# Measuring Cancer Drug Sensitivity and Resistance in Cultured Cells

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Measuring the potencies of small-molecule drugs in cell lines is a critical aspect of preclinical pharmacology. Such experiments are also prototypical of high-throughput experiments in multi-well plates. The procedure is simple in principle, but many unrecognized factors can affect the results, potentially making data unreliable. The procedures for measuring drug response described here were developed by the NIH LINCS program to improve reproducibility. Key features include maximizing uniform cell growth during the assay period, accounting for the effects of cell density on response, and correcting sensitivity measures for differences in proliferation rates. Two related protocols are described: one involves an endpoint measure well-suited to large-scale studies and the second is a time-dependent measurement that reveals changes in response over time. The methods can be adapted to other types of plate-based experiments. 

Keywords: dose-response • $GR_{50}$ • $GR_{max}$ • growth rate inhibition • cancer • drug sensitivity • drug resistance

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## INTRODUCTION

The measurement of drug dose-response in cultured cells is a cornerstone of pre-clinical assessment of cancer drugs. Assays in which immortalized cell lines are exposed to drugs have been used for decades to calculate drug potency and efficacy (Yoshida, Hoshi, Kuretani, Kanai, & Ichino, 1975). Recent studies have scaled up such measurements to large panels of cell lines and large numbers of drugs as a means to identify genetic, transcriptional, and protein biomarkers of drug sensitivity and resistance (Barretina et al., 2012; Garnett et al., 2012; Haverty et al., 2016). Routine measurement of drug response in primary human tumor cells has also been proposed as a means to personalize therapy for individual patients, by analogy to antibiotic susceptibility testing (Crystal et al., 2014; Yuan et al., 2012). However, irreproducibility in the measurement of drug response remains an issue, as witnessed by reports on the discrepancy between large pharmacogenomic screens (Haibe-Kains et al., 2013; Haverty et al., 2016; Safikhani et al., 2016; The Cancer Cell Line Encyclopedia Consortium & The Genomics of Drug Sensitivity in Cancer Consortium, 2015).

Two things are required for the generation of reproducible and useful drug dose-response data. First, experiments must be designed and conducted so as to minimize the impact of variation in cell plating density, culture conditions, and other aspects of experimental procedure. Protocols to minimize these effects are described in detail below. Second, the






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data must be parametrized in a way that corrects for known confounders, in particular the impact of natural variation in cell division time. This has been a topic of a recent publication (Hafner, Niepel, Chung, & Sorger, 2016) and is discussed in detail in a companion article focused on processing and interpreting dose-response data (Hafner, Niepel, Subramanian, & Sorger, 2017).

Time is a variable rarely considered in drug dose-response studies, but there is growing evidence of dramatic temporal changes in drug sensitivity, arising, for example, from adaptive resistance to drug treatment (Lito et al., 2012; Manchado et al., 2016). Such variation is ignored in typical drug-response measurements that report only on time-averaged cellular phenotypes. Moreover, it is not uncommon for phenotypic data to be collected at different time points from transcriptional or proteomic data, with the potential for missing differences between the acute and longer-term effects of drugs. Recently, two methods that properly quantify time-variable drug sensitivity were introduced (Hafner et al., 2016; Harris et al., 2016). We describe, here and in the companion article (Hafner et al, 2017), how to collect and analyze time-dependent drug sensitivity data using one of these methods.

Even if data are collected as described below and correctly analyzed as described in Hafner et al. (2017), it is important to note that key aspects of cellular responsiveness to drugs may be missed or obscured. Well-average cell counts or whole-well surrogate assays such as CellTiter-Glo (Promega) or resazurin (Crouch, Kozlowski, Slater, & Fletcher, 1993; Mosmann, 1983; O'Brien, Wilson, Orton, & Pognan, 2000; Tolliday, 2010) implicitly assume that cells in a population react uniformly to a drug at any given concentration. Assays, such as those described here, report only on the average responsiveness of all cells in a population; however, it is known that even in a genetically uniform cell populations, actual drug response is heterogeneous at a single-cell level (Flusberg, Roux, Spencer, & Sorger, 2013; Spencer, Gaudet, Albeck, Burke, & Sorger, 2009). This heterogeneity has a significant impact on the shape of dose-response curves (Fallahi-Sichani, Honarnejad, Heiser, Gray, & Sorger, 2013), and it is particularly important when drugs affect only some cells in a population, resulting in incomplete maximum effect. Single-cell assays are more informative than population average data, but they are substantially more laborious to collect and require different analytical methods. The population average approaches described here have the merit of being robust and easy to perform with only minor changes in protocol, and are applicable on both large and small scales.

A more general limitation of most contemporary analysis of drug response is the mismatch between highly sophisticated genomic, proteomic, and epigenomic data that are available on the pre-treatment state of many cells and tissues (these constitute dependent variables in regression and machine-learning methods) and the relatively crude approaches to analyzing phenotypes arising from response to drug treatment (which constitute independent variables). Response phenotypes are diverse and include quiescence, senescence, adaptation, apoptosis, and other forms of programmed cell death. Pharmacogenomic approaches and mechanistic studies of drug response would almost certainly be more effective if different phenotypes were distinguished from each other, for example, using biochemical or immunofluorescence assays. The approaches described in this protocol represent a first step in obtaining better independent variables.

The major steps of this protocol are outlined in Figure 1. They are applicable to adherent cells proliferating in conventional 2-D tissue culture that are exposed to a range of perturbagens, such as small-molecule drugs, therapeutic antibodies, natural ligands, and cytokines. With some modifications, primarily the addition of centrifugation steps, the protocol can be adapted for analysis of non-adherent cells. For purposes of illustration, we

