that should be propagated to the downstream analysis (e.g., cell line, treatment duration)
- Any plate-based *confounder variable* (e.g., plate model and manufacturer, treatment date)

Jupyter notebook (or Python script) called *experimental design notebook* that will serve as a record of the experimental design. Exemplar notebooks are provided on the `datarail` GitHub repository (*https://github.com/datarail/datarail*).

An external reference database if the user wants to include links to reagent identifiers stored externally (e.g., PubChem, *https://pubchem.ncbi.nlm.nih.gov*; or LINCS, *http://lincs.hms.harvard.edu/db/*)

1. Creating well annotation files.

   a. Select an exemplar experimental design notebook for the desired experiment such as multiple dose-response curves (Fig. 3A) or pairwise drug combinations. In the case of a dose-response experiment, create a tsv file with the list of drugs, their highest tested concentration, and the number of doses in the dilution series and their role in the experiment (Fig. 3B).
   b. Record the treatment conditions and describe the properties of the reagents in the experimental design notebook.
   c. Select the number of randomized replicates. We typically perform three replicates for drug dose-response studies.
   d. Save the treatments as *well annotation files* (in a tsv file or xarray object).

   Designs can be visualized as images using plotting functions in the module `experimental_design`.

   Alternatively, advanced users can design the treatments using the functions in the module `experimental_design`. *These functions can:*

   Generate single agent dose response.

   Design pairwise combination treatments.

Specify well-based conditions.

Generate multiple randomizations of the treatments.

Stack group of wells with defined pattern to generate full plates.

2. Creating plate information files.

   a. Create a *plate information file* (tsv format) either manually or through the *experimental design script* (recommended). The *plate description file* is a table containing the following columns:

      `plate_identifier`, matching the plate identifier (barcode or ID number) that will be reported in the scanner output file.

      `well_file`, that contains the name of a well annotation file used to treat a given plate, or a dash (−) if the plate is not to be exposed to treatments.

      `treatment_duration`, duration of the treatment in hours. The untreated plates fixed at the time of treatment should have a value 0.

      Any plate-level model or confounder variables that will be used for downstream analysis such as (see Table 1):

      Identity of the cell line (if not specified in the well annotation file).
      Conditions not specified in the well annotation file (e.g., describing CO_2 levels or oxygen tension under which cells were cultured).
      Other metadata and *confounder variables* such as day and time of plating, plate manufacturer, or batch number.

3. Creating the treatment files.

   a. Create the *treatment files* for the D300 drug dispenser (hpdd format) using the functions in the module `experimental_design` to merge the *treatment design files* and the *plate description file*.
   b. Create the mapping for master plate dispensing using the functions in the module `experimental_design` to generate the mapping for transferring drugs from a source plate into master plates. This function takes as inputs the well annotation files and the layout of the treatments to be dispensed. The output will be a two-column table mapping wells from the source plate to the destination plate.

PROCESSING DATA FILES

BASIC PROTOCOL 2

Upon completion of Basic Protocol 2, a user will have:

- One Jupyter notebook that serves as an record of the data processing procedures
- One result file that comprises model and readout variables for all measurements in the experiment
- Quality control reports

Introduction

This protocol describes how to process the data file containing the number of viable cells for each well into a standard table file annotated with the model variables of the experiment. A variety of instruments (which we refer to as “scanners”) can be used to collect this data, ranging from a plate scanner (for well-average values generated by CellTiter-Glo assays) to high-content microscopes. We have written import modules for files that are generated by Columbus Software (PerkinElmer) and IncuCyte ZOOM in-incubator microscopes (Essen Bioscience), but the output from any image-processing software can be converted into a standardized table, called a *processed file*, which can be passed to downstream analysis functions.

The data processing procedure is recorded in a *data processing notebook*. This notebook can also include any alteration in the original experimental design to account for potential issues that occurred during performance of the experiment, such as failed wells, errors in agent concentrations, etc. For experiments in which data files need to be split or merged, we strongly recommend scripting the procedure in the Jupyter notebook instead of manually handling result files (Fig. 4A). We provide exemplar Jupyter notebooks in the GitHub repository (*https://github.com/datarail/datarail*).

