Preface

High-throughput screening (HTS) has seen over a decade of deployment in the pharmaceutical industry as an early discovery platform to feed lead compounds into pipelines culminating in the development of therapeutic agents. In recent years, interest in HTS within the academic community has increased dramatically, as an engine of discovery for potential drug candidates, for novel probes to understand fundamental biological processes, and for developing cheminformatic rules governing the interaction between chemistry and biology. A number of academic screening centers have existed for several years, including those members of the NIH-sponsored Molecular Library Probe Production Centers Network and the NCI-sponsored Initiative for Chemical Genetics, with many more facilities in the planning and start-up stages. As HTS develops into a legitimate academic pursuit, the need for reference materials regarding the philosophy and practice of screening becomes ever more pressing. Although a large proportion of screening experiments reported in the literature has been based on biochemical interactions between small molecules and purified proteins, a rich body of literature has developed around cell-based assays. We have built upon this foundation to create an easily accessible reference volume for cell-based phenotypic screening.

We encourage the reader to view this reference in a modular way. Although each chapter presents an individual protocol peculiar to the assays being discussed, in groups they represent the four governing principles of this text: (1) model biological systems, (2) screening modalities and assay systems, (3) detection technologies, and (4) approaches to data analysis. Each chapter begins with an overview of the relevant component of HTS, providing examples of its use as well as appropriate considerations and caveats. Each chapter then presents state-of-the-art methods in terms of actionable protocols; we anticipate that the reader will be interested in direct application of the methods presented.

Taken together, the methods presented in this reference can further be used in a modular fashion, culling one chapter per section to design new screens on the basis of published methods. For example, a researcher considering a fluorescent dye-based assay in mammalian cell culture might consult both Chap. 3 (Mayer et al.) on screening with mammalian cells, and Chap. 7 (An) on fluorescent dyes. Similarly, one interested in high-content imaging of zebrafish might benefit most from chapters 4 (Hong) on zebrafish screening, and 14 (Carpenter) on extracting rich information from images. We feel that this reading technique will allow researchers to take a modular approach to the design of their assays. Rather than mimic a particular screen of interest exactly, researchers might apply theory and design principles from several sections of this reference to the development of novel and creative ways of addressing biological questions using HTS.

In summary, we expect that this reference will serve three purposes. First, each chapter will present an overview of relevant approaches taken in this relatively young field. Second, each chapter will provide sufficient methodological detail to enable direct application of existing methods to new discoveries. Third, the book itself will inspire researchers to approach their screening projects in a conceptually modular fashion, enhancing the power for discovery through new combinations of existing approaches.

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Chapter 2

High-Throughput Screening of Model Bacteria

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Summary

Small-molecule screening campaigns of model bacteria have been conducted extensively in biotechnology and pharmaceutical companies to search for novel compounds with antibacterial activity. Recently, there has been increasing interest in running such high-throughput screens within academic settings to answer questions in biology. In this respect, whole-cell screening has the particular advantage of identifying compounds with physical and chemical properties compatible with microbial cell permeation, thereby providing probes with which to study diverse aspects of microbial cell physiology and biochemistry. The focus of this chapter is to describe a general method of running a high-throughput screen against a model bacterium to identify small molecules with growth inhibitory activity. Once the primary bioactives have been identified, the determination of their dose–response relationships with the target microbe further characterizes their growth inhibitory effect.

Key words: Small-molecule screening, Model bacteria, Small-molecule library, Growth inhibition, Primary screening, Minimum inhibitory concentration, EC_{50}, Median effective concentration, *E. coli*.

1. Introduction

1.1. Small-Molecule Screens of Model Bacterial Systems

We restrict our scope in this chapter to small-molecule screening of model bacteria, e.g., *Escherichia coli* and *Bacillus subtilis*. The study of these model microbes has contributed greatly to fundamental knowledge in cell biology as well as an understanding of bacterial physiology, infectious disease, immunology, and genetic engineering. Owing primarily to ease of culture and genetic tractability, model microbes have attracted enormous experimental attention and have provided extraordinary insights into basic biology.