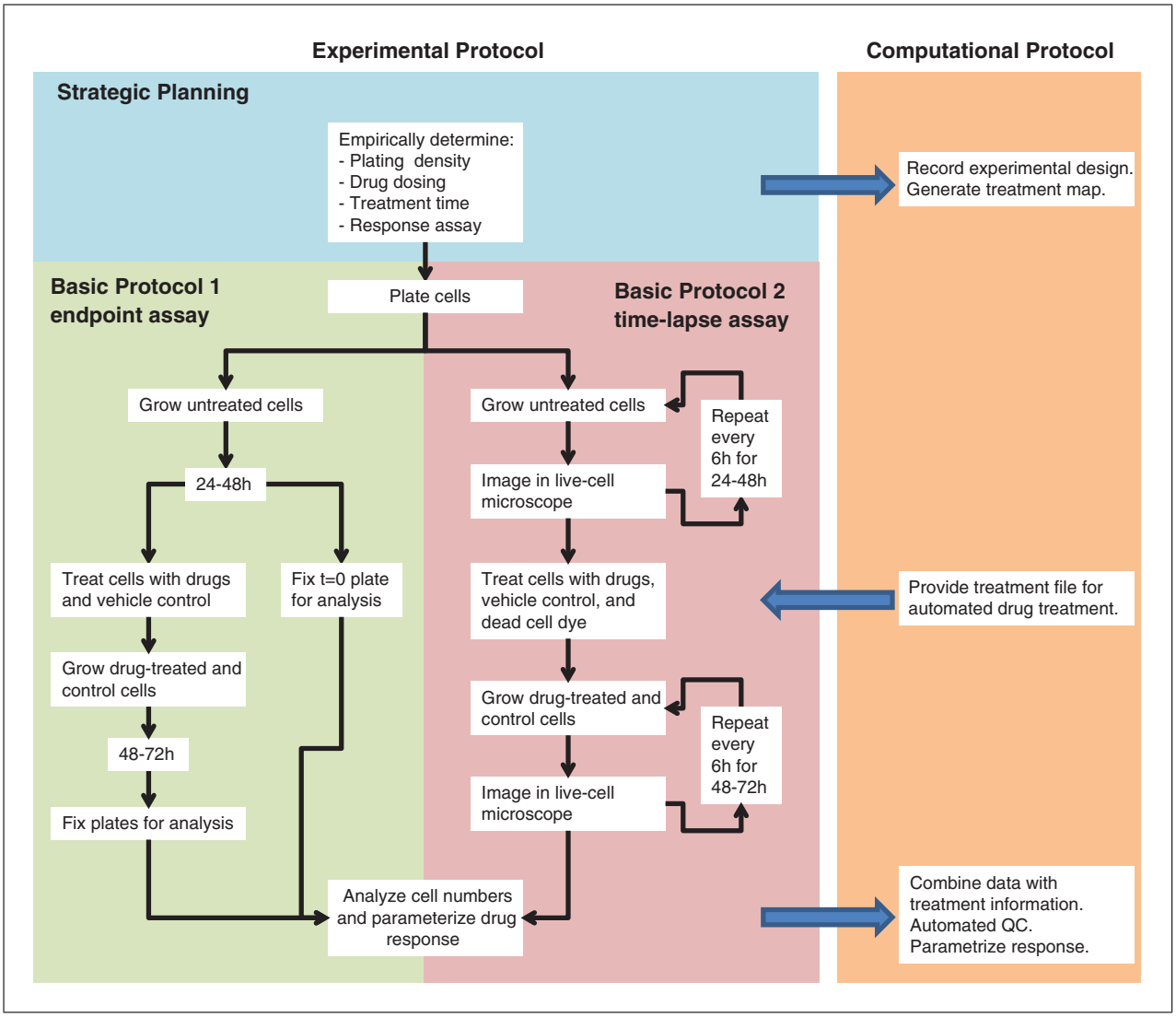


**Figure 1** Flowchart of the two basic protocols to measure drug response. The flowchart outlines how to run endpoint drug response assays (Basic Protocol 1) or time-dependent drug response assays (Basic Protocol 2).

show data collected using the non-transformed mammary epithelial MCF 10A cell line transfected with fluorescently tagged histone H2B as a means to facilitate cell counting over time (Basic Protocol 2). The protocols described below are applicable, of course, to virtually any other types of cells that grow in culture.

*NOTE*: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

*NOTE*: All culture incubations are performed in a humidified (constant 95% humidity), 37°C, 5% $CO_2$ incubator unless otherwise indicated.

## STRATEGIC PLANNING

Performing reliable assays on the effects of drugs on cell proliferation and survival requires careful control over parameters that cause proliferation rates to vary independently of the deliberate perturbation. These parameters include the growth medium, culture substrate, plating density, and conditions of the culture at the time of plating. It is self-evident that growth medium and serum should be held constant throughout a study, and the ATCC provides helpful information on standard growth conditions. These conditions should

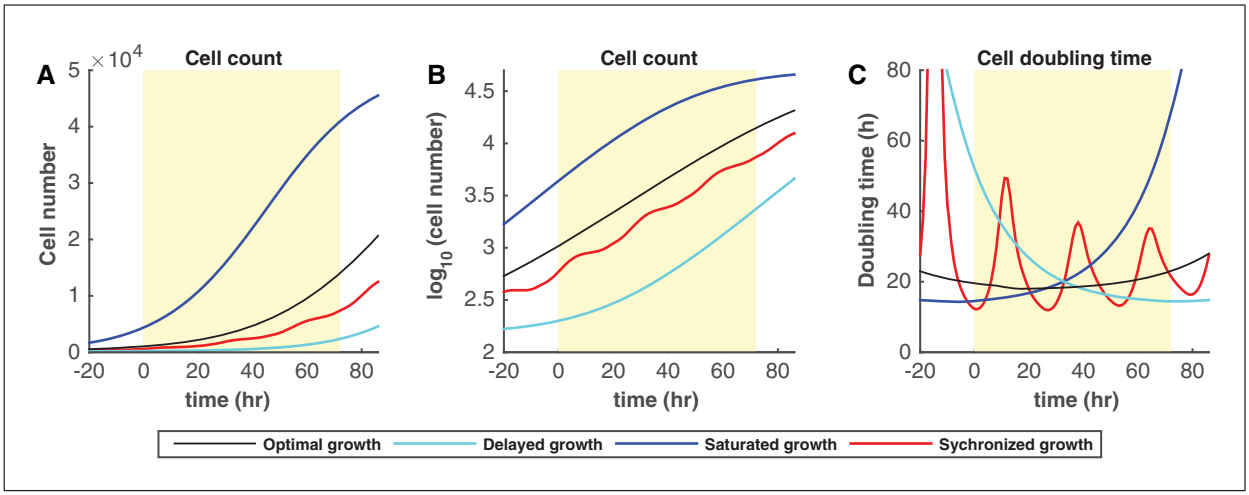


**Figure 2** Illustration of how different experimental conditions can affect growth rates over time. Cell number (left) and $\log_{10}$ of the cell number (middle) over time for a cell line showing optimal, constant growth (black), delayed growth where the growth rate is very low at the beginning of the experiment (light blue), saturated growth where growth slows down significantly at the end of the experiment (dark blue), and synchronized growth where cells divide approximately at the same time, leading to greatly fluctuating growth rates (red). Under optimal conditions, the growth rates are relatively constant during the window of a typical experiment (0 to 72 hr; shaded yellow), but rates change significantly over time under the other three regimes.

always be recorded and reported when a study is published. The quality of multi-well plates, even those from major vendors, can be surprisingly variable, and uneven growth across a plate is not uncommon. We typically ask vendors for samples from multiple batches of plates and then check each batch for its ability to sustain uniform growth. The state of a culture at the time of plating, the density of the initial culture, and the volume of medium in each assay well are all variables with a substantial impact on cell proliferation. Prior to plating, cells should be asynchronously dividing and, insofar as it is possible, their growth should remain uniform and not be limited by culture conditions during the assay period.

The duration of a typical assay involving conventional immortal cancer cell lines is 3 days, and no change of medium is required, but in longer assays acidification of the medium and depletion of nutrients are frequently observed. In principle, this can be overcome by refeeding cells and adding fresh drug, but this too has its complications since it causes abrupt changes in the experimental conditions, which may affect different cells to different degrees. Withdrawing one-third to one-half of the depleted medium and then adding fresh medium to re-feed cells represents a reasonable compromise.

Cell division time, also called cell growth rates, varies between cell lines for fundamental biological reasons. The growth rate (GR) metrics described here and in the accompanying article (Hafner et al., 2017) correct for the confounding effects of variable cell division times on the estimation of drug response by computing response metrics on a per-division basis. When growth conditions vary significantly with time (Fig. 2), the final estimate of drug sensitivity necessarily represents an average over different conditions. In such cases, we suggest determining time-dependent growth rate inhibition to resolve variation in time (Hafner et al., 2016).

The microenvironment is known to have a significant impact on the responsiveness of cells to some classes of drugs, independent of the effect that microenvironment has on growth rate (Straussman et al., 2012; Wilson et al., 2012). There is no a priori way to determine whether variation in experimental conditions will materially change drug response for a particular cell line: some empirical testing is required. As a rule, we suggest identifying culture conditions (plating density, medium volume, medium