File Types

Basic Protocol 2 converts the output file from the scanner or microscope, called *unprocessed result file*, into a standardized file called *processed file*. The latter is merged with files created in Basic Protocol 1 (well annotation files and plate information file) to generate the *annotated file*, which contains all model and readout variables from the experiment. The quality-control step generates reports in the form of pdf files.

Materials

The following modules of the `datarail` python package (*https://github.com/datarail/datarail*):
- `import_modules`: functions to import and convert the output data file of a particular scanner into a processed file with standardized format
- `data_processing.drug_response`: functions to merge the results and the design files into a single annotated result file

Output data from the scanner saved as a tsv file (other formats will require writing new import functions)

The plate description file and well annotation files generated in Basic Protocol 1 (or equivalent files generated manually or through other means)

Jupyter notebook (or Python script) called *data processing notebook* that will serve as a record of the data processing. Exemplar notebooks are provided on the `datarail` GitHub repository (*https://github.com/datarail/datarail*)

1. Converting the scanner specific data file into a processed file.

   This step converts the scanner-specific data file into a processed file containing: (1) the column `cell_count`*, which is the readout of interest; (2) the columns* `plate_identifier` *(barcodes or ID numbers) and* `well` *(e.g., B03, F12), which uniquely define the sample; and (3), for time-course experiments, the column time, which contains the scanning timestamp. Additional readout values such as* `cell_count__dead` *or* `cell_count__dividing` *can be included based on the readout of the scanner. We have written import modules for files output by Columbus Software and IncuCyte microscopes, but the output from any scanner with a non-proprietary file format can be converted into a processed file.*

   a. Use the functions in the module `import_modules` to import the file output by the scanner. Each scanner-specific function can include processing steps to correct for artifacts. For example, the function to convert the file from Columbus will correct for wells in which fewer fields were analyzed due to scanning errors.

   b. Change the column headers or perform the necessary arithmetic operation to obtain the number of viable cells (or a related measure), labeled as `cell_count` (see accompanying article (Niepel et al., 2017), and exemplar notebooks: *https://github.com/datarail/datarail*). This can be done automatically by passing the right arguments to the import function of step 1a.

c. If necessary, script the operations needed to correct for failure of the experiments in the data processing notebook. For example, this can involve removal of wells in which an experiment failed or correction of barcodes that were misread.

d. Save the results into a tsv file called *processed file*.

2. Merging data with treatment design.

This step merges the processed file with the experimental design files generated in Basic Protocol 1. The resulting file, called the annotated file, is a simple table in which each row is a measured sample and all model variables describing the experiment (treatment, readout, . . .) as well as the readout variables are columns. Saved as a tsv file, the annotated file serves as the permanent record of the experimental results.

a. Use the functions in the module `data_processing.drug_response` to combine the processed file with the plate description file and the treatment design files generated in Basic Protocol 1 (note that all tables can be kept in memory and passed as arguments instead of as file names). The output is a table that contains the model variables and readout values for each sample. For time-course experiments, it is important to specify the time of treatment in either the plate description file or as input parameter so that the algorithm can properly calculate the treatment time based on the scanner timestamps.

b. If necessary, script the operations needed to correct for failure of the experiments in the data processing notebook. For example, this can involve changing the name of a reagent that was used by mistake, an incorrect stock concentration for a drug, or the annotation of wells that were not in fact treated.

For plates in which the outermost rows and columns are used, bias can be corrected by multiplying the values of wells on the edge by the ratio of the average values for untreated controls on the edge and the average value of the ones in the center of the plate. We suggest correcting a potential bias only if enough controls are positioned on the edge and in the center and the difference is significant by t-test.

c. Save the data in a tsv file called *annotated file*.

3. *Optional:* Controlling the quality of the data.

These steps use scripts to perform basic quality-control analysis on the data (Fig. 4B) such as identification of edge bias or other potential artifacts described in the accompanying article in Current Protocols in Chemical Biology (Niepel et al., 2017). If the results of the quality control are not satisfactory, it is up to the user to update step 1c to discard low-quality plates or wells. It is important to record all operations in the data processing notebook, in particular, filtering of low-quality results, such that the notebook becomes a literate record of how data were processed and thus ensures reproducibility of the analysis.

a. Test potential bias within each plate by using the functions in `data_processing.drug_response`. This test is useful for identifying edge effects or issues with cell plating and non-uniform growth across a plate.

b. Test potential bias across plates based on the untreated controls by using the functions in `data_processing.drug_response`. This test is useful for identifying differences between replicates and evaluating the variance of the readout.