Among the prokaryotes, *E. coli* and *B. subtilis* represent highly tractable models for Gram-negative and Gram-positive bacteria,

P.A. Clemons et al. (eds.), *Cell-Based Assays for High-Throughput Screening, Methods in Molecular Biology, Vol. 486*
© Humana Press, a part of Springer Science+Business Media, LLC 2009
DOI: 10.1007/978-1-60327-545-3_1

respectively. While the study of *E. coli* has fundamentally transformed the field of molecular biology and genetic engineering, it has likewise provided extraordinary advances in our understanding of basic bacterial physiology. *E. coli* has also proven to be an ideal model for the study of processes fundamental to all life, including DNA replication, protein translation, and general metabolism. *E. coli* was among the first organisms for which genome sequence information became available. In addition to classical genetics, state-of-the-art tools for its study continue to emerge. These include highly annotated sequence information *(1)* as well as genome-scale gene-deletion *(2)* and protein-expression libraries *(3)*. These new tools will become increasingly important in forward chemical-genetic studies to sort out mechanisms of action of biologically active molecules.

While screening in bacterial systems has most often been implemented to find small molecules that inhibit growth, such efforts have been limited for the most part to researchers in pharmaceutical and biotechnology companies. Only relatively recently has small-molecule screening emerged as a tool in academic biological research *(4, 5)*. Therefore, we have focused here on screening for molecules that are growth inhibitory to model bacteria, where the goal is to yield a rich supply of bioactive molecules that could be further characterized with mechanistic studies.

Below we describe methodologies outlining (i) the development of a sensitive and robust growth-inhibition assay; (ii) a screening campaign against a library of small molecules; and (iii) follow-up studies of active compounds with the target bacteria. Note that active compounds from the primary screen can be subjected to a number of different tests before moving to the more challenging task of identifying their cellular targets and their mechanism of action. Of these tests, we describe the determination of the dose-response relationship and the associated parameters MIC and EC_{50}.

1.2. Preamble to the Methodology Provided

The instructions herein describe how to screen a library of small molecules against a model microbe. For this purpose, the following assumptions have been made:

1. Any necessary subcloning of the microbe of interest has been completed.
2. Appropriate control assays have been established.
   (a). Negative control: a measure of the assay signal in the absence of any interfering small molecules;
   (b). Positive control: a measure of the assay signal in the presence of a small molecule with the desired effect (i.e., this effect could be a decrease or increase in assay signal, depending on whether inhibitors or activators, respectively, are sought) (*see* **Note 1**).

3. The assay has been adapted to microtiter plates.
4. An automated liquid handler will be used for all liquid transfers. Specific methods for such handlers are not included here.
5. Library compounds are dissolved in dimethylsulfoxide (DMSO).
6. The final concentration of compounds to be tested in each assay has been established. For microbial systems, typical concentrations range from 1 to 5 μM for natural products and known bioactive compounds, to 10–20 μM for diverse synthetic molecules.

For these instructions, the following story is used as an illustrative example. A strain of *E. coli* subcloned with a vector that expresses a gene conveying resistance to kanamycin is screened to identify novel growth inhibitors. The assay is performed in duplicate in separate 96-well clear microtiter plates with a final volume of 100 μL (2 μL of 1 mM tested compound or neat DMSO, plus 98 μL of bacterial culture), with 20 μM final compound concentration tested. The assay is monitored for bacterial growth, as determined by absorbance at 600 nm (i.e., A_{600}). Negative controls (100% growth) contain *E. coli* and neat DMSO; positive controls (0% growth) contain *E. coli* and a lethal drug, in this case ampicillin (100 μg/mL) or chloramphenicol (25 μg/mL) in DMSO. All assays contain 2% DMSO (v/v). Library compounds are housed in storage plates in neat DMSO within columns 2–11 only; columns 1 and 12 are empty. All liquid transfers are carried out with a Biomek FX (Beckman/Coulter) using disposable tips.

2. Materials

2.1. Preparation of Bacterial Culture

1. Appropriate frozen bacterial stock.
2. Sterile test tubes.
3. Luria-Bertani (LB) media: 10 g of bacto-tryptone, 5 g of bacto-yeast extract, and 10 g of NaCl per liter of deionized water. The media is distributed into 100–500 mL aliquots in screw-cap glass bottles or capped glass flasks, then sterilized for 45 min by autoclaving on liquid cycle. Once the media cool down, supplement it with, if applicable, appropriate concentration of drug used for selection (for this example, 50 μg/mL kanamycin) *(6)*.
4. Agar plates: 1.5% bacto-agar in LB media is sterilized by autoclaving for 45 min on liquid cycle. Before pouring the agar plates into Petri dishes, allow the media to cool to ~50°C, supplement it with any necessary drugs used for selection

(for this example, 50 μg/mL kanamycin), mix well by swirling, and then pour into Petri dishes. Allow a period of 20–30 min for the agar to harden. Any unused plates can be stored at 4°C *(6)*.

5. Sterile wooden stick (toothpick) or loop for colony picking.
6. Sterile test tubes or flasks for liquid culture.
7. Bunsen burner.
8. Temperature-controlled incubator.
9. Temperature-controlled shaker incubator.