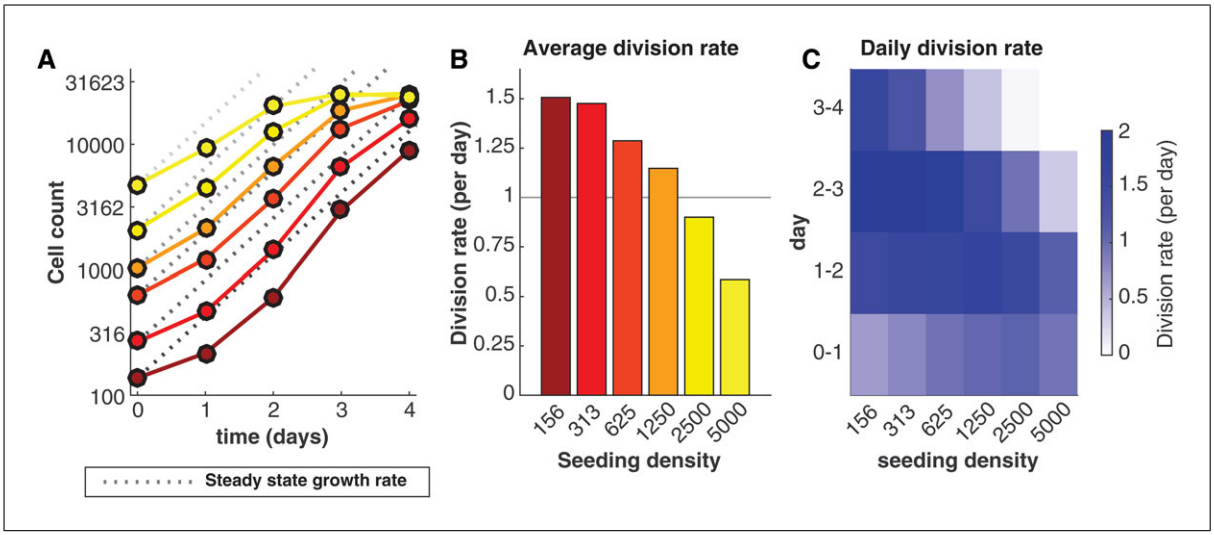


**Figure 3** Optimization of seeding density and assay timing. MCF 10A cells are plated at six different densities in five different plates and incubated. Individual plates are prepared for cell counting at 24-hr intervals thereafter. The cell count is plotted over time (left), average division rate (middle), and daily division rate (right) for each plating density. The data is used to identify a seeding density and experimental window where the growth rate is relatively constant. Based on the results of this experiment, a seeding density of 500 to 1500 cells per well for a 48-hr experiment would be judged to be optimal.

type) that have as little effect on growth rates as possible over the assay period. These conditions must be recorded as part of the dose-response measurement itself. In the context of the accompanying article, factors such as drug dose usually constitute model variables whereas plating density and medium composition usually constitute confounder variables. However, in cases in which drug sensitivity appears to vary from one repeat to the next, it can be valuable to study the impact of confounder variables directly (which then become model variables).

### Ensuring Uniform Growth Over Time

The impact of medium composition, recovery time between plating and perturbation, plating density, and duration of the experiment is determined empirically for each cell line prior to large-scale studies. While this sounds relatively laborious, it can be performed efficiently in multiwell plates. In a typical experiment with the MCF 10A line, cells were plated over a range of densities, typically 150 to 5000 cells per well, in five 384-well plates (Fig. 3; also see Supplemental Data 1). Two rows were plated at each density to create multiple replicate assays, something that is easily done using plate fillers such as the Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific). Cell numbers were then measured at 24-hr intervals starting 24 hr after initial plating, and the effect of plating number on growth rate per day was computed. From these data, a plating density is chosen that minimizes both the delay in return to proliferation that often occurs post-plating as well as changes in division rate at later times, which typically, but not always, involve slower proliferation due to contact inhibition or nutrient limitation. In some cases, cancer cells actually proliferate more rapidly when denser (Hafner et al., 2016), perhaps as a consequence of autocrine conditioning of the medium. If multiple plating densities and timeframes are nearly equivalent, we choose intermediate values on the premise that they are more likely to be stable to experimental variation.

In the case of MCF 10A cells, the best protocol is judged to involve a plating density of 750 cells per well, exposure of cells to drug 48 hr after plating, and measurement of final cell number 48 hr after drug addition. Ideally, the duration of the drug treatment should allow cells to divide twice over the course of the assay. This can be reduced to about one cell division (and possible even less), an advantage in the case of slowly

or unevenly growing cells such as primary tumor cells. However, reliable data can be obtained under these circumstances only if the population is asynchronously dividing, allowing for a full cell division cycle to be reconstructed. For some cells lines and growth conditions, a long lag is observed in proliferation post-plating, potentially leading to partial synchronization. This can impact the assessment of responsiveness to drugs that act on specific cell cycle phases (e.g., DNA replication or mitosis). When synchronization is observed and unavoidable, it is vital to ensure that the whole population divides multiple times over the course of the assay.

### Choosing a Drug Concentration Range

The potency of drugs varies dramatically from a midpoint value ($IC_{50}$ or $GR_{50}$) of pM to μM, depending on the compound, cell line, and experimental conditions. It is not feasible to cover such a large span of concentrations with the relatively fine spacing needed for accurate curve fitting (~3-fold difference from one concentration to the next). Thus, it is often advisable to run a trial experiment to determine the approximate range of drug sensitivity for cell lines under study; 10-fold spacing in drug concentration is usually adequate for this test. Dose-ranging studies are followed by analysis of more narrowly spaced concentrations around the responsive range. With the increasing available of large-scale dose response datasets, it is also possible to look up appropriate concentration ranges for lines of interest (Barretina et al., 2012; Garnett et al., 2012; Haverty et al., 2016). When no prior information is available, our standard approach is to perform a 9-point dose-response assay ranging from 1 nM to 10 μM with half-$\log_{10}$ steps.

The range of concentration over which a drug needs to be assayed depends not only on $IC_{50}$/$GR_{50}$ value, but also on the slope of the curve, since with very shallow response curves (Hill Slope $<1$), the range is necessarily greater than for steep curves (Fallahi-Sichani et al., 2013). Ideally, the two lowest doses should elicit little or no response and the two highest doses should elicit maximal response. This facilitates robust curve fitting and estimation of $GR_{inf}$. It is not necessary for the spacing between concentrations to be equal across the dose range: by spacing concentrations closely within one order of magnitude above and below $GR_{50}$ and more widely thereafter, curve fitting is improved. With drug-resistant cell lines, however, the highest drug dose is usually limited by solubility, cost, or biological relevance, and may still lie in a steep part of the response curve. In such cases, the fitted values for $GEC_{50}$ and $GR_{inf}$ are unreliable and should be interpreted with caution, a point we discuss in more details in the accompanying article (Hafner et al., 2017).

The process of reliably and accurately dosing cells with drug over a wide concentration range is not trivial. We discourage researchers from using manual dilution unless absolutely necessary, as this has an unexpectedly large potential for error. The method of choice for large numbers of compounds and multiple cell lines is pin transfer, which is readily available at most high-throughput facilities. For fewer compounds, the Hewlett Packard D300 Digital Dispenser is a good choice. This instrument makes it possible to dispense as little as 11 pl of a stock solution in an individual well of a multi-well plate, thus obviating the need to create a dilution series (and thereby enhancing data integrity). Use of a drug dispenser makes it possible to randomize treatments across plates, which helps to correct for systematic experimental artifacts such as edge effects. The companion article describes computer scripts for automatically generating dose-series using the D300 dispenser as a way of saving time and reducing errors introduced by manual procedures (Hafner et al., 2017).

### Choice of Assays for Determining Viable Cell Number

To quantify the effects of perturbations on cell viability, for example for anti-cancer drugs, it is necessary to determine the number of living cells at the end of the assay.

While the simplest and most direct method for accomplishing this is to count viable cells with a microscope, the use of proxy measurements is common. Proxy measurements include quantification of cytosolic reducing potential with resazurin (O'Brien et al., 2000), activity of NADPH-dependent oxidoreductase enzymes using tetrazolium compounds (Mosmann, 1983), ATP concentrations using CellTiter-Glo (Tolliday, 2010), or DNA content using crystal violet, PicoGreen, Hoechst, or CyQuant, most of which are available from multiple commercial vendors. Under most circumstances, these measures correlate well with each other and with cell count, but they are not identical, and, not surprisingly, will give discrepant results with drugs that block cell proliferation but not cell growth (Chan, Kleinheinz, Peterson, & Moffat, 2013; Quent, Loessner, Friis, Reichert, & Hutmacher, 2010). The alternative and very cost-effective approach is simply to count cells using a high-throughput microscope. This method has excellent sensitivity (down to a few cells per well), a dynamic range that routinely exceeds $10^4$ in 384 well plates, is inherently linear over the complete range of the assay, and can report on the total number of cells in a well if the complete well surface is imaged. Therefore, direct counting is our preferred means of determining cell number.