BASIC PROTOCOL 3

EVALUATING SENSITIVITY METRICS

Upon completion of Basic Protocol 3, a user will have:

- One *normalized file* which contains the GR values and relative cell count for all treated samples
- One *response metric file* which contains the drug sensitivity metrics for each dose-response curve

Introduction

This article provides scripts to normalize drug response values and evaluate sensitivity metrics based on annotated result files. The first step is to normalize the treated samples to the untreated controls. The simplest normalization is to calculate the relative cell count $\bar{x}$, defined as the cell count value measured in the treated conditions $x(c)$ divided by the cell count value of an untreated (or vehicle-treated) control x_{ctrl}: $\bar{x}(c) = x(c)/x_{ctrl}$. Relative cell count is valid for experiments where the untreated controls do not grow over the course of the experiment, but it is not suitable for comparing cell lines or conditions where the division rate differs (Hafner et al., 2016). An alternative normalization based on growth rate inhibition (GR) corrects such variation:

$$GR\,(c) = 2^{\frac{\log_2(x(c)/x_0)}{\log_2(x_{ctrl}/x_0)}} - 1,$$

where x_0 is the cell count value measured at the time of treatment. Given the proper controls x_{ctrl} and x_0 for a set of conditions, both the relative cell count and the GR values are calculated. By default, values for untreated controls are averaged using a 50%-trimmed mean and replicates for treated samples on the same plates are averaged before calculating the normalized values. Values across multiple plates are aggregated to yield the *normalized file*.

Experiments with multiple time points (either time-course experiments or multiple plates fixed at different time points) can be parameterized using time-dependent *GR* values defined as:

$$GR\,(c, t) = 2^{\frac{\log_2(x(c,t+\Delta t)/x(c,t-\Delta t))}{\log_2(x(c,t+\Delta t)/x(c,t-\Delta t))}} - 1,$$

where $x(c,t \pm \Delta t)$ are the measured cell counts after a given treatment at times $t + \Delta t$ and $t - \Delta t$, and where $x_{ctrl}(t \pm \Delta t)$ are the 50%-trimmed means of the cell count of the negative control wells on the same treated plate at the same times $t + \Delta t$ and $t - \Delta t$. The interval $2 \cdot \Delta t$ should be long enough for the negative control to grow at least 20% (a fourth of division time). We have found 8 to 12 hr to be a reasonable range for $2 \cdot \Delta t$ capture adaptive response while keeping experimental noise low.

The second step evaluates sensitivity metrics by fitting a sigmoidal curve to dose-response data:

$$y(c) = y_{inf} + \frac{t - y_{inf}}{1 + (c/x_{50})^h},$$

where y corresponds to *GR* values or the relative cell count across a range of concentrations. By default, fits are performed if there are at least five different concentrations for a given treatment and unique set of conditions. The script yields a *response metric file* which contains, for each dose-response curve, the *GR* and traditional metrics based on *GR* values and, respectively, relative cell count values (Fig. 6):