2.2. Determination of Appropriate Incubation Time

1. Working bacterial culture as described in **Subheading 3.1.**, **step 8a**.
2. Neat DMSO.
3. Appropriate antimicrobial compounds as negative controls: for this example, ampicillin (5 mg/mL) or chloramphenicol (1.25 mg/mL) in neat DMSO stored in aliquots at –20°C. If an appropriate antimicrobial is not available, 15 mL LB media is required.
4. A sterile, lidded, 96-well, flat-bottom, clear plate (Corning; Lowell, MA).
5. An appropriate detector: for this example, a plate reader that measures absorbance at 600 nm.
6. Temperature-controlled incubator.

2.3. Primary Screen

Note that all microtiter plates and tips must be compatible with the chosen liquid handler.

1. Working culture from **Subheading 3.1.**, **step 8b**.
2. Kanamycin (50 mg/mL) stored in aliquots at –20°C.
3. Appropriate antimicrobial compounds~ as positive controls; for this example, ampicillin (5 mg/mL) or chloramphenicol (1.25 mg/mL) in neat DMSO stored in aliquots at -20°C. If an appropriate antimicrobial is not available, one 96-well, –2 mL high-profile plate and fresh LB media are required.
4. Neat DMSO. Use fresh daily, as this solvent is very hygroscopic. Store at room temperature.
5. Library compounds in 96-well storage plates (1 mM in DMSO). Store at –20°C. Take care to minimize exposure of compounds to air, as DMSO is very hygroscopic.
6. Sterile, lidded, 96-well microtiter plates appropriate for the assay: in this example, clear plates are used (Costar 96-well flat-bottom sterile polystyrene plates) (*see* **Note 2**).

7. A high-profile DMSO-resistant storage block (Corning 0.5 mL polypropylene plate).
8. Reservoirs that hold ~300 mL of reagent (Nalge Nunc International; Rochester, NY);
9. Twenty-microliter, 96-racked tips for the liquid handler (Molecular BioProducts: San Diego, CA) (*see* **Note 3**);
10. Two hundred-microliter, 96-racked tips for the liquid handler (Beckman Coulter; Fullerton, CA).
11. Humidity- and temperature-controlled incubator (*see* **Note 4**);
12. Spray bottle containing 95% ethanol;
13. Water with a minimum resistivity of 17.5 MΩ cm (hereafter referred to as "water").

2.4. Follow-Up on Primary Actives: Dose-Response Determination

1. Any necessary drugs used for selection (for this example, 50 μg/mL kanamycin);
2. LB media from **Subheading 2.1.**, **step 2**.
3. Working culture from **Subheading 3.1.**, **step 8c**.
4. Neat DMSO.
5. Appropriate antimicrobial for positive controls: for this example, ampicillin (5 mg/mL) or chloramphenicol (1.25 mg/mL) in neat DMSO stored in aliquots at –20°C. If an appropriate antimicrobial is not available, one 96-well, 2-mL, high-profile plate and fresh LB media are required.
6. Compounds identified as actives from **Subheading 3.3**, at 5 mM, or the highest concentration available.
7. Low-volume polypropylene plates to reformat active compounds.
8. Sterile, lidded, 96-well microtiter plates appropriate for the assay: in this example, clear plates are used (Costar 96-well flat-bottom sterile polystyrene) (*see* **Note 2**).
9. Reservoirs that hold ~300 mL of reagent (Nalge Nunc International).
10. Twenty-microliter 96-racked tips for the liquid handler (Molecular BioProducts) (*see* **Note 3**).
11. Two hundred-microliter, 96-racked tips for the liquid handler (Beckman Coulter).
12. Humidity- and temperature-controlled incubator (*see* **Note 4**).
13. Spray bottle containing 95% ethanol.
14. Water.