For all proxy measures, take care that assays operate in the linear range and are calibrated against samples containing a known number of cells. It is particularly important to discriminate between the level of signal corresponding to the initial number of cells in the assay (which might be quite low) and a true no-cell control. This difference represents the dynamic range for the measurement of cytotoxic responses. It seems likely that a failure to make this distinction (often due to inadequate calibration of surrogate assays) is one reason why cytostatic and cytotoxic responses are not clearly distinguished in much of the literature. Direct cell counting suffers no such limitations and makes these differences self-evident. It is also possible to increase the information content of phenotypic assays, for example, by measuring the fraction of senescent or apoptotic cells using dyes or immunofluorescence (Tang, 2014). However, methods for performing such assays reliably in multi-well plates are beyond the scope of the present article.

### Choosing Endpoint or Live-Cell Assay

In most cases, drug responses are measured using Basic Protocol 1, which only uses cell number at the time of drug addition and at the endpoint to estimate effects averaged over the duration of the assay. To make Basic Protocol 1 effective and reliable in any particular setting, the experimental conditions should be adjusted such that cell growth remains roughly constant during the measurement period (see above). This basic protocol is simple to run with only minor modifications to existing practice, and is well suited to high-throughput measurement and screening.

If either the growth rate or response to perturbation varies significantly over time even with optimized conditions, Basic Protocol 2 is used to estimate a time-dependent drug sensitivity (Fig. 4). This can be undertaken in two ways. The first is simply to repeat Basic Protocol 1 with multiple plates, each of which is withdrawn from the incubator at a discrete point in time. This approach has the disadvantage that it increases the number of cells, drugs, and reagents in proportion to the number of time points. The data are also less reliable than with continuous time measurement. The alternative is to measure cell number by repeatedly withdrawing the same plate from an incubator and measuring cell number in a non-destructive way. Repeated measurement of a single plate has the benefit of reducing experimental variability arising from inevitable differences in the number of cells in each plate. In the time-lapse approach, cell number is typically measured every 4 to 12 hr, which is sufficient to reliably determine the division time of the cells. Drug response is then computed using time-dependent GR values (Hafner et al., 2016) or other approaches (Harris et al., 2016; Tyson, Garbett, Frick, & Quaranta, 2012). The use of robotic handling or environmental chambers obviates the need for frequent human

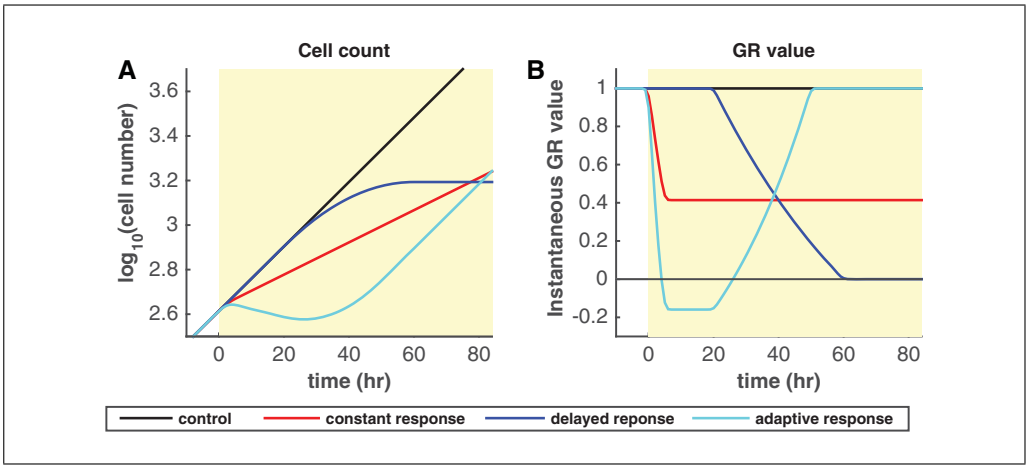


**Figure 4** Examples of GR values over time. $Log_{10}$ of cell number (left) and the resulting GR value (right) over time, showing the constant growth of an untreated control (black) and the reduced growth of a typical drug treatment that reduces the growth rate (red). A delayed response where a cell line initially does not respond to the drug, but later switches to complete growth inhibition (dark blue), or an adaptive response where a cell line responds early to the drug, but then recovers normal growth (light blue) demonstrate how the GR value can be time dependent. Note that end-point GR values at 72 hr are similar.

intervention and makes such an approach feasible. We are not aware of any large-scale datasets that report on time-dependent drug responses, but growing interest in real-time measurement of drug response suggests that rapid innovation can be expected in this area.

We generally distinguish two methods for deriving viable cell number in live-cell time-lapse experiments: cell counting with a fluorescent marker or confluence-based measurements. Confluence is typically assessed by bright-field or phase-contrast microscopy, but can also be measured by other means, such as the electrical impedance across microelectrodes in special tissue culture plates in the xCELLigence platform (ACEA Biosciences). In general, there is a reasonable correlation between degree of confluence and cell number, as long as the wells are sub-confluent. At high density, however, many cell lines continue to divide, with cells either becoming smaller or overgrowing each other. In these cases, proliferation is not accompanied by an increase in the degree of confluence. Furthermore, some perturbations can cause cells to change size (Chan et al., 2013), which also leads to discrepant results between confluence measurements and cell counts. For the highest accuracy in counting cells, we use cell lines labeled with fluorescent markers of the nucleus, such as histone H2B, using a fluorescence microscope suitable for live-cell measurements, such as the Operetta microscope with an incubator and robotic arm (Perkin Elmer) or an IncuCyte ZOOM (Essen Biosciences). Some specialized instruments, such as the IncuCyte ZOOM (or xCELLigence system), have the advantage that they can hold six micro-well plates at once and can be placed directly into a tissue culture incubator. They do not require any robotic automation or environmental chambers and minimize unwanted perturbation of growth conditions.

## The Value of Automation

Recent attempts in our lab to increase the reproducibility of dose-response assays have revealed the surprisingly important role played by automation in the original plating and treatment steps: even experienced bench scientists are not particularly good at repeatedly diluting drugs and pipetting cells. In some cases, the advantages of automation are obvious: plate fillers are much better than humans at ensuring an equal number of cells across a 384-well plate. In other cases, the advantages are subtler: the use of digital compound dispensers not only eliminates the error-prone process of diluting

drug libraries, but also allows geographic randomization of drug and dose across the plate. Cell-based assays in multi-well plates are subject to a variety of systematic errors involving edge and area effects, and the use of technical replicates each based on a different randomization pattern ensures that uneven growth will not correlate with dose across replicates. The cell counters, plate fillers, compound dispensers, and plate washers described in the current article are generally simple to operate and have prices in the range of 15,000 to 35,000 USD. Unlike sophisticated multi-purpose laboratory robots such as the BioMek (Beckman), these instruments do not require complex programming or staff scientists to operate. They are highly recommended for any laboratory or organization wishing to assay the responses of mammalian cells to perturbation on a regular basis.

## ENDPOINT MEASUREMENT OF DRUG SENSITIVITY

***BASIC PROTOCOL 1***

Basic Protocol 1 can be divided four general procedures: (1) seeding cells into multi-well plates and allowing them to adhere and proliferate, (2) measuring cell number just prior to drug addition, (3) adding drug to wells across a range of concentrations, and (4) measuring cell number at the end of a defined period, typically 3 days.