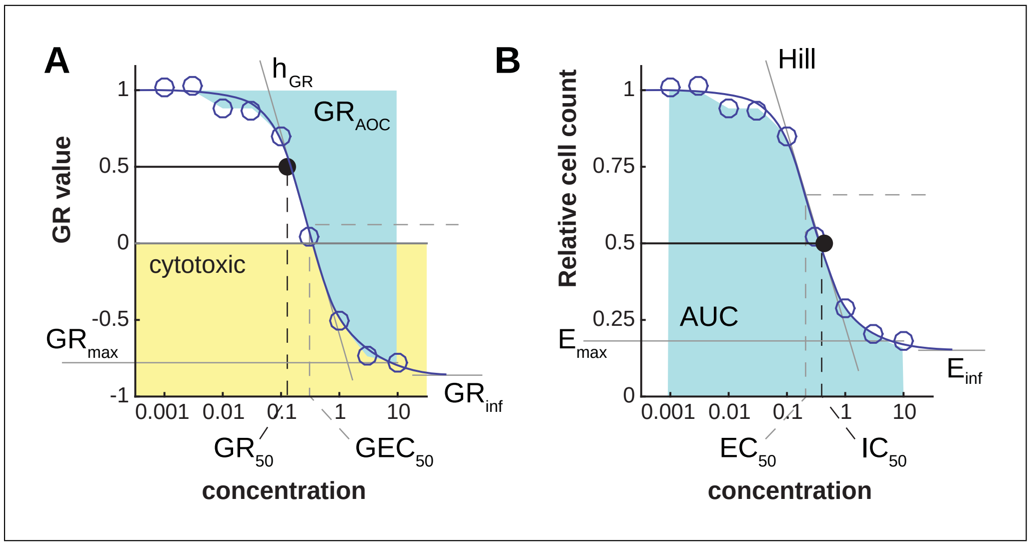


Figure 6 Parameters of a dose-response curve. Parameters of a dose-response curve for GR value (left) or relative cell count (right). Negative GR values correspond to a cytotoxic response (yellow area).

- ***GR_{inf}*** or ***E_{inf}***: The asymptotic value of the sigmoidal fit to the dose-response data ($GR_{inf} \equiv GR(c \to \infty)$, respectively $E_{inf} \equiv \bar{x}(c \to \infty)$). When a drug is potent and tested at a high concentration, GR_{max} (the highest experimentally tested concentration) and GR_{inf} will be quite similar, but in many cases the convergence to the asymptote occurs only at concentrations well above what are experimentally testable. GR_{inf} lies between –1 and 1; negative values correspond to cytotoxic responses (i.e., cell loss), a value of 0 corresponds to a fully cytostatic response (constant cell number), and a positive value corresponds to partial growth inhibition. E_{inf} is bound between 0 and 1; in case of strong toxic response, it can be interesting to study E_{inf} in the log domain to have better resolution of the residual population.
- ***h_{GR}*** or ***h***: Hill coefficient of the fitted curve. It reflects the steepness of the dose-response curve. By default, the value is constrained between 0.1 and 5.
- ***GEC_{50}*** or ***EC_{50}***: Drug concentration at half-maximal effect. By default, GEC_{50} and EC_{50} values are constrained within two orders of magnitude higher and lower than the tested concentration range.
- ***GR_{50}*** or ***IC_{50}***: The concentration of drug at which $GR(c = GR_{50}) = 0.5$, or respectively $\bar{x}(c = \mathrm{IC}_{50}) = 0.5$. If the GR_{inf} or E_{inf} value is above 0.5, the corresponding GR_{50} or IC_{50} value is not defined, and therefore set to $+\infty$.
- ***GR_{max}*** or ***E_{max}***: The maximum effect of the drug. For dose-response curves that reach a plateau, the GR_{max} or E_{max} value is close to the corresponding GR_{inf} or E_{inf} value. GR_{max} and E_{max} can be estimated from the fitted curve or obtained directly from the experimental data; the latter approach is implemented in our package.
- ***GR_{AOC}*** or ***AUC***: the area over the GR curve or, respectively, under the relative cell count curve, averaged over the range of concentration values.

If the fit of the curve fails or is not significantly better than that of a flat curve based on an F test (see below), with cutoff of $p = 0.05$, the response is considered flat. In such a case, the parameter GEC_{50} *or* EC_{50} is set to 0, the Hill coefficient is set to 0.01, and the GR_{50} or IC_{50} value is not defined. In such case, if GR values are above 0.5, the GR_{50} *or* IC_{50} value is set to $+\infty$ or, if all measured GR values are below 0.5, to $-\infty$.

For experiments with multiple time points, all GR metrics can be evaluated at each time point at which time-dependent GR values were calculated. All considerations discussed above are relevant for time-dependent GR metrics.

File Types

From the annotated file generated in Basic Protocol 2, the first step of Basic Protocol 3 generates the *normalized file*, which contains all result values. Step 2 generates the *sensitivity metric file*, which contains the parameters of the dose-response fits. Plots of these data can be generated and saved as pdf files.