3. Methods

3.1. Preparation of Bacterial Culture

1. Next to a flaming Bunsen burner, open the frozen strain stock, scrape up a small amount using a sterile loop or toothpick, and streak it on an agar plate (*see* **Note 5**).
2. Incubate plate overnight at 37°C. This plate can be used over 7 days to pick colonies.
3. The next day, next to a flaming Bunsen burner, inoculate 5 mL of LB media in a sterile test tube with a single colony from the agar plate using a sterile toothpick (*see* **Note 6**).
4. Grow the liquid culture overnight in the incubated shaker at 37°C, 250 rpm (*see* **Note** 7).
5. The next day, next to a flaming Bunsen burner, dilute the overnight culture 1:100 into fresh LB media. Typically, this dilution is 30 μL into 3 mL in a sterile test tube. This is the *subculture* (*see* **Note 8**).
6. Place the subculture in the incubated shaker at 37°C, 250 rpm, and grow until it reaches mid-log phase (A_{600} = 0.4 – 0.5): for this example, ~3 h (*see* **Note 9**).
7. Next to a flaming Bunsen burner, prepare the *working bacterial culture* by diluting the subculture 1:10,000 into one of the screw-cap bottles or capped flasks containing LB media and mix well.
8. The number and volume of working bacterial cultures to be prepared is dependent on the procedure to be completed next:
   a. If determining growth/signal over time, one flask of 100 mL culture is sufficient.
   b. If undertaking a primary screen, three separate 500 mL working bacterial cultures are needed each day. Inoculate each culture, as in **step** 7, from three separate subcultures, as in **step 5**, that have been prepared every 2 h; this step will ensure that fresh working culture is used throughout the day (*see* **Note 10**).
   c. If undertaking follow-up studies, the number of cultures and volumes needed will vary with the number of compounds to be tested. Follow method as in **Subheading 3.1.**, **step 8b**, preparing one 500 mL bacterial culture for every 48 assay plates.

3.2. Determination of Appropriate Incubation Time

If an appropriate antimicrobial for positive controls is available (*see* **Subheading 2.2.**, item 3), follow **steps 1–5**. Otherwise, start with **step 6**.

1. Prepare a working bacterial culture as in **Subheading 3.1.**, **step 8a**.
2. Using either a multichannel pipettor or a liquid handler, aliquot 98 μL of the culture to two 96-well plates.
3. Add neat DMSO to one half of the wells of each plate (negative controls yield 100% growth). The final concentration of DMSO is the same as what will be used in the primary screen (typically, 0.2–5%).
4. Add the antimicrobial reagent to the other half of each plate (positive controls yield 0% growth). Ensure the same final concentration of DMSO is used as in the previous step.
5. Proceed to **step 9**.
6. Prepare a working bacterial culture as in **Subheading 3.1.**, **step 8a**.
7. Using either a multichannel pipettor or a liquid handler, aliquot 98 μL of the culture to one half of the wells of two 96-well plates (positive controls) and 98 μL of LB media to the other half (negative controls).
8. Add neat DMSO to all wells of each plate. The final concentration of DMSO is the same as what will be used in the primary screen (typically, 0.2–5%).
9. Measure the absorbance at 600 nm, and any other signal as appropriate, for each plate. Note that if it is necessary to measure a signal in end point fashion, quench three positive controls and three negative controls for each measurement.
10. Incubate both plates at the appropriate temperature (e.g., 37°C). Take absorbance and any other necessary readings every 2 h or as manageable, until the growth of the microbe reaches the stationary phase, as indicated by little comparable change in growth over several hours.
11. Plot growth/signal versus time. Set the most appropriate *incubation time* for use in the primary screen by choosing the time at which microbial growth is still in log phase, and any secondary signals are maximized (*see* **Note 11**). At the selected incubation time, the calculated statistical parameter Z' should be >0.5 if looking to identify target inhibitors in the primary screen (*see* **Notes 12** and **13**).

3.3. Primary Screening

3.3.1. Labware Preparation

1. Allow library compounds to come to room temperature while still sealed in the storage plates. Unseal compounds immediately prior to use and reseal as soon as possible after use to minimize uptake of atmospheric water (*see* **Note 14**).
2. Label all assay plates appropriately; the number of the compound plate to be tested and replicate number are usually sufficient (*see* **Note 15**). If using an antimicrobial agent

for negative controls, follow **steps 3–5**. Otherwise, skip to **step 6**.

3. Add neat DMSO to the positive and negative control wells of each assay plate in the same volume as the library compounds for testing (in this example, 2 μL). To the 0.5 mL DMSO-resistant storage block, add neat DMSO to wells A1, C1, E1, G1, B12, D12, F12, and H12 in an appropriate volume for at least several runs of the liquid handler (*see* **Note 16**). Add the same amount of ampicillin (5 mg/mL) or chloramphenicol (1.25 mg/mL) in DMSO to wells B1, D1, F1, H1, A12, C12, E12, G12 (*see* **Note 17**). This is the *control block*.
4. Add an appropriate volume of microbe to one of the 300 mL reservoirs.
5. Skip to **step 8**.
6. Add neat DMSO to the positive and negative control wells of each assay plate in the same volume as the library compounds for testing (in this example 2 μL). To the 0.5 mL DMSO-resistant storage block, add neat DMSO to columns 1 and 12 in an appropriate volume for at least several runs of the liquid handler. This is the *control block* (*see* **Notes 16** and **17**).
7. Add LB media to wells B1, D1, F1, H1, A12, C12, E12, and G12 of the 2 mL high-profile plate. Add working bacterial culture to all other wells.
8. Add 300 mL water to the second 300 mL reservoir.