Basic Protocol 1 measures drug sensitivity using a classical endpoint assay in which final cell number relative to initial cell number is determined at a single point in time. Perhaps surprisingly, it has become common to collect data from control and drug-treated cells only at the end of the experiment, omitting a $t = 0$ control (Barretina et al., 2012; Garnett et al., 2012; Haverty et al., 2016). Data collected in this way is typically used to determine $IC_{50}$, $E_{max}$, or AUC values, but such metrics confound measures of drug sensitivity with natural variation in cell division rates (Hafner et al., 2016). Measurement of cell numbers at the time of drug addition, in contrast, enables calculation of GR (Hafner et al., 2016) and GI (Heiser et al., 2012) response metrics, which are much less affected by variation in division rates. It is also possible to estimate the cell number at $t = 0$ from endpoint data in control cells using existing division rate data for each cell line used *under the same conditions* as the drug response assay (Hafner et al., 2016). However, there are so many variables in collecting good dose-response data that we strongly believe in collecting contemporaneous $t = 0$ data. Such data also provide important information on cell density and should be reported as part of the dose-response measurement. In a subset of drug-cell line pairs, cell density has a substantial impact on drug sensitivity independent of its impact on proliferation rate; the reasons for this remain largely unexplored but appear to involve biology related to drug mechanism of action (Chauffert et al., 1998; Dimanche-Boitrel, Garrido, & Chauffert, 1993; Fang, Sullivan, & Graham, 2007; Garrido et al., 1995; Hafner et al., 2016).

### *Materials*

MCF 10A cells (ATCC #CRL-10317) labeled with H2B-mCherry (Hafner et al., 2016)
Complete growth medium for MCF 10A cells (see recipe)
Phosphate-buffered saline (PBS; Thermo Fisher Scientific, cat. no. 21-040-CV)
0.25% Trypsin/2.21 mM EDTA in HBSS (Thermo Fisher Scientific, cat. no. 25-053-CL)
0.4% trypan blue (Thermo Fisher Scientific, cat. no. 15250-061)
10 mM stock solutions in DMSO of drugs for treatment (also see recipe):
  Etoposide (Selleck Chemicals, cat. no. S1225)
  Taxol (Selleck Chemicals, cat. no. S1150)
  Trametinib (Selleck Chemicals, cat. no. S2673)
  Neratinib (Selleck Chemicals, cat. no. S2150)
Staining solution (see recipe)
Fixation solution (see recipe)

15-cm tissue culture–treated Petri dishes
50-ml conical polypropylene tubes (e.g., Corning Falcon)
TC20 Automated Cell Counter (BioRad; preferred) or hemacytometer and suitable microscope (also see Phelan & May, 2015)
CellCarrier tissue culture–treated optical clear-bottom 384-well plates (PerkinElmer, cat. no. 6007558)
Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific, cat. no. 5840300; preferred) or multi-well pipettors
D300 Digital Dispenser (Hewlett-Packard; preferred) or multiwell pipettors
Operetta High-Content Imaging System (Perkin Elmer) or other microscope system able to read multi-well plates, such as the IN Cell Analyzer (General Electric) or the ImageXpress System (Molecular Devices)
EL406 Washer Dispenser (BioTek; preferred) or other automated plate washer/dispenser
Microseal 'F' Sealing Foil plate seals (Bio-Rad, cat. no. MSF1001)
Columbus Image Data Storage and Analysis System (PerkinElmer) or alternative software to automate counting of viable cells, such as Cell Profiler (Carpenter et al., 2006)

Additional reagents and equipment for cell culture including trypsinization and cell counting (Phelan & May, 2015)

*NOTE:* The materials listed here were used to create the MCF 10A data presented in this protocol, but different cell lines require the use of different reagents. We encourage users, if possible, to run control experiments with MCF 10A cells as a means of calibrating their results against ours.

***Seed cells into multi-well plates***

1. Grow cells in complete growth medium to mid–log phase in 15-cm tissue-culture-treated Petri dishes (Phelan & May, 2015).

   *The cells should be actively dividing and not confluent.*

   *Whenever possible, we follow the recommendations for cell growth provided by the ATCC for each cell line.*

2. To harvest cells, remove growth medium, wash cells once with 10 ml of PBS, and incubate in 1.5 ml of 0.25% trypsin/2.21 mM EDTA at 37°C until cells detach from the plate (also see Phelan & May, 2015).

   *Whenever possible, we follow the recommendations for cell harvesting provided by the ATCC for each cell line.*

3. Pellet cells 3 min at 200 × *g*, 25ºC, in a 50-ml sterile, conical tube.

4. Carefully remove supernatant and resuspend cells in 10 ml of complete growth medium by pipetting gently.

5. Pellet cells 3 min at 200 × *g*, 25ºC.

6. Carefully remove supernatant and gently resuspend cells in 1 to 3 ml of complete growth medium to achieve a final concentration of approximately $10^6$ cells/ml.

7. Remove 10 µl of cells and counterstain dead cells with 10 µl of 0.4% trypan blue (also see Phelan & May, 2015).

8. Count the number of live cells using a TC20 cell counter or hemacytometer and suitable microscope (live cells are those that are trypan blue negative; also see Phelan & May, 2015).

9. Dilute cells to 12,500 viable cells/ml in growth medium.

   *This cell number should be determined in preliminary experiments as described above. The starting cell density for the experiment is dependent on multiple parameters of the experimental setup, such as the cell line, growth conditions, and length of the experiment. 12,500 cells per ml is appropriate for MCF 10A cells and yields 750 cells/well in 60 µl of medium.*

10. Seed four CellCarrier 384-well plates with 750 cells/well in 60 µl complete growth medium.

    *For cell counts performed with the Operetta High-Content Imaging System, we use PerkinElmer CellCarrier 384-well plates. For cell counts performed with the IncuCyte ZOOM System, we use Corning 3712 384-well plates. Other plates also work, but care needs to be taken that the chosen plates are compatible with the downstream assay (e.g., clear-bottom for microscopy assays).*

    *Experimentation with different types of plates and different vendors is highly recommended. We test individual lots of plates and purchase only lots that pass QC for even growth in all wells.*

    *We generally run all our drug-response assays in at least three technical triplicates. Technical repeats should be performed on at least two different plates.*

11. Incubate cells for 48 hr.

    *The duration of the initially growth step needs to be determined empirically in preliminary experiments such that at the end of the incubation period cells are asynchronously dividing and not confluent.*

### *Perform drug treatment*

12. At the time of drug treatment, retain one untreated plate to determine the cell number at $t = 0$, which is defined as the time of drug addition (see 'Perform cell count', below).

    *Counting $t = 0$ plates is often omitted, but we very strongly recommend collecting this data.*

13. Treat cells in the remaining three plates with the four drugs (etoposide, taxol, trametinib, neratinib) ranging over approximately four orders of magnitude using a D300 drug dispenser (see Supplemental Data 2 for the concentrations used here).

    *To lessen the impact of uneven growth, researchers can leave the outermost one or two rows and columns untreated. In addition, drug concentrations should be randomized across the plate. Automated scripts to generate randomized layout are described in the companion manuscript (Hafner et al., 2017).*

    *Drugs can be dispensed by other means, such as via multichannel pipettors. We have examined the consequences of this with care and determined that manual dispensing substantially increases the potential for error. Furthermore, manual pipetting is not recommended because it does not allow for randomization.*

14. Incubate cells in the presence of drug for 48 hr.

    *The duration of the drug incubation step needs to be determined empirically in preliminary experiments. Untreated control cells should divide at least once in the period and the growth rate should be uniform over the course of the assay.*

15. At the end of the incubation period, determine the cell number (see 'Perform cell count', below).

***Perform cell count***

16. At the times indicated above ($t = 0$ and at the end of drug incubation), count the cells using high-throughput microscopy as described in steps 17 to 24, below.