Materials

The `gr_metrics` Python package (*https://github.com/datarail/gr_metrics*)
Annotated *result file*, which contains the model and readout variables for each sample. This file can be the output of Basic Protocol 2, or a file that has tabular format with the following columns:
  Model variables (descriptions of the perturbation such as drug name and concentration)
  Readout (cell count or a surrogate) in a column labeled `cell_count`

1. Calculating relative cell count and GR values.

   Use the function `add_gr_column.py` with the processed data file as input. By default, the match between treated samples and controls is determined based on the model variables: x_{ctrl} values have the same model variables as corresponding treated samples, but no drug or reagent (and consequently a value of 0 for concentration); x_0 values have the same model variables as the corresponding untreated and treated samples but a treatment time of 0 and no drug or reagent (concentration value equals 0). For a more complex scheme, the function `add_gr_column.py` can take as input a table with the controls already assigned to the treated samples in two columns: `cell_count__ctrl` and `cell_count__time0`.

   For evaluating time-dependent GR values, use the function `add_time_gr_column.py` with the processed data file as input (Fig. 5). By default, the match between treated samples and controls is determined based on the model variables: x_{ctrl} values have the same model variables as corresponding treated samples, but no drug or reagent (and consequently a value of 0 for concentration). It is not necessary to have a measurement at time 0, as the time-dependent GR values can be evaluated on any time interval even during treatment.

   Technical replicates can be averaged at this stage using the functions in the module `data_processing.drug_response`. For time-dependent GR values, the function can average technical replicates that were collected at slightly different time points to account for sequential plate scanning.

2. Fitting dose-response curve and evaluating sensitivity metrics.

   Use the function `compute_gr_metrics.py` to fit and plot the dose response curves (Fig. 5). By default, the fitting function performs a significance test on the fit and replaces non-significant fits by a flat line. More options, such as capping of values or default range for parameters, can be selected by the user.

   The significance of the sigmoidal fit is evaluated using an *F*-test. The sigmoidal fit, which is the unrestricted model, has $p_2 = 3$ parameters (GR_{inf}, h_{GR}, and GEC_{50}), whereas the flat line, which is the nested model, has $p_1 = 1$ parameters (GR_{inf}). Given the number of data points n and the residual sums of squares RSS_2 and RSS_1 of the sigmoidal fit and the flat line respectively, the *F*-statistic is defined as:

$$F = \frac{\left(\frac{RSS_1 - RSS_2}{p_2 - p_1}\right)}{\left(\frac{RSS_2}{n - p_2}\right)}.$$

The null hypothesis, meaning that the sigmoidal fit is not better than a flat line, is rejected if the value F is greater than a critical value (e.g., 0.95 for a significance of $p < 0.05$) of the cumulative F-distribution with $(p_2 - p_1, n - p_2)$ degrees of freedom.

The interactive Web site http://www.grcalculator.org offers the same data analysis and simple visualization widgets and is a good place to explore datasets calculated using the GR method.

Both the function `compute_gr_metrics.py` *and http://www.grcalculator.org yield a tabular file with sensitivity metrics and quality of the fit.*

COMMENTARY

Importance of Processing Data Using Computer Scripts

In the protocols above, we emphasize the value of processing data using scripts. Manual steps or cut-and-paste operations in spreadsheets risk corrupting data. Operations (e.g., transformation, filtering, scaling operations, normalization) that are not scripted are not recorded and therefore not reproducible. While the scripts and functions from the `datarail` GitHub repository focus on dose-response assays, they are a model for programmatic manipulation of data in other contexts as well. We strongly encourage users to use these functions and Jupyter notebooks for all aspects of data processing.

Critical Parameters and Limitations in the Design and Experiment

Four critical parameters must be considered in the design of perturbation experiments:

Control wells: Both relative cell count and GR values are sensitive to the value of the negative control (generally untreated or vehicle-treated wells). The experimental design must accommodate sufficient negative controls. We recommend >12 control wells for each 384-well plate (>8 if not using the outermost wells) and >4 for each 96-well plate. For experiments with combinations of conditions and perturbations, we recommend >4 control wells per condition independently of plate size.

Number of concentrations for dose-response curves: For proper fitting of a dose-response curve, at least five different concentrations should be tested, and these must span the high and low end of the phenotypic response. Ideally, the readout at the lowest and highest concentrations should reach plateaus. We suggest using at least seven doses spanning three orders of magnitude if the response range is known in advance, and at least nine doses over four orders of magnitude if the response is unknown or if the same range of doses will be used for multiple cell lines across different experiments. We have found that a 3.16-fold dilution (i.e., two concentrations per order of magnitude) is a good compromise between range and dose density; however, smaller spacing between doses will allow more precise evaluation of GEC_{50} or EC_{50} and GR_{50} or IC_{50} values, whereas a wider range will improve the precision of the GR_{inf} or E_{inf} values. Note that there is no requirement that the spacing between doses be constant across the entire range; non-uniform spacing is particularly easy to achieve using an automated dispenser.

Number of replicates: Readout of cell number or a surrogate such as ATP level is relatively noisy when performed in multi-well plates. Replicates are therefore essential for improving the estimation of cell counts and GR values. Repeats are particularly important when assaying small changes such as those resulting from low-efficacy drugs or short-duration assays. When evaluating time-dependent GR values, it is important that negative controls be sufficiently accurately determined and assay duration long enough such that differences in cell count between time points are larger than the experimental noise.

Edge and plate-based effects: Cell viability is frequently altered by differences in temperature and humidity at the edge of the plate. Therefore, we suggest not using the outermost wells unless preliminary experiments confirm that edge effects are small. Performing multiple technical replicates with different randomization schemes helps to control artifacts arising from non-uniform growth across plates. Our software enables pseudo-randomization to ensure that replicates in different plates are positioned at different distances from the edge to avoid systematic error. If all wells are used in a plate, this also makes it less problematic to disregard some edge wells *a posteriori* if problems become evident (e.g., low media volume from excess evaporation), since data from an entire condition will not be lost.

Positive Controls and Fingerprints for Master Plates

In addition to negative controls (vehicle only wells), we suggest adding positive controls to check that cells are responding to perturbation and that data acquisition and processing steps are performing correctly. We find that drugs such as staurosporine or actinomycin D make good positive controls for cytotoxicity. Positive controls can be labeled in the column role with the flag `positive_control`. The quality-control functions in the module `data_processing.drug_response` will process results for positive controls separately and include them in the quality-control report.

For master drug plates that are meant to be reused over months or years by different experimentalists, we recommend using negative and positive controls to hard code the identity of the master plate. If the outermost wells are generally not used for treatments, these wells can include cytotoxic compounds in a unique pattern. Functions in the module `experimental_design` can encode and decode an 11-character name (5 characters for 96-well plates) in a set of positive and negative controls and position them on the edge on a plate (Fig. 7). Treatments used for the fingerprint wells will be labeled with the flag `fingerprint` as role. Quality-control scripts decode the fingerprint on the edge wells and check for consistency with the name of the master plate used for treatment as labeled in the *plate information file*.

Experimental Implementation

By default, only *treatment variables* are exported in the files used to describe the pin-transfer procedure or to control drug dispensers; this is appropriate for experiments in which a single factor is varied from well to well (e.g., drug dose and identity). However, we include a software flag such that *condition variables* can also be included in the treatment file. This is necessary when two factors are being altered. For example, when exploring the effects of varying the concentration of a soluble ligand on drug response, ligand identity and concentration are a *condition variable* but flagged as being a reagent for robotic dispensing; drug remains a *treatment variable* and is thus dispensed by default.

Complex Designs

The protocols described above and Jupyter notebooks available on GitHub are designed for common single-agent dose-response experiments and pairwise testing of drugs. However, the flexibility of the D300 drug dispenser makes it possible to design much more complex experiments in which wells are exposed to multiple drugs or ligands and multiple cell lines are present per plate. The data structures and packages used within datarail support the design of complex experiments that have two or more treatments per well. The current set of templates does not include complex experiments with multiple conditions or combined treatments. Moreover, our curve-fitting algorithm assumes a dilution series across a single variable. In more complex cases, we leave it to the user to write personalized scripts that rely on sub-functions from the package. However, we expect such design and processing scripts to be developed and published on our GitHub repository in the future.