    *As described earlier, proxy measurements can be substituted to estimate the count of viable cells. These assays should be performed according to vendor specifications or papers describing the method. In all cases, care should be taken that cell numbers fall into the linear range of the assay. Since drug responses are often density dependent, reproducibility requires that values for proxy assays be converted into cell number using calibration curves.*

17. Add 20 µl of staining solution.

    *To dispense the staining solution, we recommend using a manual or electronic multichannel pipettor held at a 45° angle and touching the side of the well. This will allow the dense staining solution to sink to the bottom of the well and displace the medium for optimal staining of the cells.*

18. Incubate for 30 min at room temperature.

19. Add 20 µl of fixation solution.

    *To dispense the fixing solution, we recommend using a manual or electronic multichannel pipettor held at a 45° angle and touching the side of the well. This will allow the denser fixing solution to sink to the bottom of the well and displace the staining solution, which is necessary to avoid staining of live cells with the LIVE/DEAD fixable dye after they become permeabilized by exposure to formaldehyde.*

20. Incubate for 30 min at room temperature.

21. Remove 90 µl of supernatant and replace with 90 µl of PBS using a BioTek EL406 Washer Dispenser.

    *This step reduces imaging background noise, but is not strictly necessary for the cell lines we have worked with so far.*

    *If desired, this step can be performed manually, but automation increases the consistency and ease of the assay.*

22. Seal plates using Microseal 'F' Sealing Foil plate seals.

    *Other seals can be substituted as long as they prevent evaporation of the PBS.*

23. Scan all treated wells with a High-Content Imaging System and transfer scanned images to a Data Storage and Analysis System (e.g., Perkin Elmer Columbus or Cell Profiler).

    *For every well, we scan six non-overlapping fields with a 10× high-NA lens in two channels to detect Hoechst (excitation, 360 to 400 nm; emission, 410 to 480 nm; exposure, 100 msec) and LIVE/DEAD far red dye (excitation 620 to 640 nm; emission 650 to 700 nm; exposure 100 msec).*

    *Scanning the complete well decreases experimental noise and gives absolute numbers of cells per well.*

    *Any high-throughput microscope can be substituted, as long as it allows to accurately identifying viable cells (positive for a Hoechst-stained nucleus, but negative for the LIVE/DEAD dye).*

24. Count the number of viable cells using appropriate image analysis tools. In Columbus, the steps are as follows:

a. All detected nuclei → Find Nuclei (Hoechst channel)—Method 'B': Common Threshold = 0.4; Area > 30 μm²; Split Factor = 7; Individual Threshold = 0.4; Contrast > 0.1.
b. Calculate Texture Properties (LIVE/DEAD far red channel)—Method 'SER Features': Scale = 8 px; Normalization by Region Intensity; SER Spot.
c. Number of viable cells → Select Population (all detected nuclei)—Method 'Filter by Property': Nucleus Alexa 647 SER Spot 8 px < 0.0001.

*It is critical to verify the exact settings of image analysis, even if the same instrument is being used, as different cells, lots of dyes, or scanners may result in differing results.*

*Other software or detection algorithms can be substituted. They should be benchmarked against compounds with known activity and the results confirmed by manual counting.*

***Analysis***

25. For each small-molecule treatment, use the number of viable cells to determine the mean normalized growth rate inhibition (GR) values across all tested concentrations. Fit the results to a sigmoidal curve to extract the $GR_{50}$, $GR_{max}$, $GR_{AOC}$, $GEC_{50}$, $GR_{inf}$, and $h_{GR}$ coefficient.

    *For further details and automated scripts, please consult Hafner et al. (2017) and the original publication describing the GR metrics (Hafner et al., 2016).*

26. If required, fit the mean relative cell counts across the tested concentrations for each small molecule to a sigmoidal curve to extract the $IC_{50}$, $E_{max}$, AUC, $EC_{50}$, $E_{inf}$, and Hill coefficient.

    *Conventional response metrics such as $IC_{50}$ are valuable with drugs that are rapidly cytotoxic so that there is no appreciable cell division.*

## TIME-DEPENDENT MEASUREMENT OF DRUG SENSITIVITY

***BASIC PROTOCOL 2***

Basic Protocol 2 is designed to determine the response of cells to perturbation over time. Optimal results are achieved by automated imaging of live cells using a microscope, since this enables non-destructive monitoring. Repeated measurement of the same set of plates saves materials and decreases experimental error arising from variation in plating and treatment. Time-dependent drug sensitivity can also be determined by following Basic Protocol 1 and simply increasing the number of timepoints at which endpoint measurements are performed. The advantage of the latter approach is that it does not require any specialized instrumentation.

Time-lapse imaging can be performed using both labeled and unlabeled cells. Cell counting and tracking are greatly simplified and far more accurate if cells are transfected with a fluorescent protein label (e.g., H2B-mCherry, NLS-GFP); however, in experiments with a large number of cell lines, this might not be feasible. Plates can be manually moved from an incubator to the high-throughput microscope for scanning and then returned to an incubator. The use of a robotic workstation that transfers plates automatically from an incubator to a high-throughput microscope with an environmental chamber greatly facilitates such measurements and generally provides the most consistent data. Thus, the method described below assumes access to a microscope such as an Operetta High-Content Imaging System (PerkinElmer) with an environmental chamber, attached robotic arm, and tissue culture incubator. However, such solutions are costly, require dedicated space and maintenance, and are thus not options for many laboratories. A cheaper and more readily available method of collecting accurate time-dependent dose response data is to use a microscope that is placed in the incubator, such as an IncuCyte ZOOM System.

***Additional Materials*** *(also see Basic Protocol 1)*

Complete microscopy growth medium (see recipe)
YOYO-1 Iodide (ThermoFisher Scientific, Y3601)
Operetta High-Content Imaging System (Perkin Elmer) with an environmental chamber, attached robotic arm, and tissue culture incubator

***Seed cells into multiwell plates***

1. Steps 1 to 10, which involve growing, washing, and plating cells, are the same as in Basic Protocol 1, with the exception that only three plates are needed as the $t = 0$ plate can be omitted, and complete microscopy growth medium should be used in place of the regular complete growth medium.

   *We generally only use the microscopy medium when actually imaging the cells. The cells are cultured and expanded as described in regular complete growth medium.*

***Drug treatment***

2. Treat cells in the three plates with the four drugs (etoposide, taxol, trametinib, neratinib) ranging over approximately four orders of magnitude, as described in Basic Protocol 1, and with 250 nM YOYO-1 using a D300 drug dispenser.

   *To lessen the impact of uneven growth, researchers can leave the outermost one or two rows and columns untreated. In addition, drug concentrations should be randomized across the plate. Automated scripts to generate randomized layout are described in the companion manuscript (Hafner et al., 2017).*

   *Drugs can be dispensed by other means, such multi-channel pipetting. We have examined the consequences of this with care and determined that manual dispensing substantially increases the potential for error and manual pipetting does not allow for randomization and is not recommended.*

3. Place plates into a tissue culture incubator and set up the robotic system to scan each plate at 4-hr intervals for 48 hr using an Operetta High-Content Imaging System with an environmental chamber, and transfer scanned images to a Data Storage and Analysis System (e.g., Perkin Elmer Columbus or Cell Profiler).

   *The duration of the drug incubation step needs to be determined empirically in preliminary experiments. Once the growth rate of the untreated control cells slows down significantly, time-dependent metrics of drug response become less reliable.*

   *For every well, we scan four non-overlapping fields with a 10× high-NA lens in two channels to detect mCherry (excitation 560 to 580 nm; emission 590 to 640 nm; exposure 250 msec) and YOYO-1 (excitation 460 to 490 nm; emission 500 to 550 nm; exposure 10 msec).*

   *Scanning the complete well decreases experimental noise and gives absolute numbers of cells per well.*

   *Any high-throughput microscope can be substituted as long as it allows accurate identification of viable cells (positive for a mCherry-stained nucleus, but negative for the YOYO-1 dye).*

***Cell count***

4. Count the number of viable cells using appropriate image analysis tools. In Columbus, the steps are as follows:

   a. All detected nuclei → Find Nuclei (mCherry channel)— Method ‘B’: Common Threshold = 0.4; Area > 30 μm²; Split Factor = 1; Individual Threshold = 0.4; Contrast > 0.1.
   b. Calculate Intensity Properties (YOYO-1 channel)—Method ‘Standard’, ‘Mean’.

c. Number of viable cells → Select Population (all detected nuclei)—Method 'Filter by Property': Intensity Nucleus YOYO-1 channel < 1500.