Assignment of Control Well is Based on the Model Variables

Data processing using the scripts in this article relies heavily on keys for identifying the controls necessary to normalize the cell count values. Each treated sample needs to be matched with a negative control x_{ctrl} and, in the case of GR calculations, a cell count value at the start of treatment at $t = 0$ (i.e., x_0). By default, matching treated samples to control wells is based on the model variables: negative controls have an empty value for reagent and $t = 0$ data have a value of 0 for `treatment_duration`. Thus, Basic Protocol 3 may not be directly usable in complex experiments with multiple reagents and ambiguous normalization. In such cases, users may need to assign control wells to each sample using a custom script.

Troubleshooting and Avoiding Common Problems

We have identified three common pitfalls in this method:

Manual processing steps: Any stage in the generation of the experiment design or in data processing that is not scripted has a high potential to introduce errors that are hard to troubleshoot. In particular, files that are generated by hand are a potential source of mistakes through typos, erroneous formatting, parsing

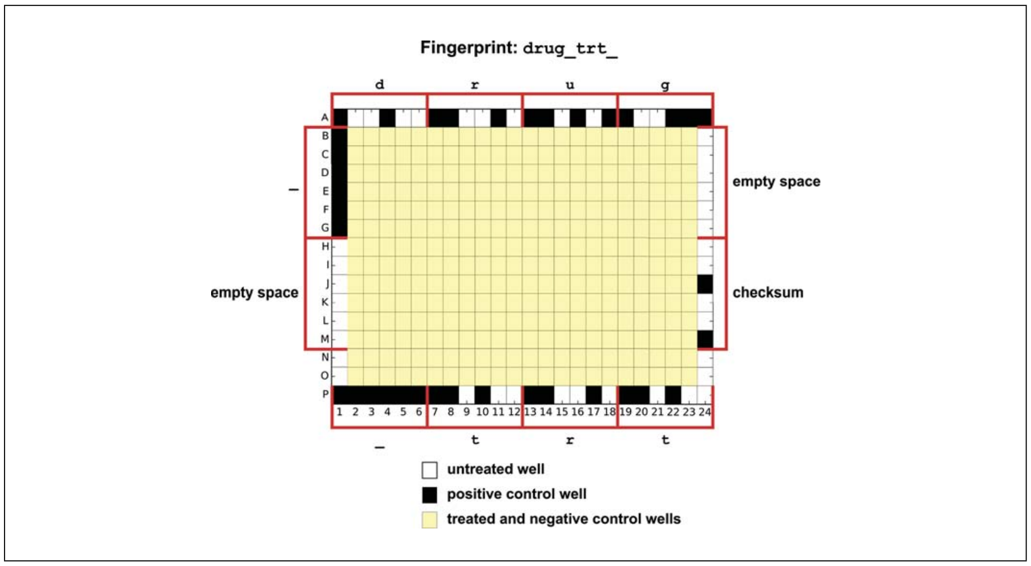


Figure 7 Plate fingerprint. Positive controls can be used to encode the name of the treatment plate in the edge wells with up to eleven characters for a 384-well plate (five for a 96-well plate). Each letter in the label `drug_trt_` is transformed into a list of 6-bit values that define treatments along row A, row P, column 1, and column 24 (one well per bit, six wells per character). The last value of column 24 is a checksum.

errors, or inconsistencies in naming conventions. In addition, we have observed issues with files generated by spreadsheet programs, such as Microsoft Excel, because in certain circumstances, numeric fields are automatically iterated and some names and numbers automatically converted into dates.

Growth of negative controls: Calculation of GR values requires that negative control samples grow over the course of the assay. As the GR acronym indicates, GR values and metrics are meant to study the inhibition of cellular growth and proliferation. If the study is focused on cell death, or growth is minimal during the assay due to the cell line tested, the incubation conditions, or the length of the assay, relative cell count and metrics such as IC_{50} and E_{max} are the correct ones to use, since GR values will be undetermined.

Fitting artifacts: The fitting of the dose-response curve is subject to several artifacts. First, enough concentrations must be measured for the fit to be reliable. Second, values greater than 1.0 for relative cell count or GR value are not accounted for in the model; this can arise if a drug actually stimulated growth. Third, noisy data may yield a bad fit and the interpolated parameters can have unwanted variability. Fourth, a fair number of drugs are associated with biphasic dose-responses that cannot be properly fitted using a sigmoidal curve. Alternative fitting functions will be implemented in the future to parameterize biphasic dose-responses. In all cases, the goodness of the fit (as defined by the r-squared and corresponding p-value) reflects how reliable the metrics are. Metrics obtained from low-quality fits should be used carefully.

Companion Web Site

The companion Web Site *http://www.grcalculator.org* can be used to replace Basic Protocol 3 for simple experiments. It also provides visualization tools and easy comparison between samples and treatments. Text is included describing the functionalities of the website and corresponding R package (see Internet Resources).

Acknowledgements

This work was funded by NIH P50 GM107618 and NIH LINCS U54-HL127365 grants to P.K.S. and by a Swiss National Science Foundation fellowship P300P3_147876 to M.H.

Literature Cited

Hafner, M., Niepel, M., Chung, M., & Sorger, P. K. (2016). Growth rate inhibition metrics correct for confounders in measuring sensitivity to cancer drugs. *Nature Methods*, *13*, 521–527. doi: 10.1038/nmeth.3853

Lundholt, B. K., Scudder, K. M., & Pagliaro, L. (2003). A simple technique for reducing edge effect in cell-based assays. *Journal of Biomolecular Screening*, *8*, 566–570. doi: 10.1177/1087057103256465

Niepel, M., Hafner, M., Chung, M., & Sorger, P. K. (2017). Measuring cancer drug sensitivity and resistance in cultured cells. *Current Protocols in Chemical Biology*, *9*(2), 1–20.

Powell, S. G., Baker, K. R., & Lawson, B. (2008). A critical review of the literature on spreadsheet errors. *Decision Support Systems*, *46*, 128–138. doi: 10.1016/j.dss.2008.06.001

Saez-Rodriguez, J., Goldsipe, A., Muhlich, J., Alexopoulos, L. G., Millard, B., Lauffenburger, D. A., & Sorger, P. K. (2008). Flexible informatics for linking experimental data to mathematical models via DataRail. *Bioinformatics*, *24*, 840–847. doi: 10.1093/bioinformatics/btn018

Stocker, G., Fischer, M., Rieder, D., Bindea, G., Kainz, S., Oberstolz, M., . . . Trajanoski, Z. (2009). iLAP: A workflow-driven software for experimental protocol development, data acquisition and analysis. *BMC Bioinformatics*, *10*, 390–401. doi: 10.1186/1471-2105-10-390

Wu, T., & Zhou, Y. (2014). An intelligent automation platform for rapid bioprocess design. *Journal of Laboratory Automation*, *19*, 381–393. doi: 10.1177/2211068213499756

Zeeberg, B. R., Riss, J., Kane, D. W., Bussey, K. J., Uchio, E., Linehan, W. M., . . . Weinstein, J. N. (2004). Mistaken identifiers: Gene name errors can be introduced inadvertently when using Excel in bioinformatics. *BMC Bioinformatics*, *5*, 80. doi: 10.1186/1471-2105-5-80

Ziemann, M., Eren, Y., El-Osta, A., Zeeberg, B., Riss, J., Kane, D., . . . Smedley, D. (2016). Gene name errors are widespread in the scientific literature. *Genome Biology*, *17*, 177. doi: 10.1186/s13059-016-1044-7

Key References

Hafner et al. (2016). See above.

Paper describing the GR method.

Niepel et al. (2017). See above.

This article in Current Protocols in Chemical Biology is a companion article to the present article, including more experimental detail.

Internet Resources

https://github.com/datarail/datarail

GitHub repository with the scripts for the experimental design and data handling

https://github.com/datarail/gr_metrics

GitHub repository with the scripts for the evaluation of the GR values and metrics.

http://www.GRcalculator.org

GRcalculator: An online tool for calculating, visualizing, and mining drug response data designed by Clark, N. A., Hafner, M., Kouril, M., Williams, E. H., Muhlich, J. L., Niepel, M., Medvedovic, M.

http://www.labautopedia.org/mw/Helpful_Hints_to_Manage_Edge_Effect_of_Cultured_Cells_for_High_Throughput_Screening

Helpful hints to manage edge effect of cultured cells for high throughput screening. Corning Cell Culture Application and Technical Notes, 7–8 (author, Allison Tanner of Corning Life Sciences, 2001).