*It is critical to verify the exact settings of image analysis, even if the same instrument is being used, as different cells, lots of dyes, or scanners may result in differing results.*

*Other software or detection algorithms can be substituted. They should be benchmarked against compounds with known activity and the results confirmed by manual counting.*

### *Analysis*

5. For each small-molecule treatment, use the number of viable cells to determine the time-dependent normalized growth rate inhibition (GR) values across all tested concentrations and all measured time points. Fit the results to a sigmoidal curve to extract the time-dependent $GR_{50}$, $GR_{max}$, $GR_{AOC}$, $GEC_{50}$, $GR_{inf}$, and $h_{GR}$ coefficient.

*For further details and automated scripts please consult the companion paper (Hafner et al., 2017), and the original publication describing the GR metrics (Hafner et al., 2016).*

*There are other ways to analyze drug response assays using time-lapse microscopy (Harris et al., 2016; Tyson et al., 2012); however, we have not tested these methods.*

## REAGENTS AND SOLUTIONS

*Use Milli-Q filtered water for all solutions.*

### *Complete growth medium for MCF 10A cells*

Prepare media according to ATCC guidelines when available. For MCF 10A cells we use the following medium:
DMEM/F12 (1:1; Thermo Fisher Scientific, cat. no. 11330) supplemented with:
5% horse serum (Thermo Fisher Scientific, cat. no. 16050-122)
20 ng/ml human epidermal growth factor (PeproTech, AF-100-15)
0.5 mg/ml hydrocortisone (Sigma-Aldrich, cat. no. H0888)
100 ng/ml cholera toxin (EMD Millipore, cat. no. 227036)
10 μg/ml insulin (Sigma-Aldrich, cat. no. I1882)
100 U/ml penicillin-streptomycin (Thermo Fisher Scientific, cat. no. 15140-122)

*Medium can be stored at 4°C for approximately 1 month.*

### *Complete microscopy growth medium for MCF 10A cells*

Standard cell culture media contain colored and fluorescent compounds that interfere with imaging. For live-cell imaging, medium lacking fluorescent compounds and specifically made for imaging should therefore be used. In the case of MCF 10A cells, DMEM/F12 (Invitrogen, cat. no. 11330-032) is substituted with a 1:1 mixture of FluoroBrite DMEM (Thermo Fisher Scientific, cat. no. A18967) and Ham's F-12 (Corning, cat. no. 10-080-CV). Medium can be stored at 4°C for ∼1 month. Fixed cell studies (Basic Protocol 1) should be used to confirm that any change in medium does not have a significant impact on drug response.

### *Drug stock solutions*

Prepare drug solutions according to guidelines provided by the seller. Most commonly, drugs are suspended at a final concentration of 10 mM in deuterated dimethyl sulfoxide (DMSO-d6; Sigma-Aldrich, cat. no. 151874). This makes it possible to confirm purity of the compounds by liquid chromatography–mass spectrometry (LC-MS) analysis. Unless otherwise indicated, compounds are stored in small aliquots at −20°C. Because cells are affected by DMSO and other solvents, it is important to keep the total concentration of the drug vehicle constant across all assay conditions (Timm, Saaby, Moesby, & Hansen, 2013). Note also that some drugs are inactivated

*continued*

by DMSO, most notoriously cisplatin, carboplatin, and other platinum complexes (Hall et al., 2014), and must be dissolved in water. Anhydrous DMSO will equilibrate with water present in the atmosphere, gradually reducing drug concentration and potentially inducing compound degradation (Lipinski, 2008).

### *Fixation solution*

3% (v/v) formaldehyde (prepare from 36.5% to 38% formaldehyde; Sigma Aldrich, cat. no. F8775-500ML)
20% (v/v) OptiPrep (Sigma-Aldrich, cat. no. D1556-250ML)
77% (v/v) phosphate-buffered saline (PBS; Thermo Fisher Scientific, cat. no. 21040CV)

*Prepare the solution on the day it is to be used.*

### *Staining solution*

10% (v/v) OptiPrep (Sigma-Aldrich, cat. no. D1556-250ML)
90% phosphate-buffered saline (PBS; Thermo Fisher Scientific, cat. no. 21040CV)
Add 1:1000 LIVE/DEAD Fixable Far Red Dead Cell Stain (Thermo Fisher Scientific, L-34974) and 2 μM Hoechst 33342 (Thermo Fisher Scientific, 62249) to the solution of 90% PBS with 10% OptiPrep. Prepare the solution on the day it is to be used.

## COMMENTARY

### Discriminating Between Sensitive and Resistant Cell Lines

Application of many machine-learning methods, in pharmacogenomics for example, requires that cell lines or drugs be sorted into binary classes corresponding to sensitive and resistant. However, if actual dose-response data are compared across multiple cell lines (or multiple drugs in a single cell line), the distribution is usually continuous and frequently unimodal. In this case, labels such as sensitive and resistant are arbitrary. In addition, the values of response parameters such as $GR_{50}$ or $IC_{50}$ can be highly context-dependent, varying with cell density, growth conditions, components of the microenvironment, etc. The tendency to treat such contextual parameters as constants has little basis in reality. The key is to precisely measure and then understand the molecular basis of context dependence and use this information to improve the concurrence between in vitro and in vivo measures of drug response. The protocols we describe are designed to characterize, control, and record context as a means to improve the reliability of drug-response data.

### Troubleshooting

Common problems in measuring drug sensitivity in cell lines and potential ways to overcome them are described in Table 1. Common themes include: (1) perform each assay in technical replicates (three or more) and perform at least two biological replicates, (2) randomize the treatment layout, and (3) automate as many steps as possible, including the generation of treatment protocols and data handling.

### Experimental Error and Plating Artifacts

Uneven cell numbers across a plate can introduce significant errors into drug-response experiments, and it is therefore important to test the uniformity of cell plating and cell growth. To this end, prepare two plates according to the conditions best for a particular cell line but omitting drug. Count cell number at a fixed point in time and compare estimated growth rates across wells. Common plating anomalies fall into three categories: (1) edge effects, most likely caused by increased evaporation, cooling, or gas exchange at the edge of the plate; (2) inconsistency by row or column caused by inaccurate pipetting during plating, either due to poor technique or problems with instrumentation; and (3) area effects caused by pooling of cells in specific regions of the plate, uneven incubation conditions, or plate artifacts. We find that plates from major vendors differ dramatically in performance, and we therefore test each batch of plates prior to using them in large-scale studies.

Uneven cell dispensing along rows or columns can occur during plating and warrant examination of plating procedures.

**Table 1** Common Problems and Potential Solutions for Troubleshooting Drug-Response Measurements

| Problem | Potential solution |
|---|---|
| Experimental noise; experimental error | Automate treatments and analysis. Confirm accurate staining. Perform randomized technical replicates and multiple independent biological experiments. Increase cell number per well to reduce stochastic variations (see discussion below). |
| Uneven cell numbers across plates; edge effects | Automate cell plating. Incubate plates in humidified, secondary containment. Leave edge wells out of the treatment procedure. Randomize treatments across technical replicates (see discussion below). |
| Growth slows at the end of the experiment | Decrease cell number at the time of plating. Reduce the length of the experiment. |
| Delayed growth at the beginning of the experiment; synchronized cell division | Use cells that are not confluent to seed for experiment. Treat cells as gently as possible during plating. Increase the number of plated cells. Increase delay between plating and drug treatment. |

Edge and area effects across a plate that occur due to differential cell growth after plating can sometimes be ameliorated by secondary containment of the plates within the incubator; if this does not help, then the best option is to simply to skip the outer one or two rows and columns on each plate. Importantly, the impact of any plating problem can be greatly reduced by geographic randomization of doses across a plate combined with technical replicates involving different randomization patterns. Under these circumstances, position in the plate and resulting difference in growth will not be correlated with drug dose.

## Anticipated Results

The expected outcome of Basic Protocol 1 is a series of GR values across a range of drug concentrations, which can be summarized as $GR_{AOC}$ and $GR_{max}$ values or parameterized to report the $GR_{50}$, $h_{GR}$, $GR_{inf}$, and $GEC_{50}$ values (Hafner et al., 2016) as described in Hafner et al. (2017), the HMS LINCS Web site (*http://lincs.hms.harvard.edu/hafner-natmethods-2016/*), and the GR Calculator Web site (*http://www.grcalculator.org/*). Basic Protocol 2 can be analyzed in an analogous fashion to Basic Protocol 1 at each time point, so that the evolution of response parameters over time can be observed.

Careful optimization of drug dose-response experiments using the protocols described here should result in difference in response parameters of ∼2-fold between biological repeats, dramatically better than the repeatability reported in the literature (in which 10- to 50-fold variation is not uncommon). Should larger variation be observed, it is likely that a confounder is involved, such as growth condition or plating density. As an example, the results that can be achieved are shown for MCF 10A cells exposed to four common anticancer drugs measured by both endpoint measurements and time-lapse measurements (Fig. 5; Supplemental Data 3).

## Time Considerations

The full procedure will take approximately 1 week to execute, from plating the cells to collecting a parameterized response. However, the workload on successive days is low and assays are easily parallelized, so many compounds or cell lines can be tested at once. Basic Protocol 1 entails ∼1 hr for cell plating, ∼1 hr for drug treatment, and ∼1.5 hr for staining and fixation. The time required to scan plates depends on the instrument used, but is usually ∼2 hr per 384-well plate. If data is collected in triplicate on a 9-point dose-response curve, throughput should be about 200 drugs in a single cell line per week, and higher if multiple experiments can be staggered. In general, the greatest bottleneck in direct cell counting is microscopy time (our time estimates pertain to an Operetta High-Content Imaging System equipped with an automated plate stacker). Data analysis is both more rapid and more accurate if scripts are used (Hafner et al., 2017). Generating treatment files should

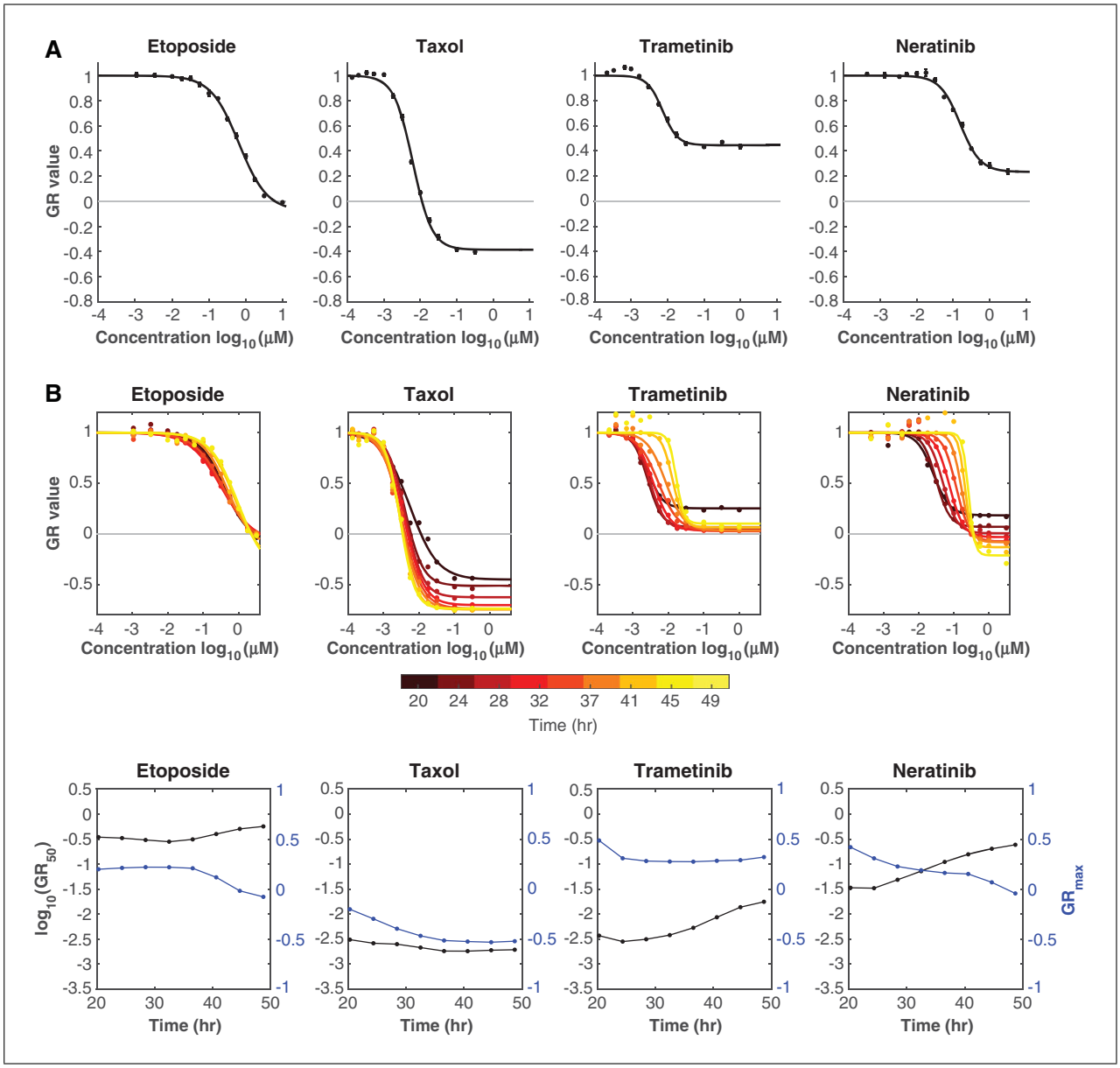


**Figure 5** Example of results. MCF 10A cells were treated with four common anti-cancer drugs (etoposide, taxol, trametinib, or neratinib) and drug responses determined at an endpoint using Basic Protocol 1 (**A**) or over time using Basic Protocol 2 (**B**). Panel A shows the GR value by treated dose at the endpoint of the experiment and a fitted sigmoid dose response curve for each drug. Panel B shows the fitted dose response curves as in A at different time points (top) and the evolution of the $GR_{50}$ and $GR_{max}$ over time (bottom).

not take more than 30 min, and analysis requires approximately 1 hr.

## Supporting Materials

All supplemental data discussed in this article can only be accessed from the online version of this article.

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## Key References

Hafner et al. (2016). See above.

*This paper describes the theory behind GR metrics as a means to parameterize drug sensitivity. Explains why they are superior to classical metrics based on relative cell counts, and shows how GR data can be applied to gain insights into biological mechanisms of drug response.*

Hafner et al. (2017). See above.

*This article is the companion to the present article and focuses on the computational pipeline needed to run drug-response experiments effectively. It can be used independently of the protocols described here, but for optimal results, both methods should be implemented together.*

Haibe-Kains et al. (2013). See above.

*This work describes inconsistencies among recently published, large-scale, pharmacogenomic studies. The authors identify significant discrepancies between the two studies.*

Haverty et al. (2016). See above.

*This manuscript explores the reproducibility of large-scale pharmacogenomic profiling efforts, paying particular attention how variations in the experimental procedure can affect the outcome of drug-response measurements.*

## Internet Resources

http://lincs.hms.harvard.edu/hafner-natmethods-2016/.

*This is the landing page on the Harvard Medical School LINCS Web site for the paper describing the GR metrics (Hafner et al., 2016). It summarizes the method, provides all data from the paper, and links out to additional tools related to calculating GR metrics.*

http://www.grcalculator.org/

*The GR Calculator Web site provides explanations of GR metrics, examples of dose-response datasets collected by the LINCS consortium, and the option of uploading a user's own data to automatically extract the GR metric parameters using the online GR calculator tool.*