3.3.2. Liquid Handling

Note that a full method on the liquid handler from start to finish without any user intervention is referred to as a "run." Using the automated liquid handler, transfer the appropriate volumes of microbe and compounds to the assay plates, using the following outline as a guide.

1. The number of assay plates that are prepared for each run of the liquid handler will be dictated by the size of the working surface of the instrument. For any liquid handler, each run should be able to access (*see* **Notes 18** and **19**):
   - *n* compound storage plates
   - 2*n* assay plates
   - The control block
   - *n* 20-μL racked tips
   - 1 × 200-μL racked tips
   - 1 × 300-mL reservoir, or 2-mL high-profile plate containing microbe culture
   - 1 × 300 mL reservoir containing water

2. Add reagents to the assay plates in the following order:
   a. Use the 20 μL liquid-handling tips to transfer the appropriate amount of library small molecules (in this example, 2 μL) from compound storage plate 1 to assay plate 1, replicate 1 (or R1) and then assay plate 1, R2. Using this same box of tips, transfer the same volume from the control block to these same two assay plates in the same order (*see* **Note 20**). Discard tips (*see* **Note 21**).
   b. Repeat **step 2a** to distribute all compound plates to the appropriate assay plates for this run.
   c. Using one box of 200 μL tips, aliquot the screening broth (98 μL) to each assay plate in the following sequence:
      - Assay plate 1, R1 (*see***Notes 22** and **23**)
      - Wash tips in 300 mL water in reservoir (*see* **Note 24**)
      - Assay plate 1, R2
      - Wash tips in 300 mL water in reservoir
      - Assay plate 2, R1
      - Wash tips in 300 mL water in reservoir
      - Assay plate 2, R2
      - Wash tips in 300 mL water in reservoir

      Use the same 200 μL tips for approximately ten assay plates, or at least one run, then discard.
3. Replace the lids of the assay plates in the reverse order to that in which they were removed. Remove assay plates from the liquid handler and stack.
4. Prepare for the second liquid-handling run by replenishing reagents as needed, and using new compound and assay plates and appropriate tips. Start the second run.
5. While the liquid-transfer of **step 4** is occurring, if applicable, read the assay plates on the appropriate detector for a $t = 0$ measurement (*see* **Note 25**). Note the time at which measurements are made (*see* **Note 26**).
6. While the liquid-transfer of **step 4** is occurring, transfer the prepared assay plates to an incubator at the appropriate temperature and humidity for the microbe (*see* **Note 27**). Note the time that each stack was placed in the incubator (*see* **Note 28**).
7. Repeat **steps 2–6**, until approximately 2 h has elapsed; a reasonable throughput is 12 compound plates (960 compounds in duplicate) per hour.
8. Continue the primary screen as above, using a fresh working culture every 2 h.

9. At the end of each day, reseal all compounds and store at –20°C.
10. Incubate the microbes for the appropriate incubation time as determined in **Subheading 3.2.**, then measure the appropriate signal, e.g., growth as determined by A_{600}.
11. Calculate the percent growth/signal of each assay by

$$\%G = \left(\frac{S - \mu_{+c}}{|\mu_{-c} - \mu_{+c}|} \right),$$

where S is the signal for either a positive, negative, or test reaction, and μ_{+c} and μ_{-c} are the average S for the positive and negative controls, respectively. Note $\%G$ for each tested compound is to be calculated using the control wells contained within the same assay plate.

12. Identify primary active compounds; typically, these are identified as those compounds that result in growth/activity 3 standard deviations below or above (for inhibitors or activators, respectively) the average of the negative controls (i.e., 100% activity).

3.4. Follow-Up Tests of Primary Actives: Dose–Response Determination

1. Prepare the working bacterial culture as in **Subheading 3.1.**, **step 8c**.
2. Reformat active compounds from **Subheading 3.3** into polypropylene plates: with one compound per well, aliquot 15 μL of 8 compounds into wells A1–H1, and 10 μL neat DMSO to all wells in columns 2–11. Using a multichannel pipettor or liquid handler, transfer 4.7 μL of the compounds in column 1 into the DMSO into column 2. Continue the serial dilution across the plate to column 11 (*see* **Note 29**).
3. Aliquot 15 μL neat DMSO to wells A12–D12.
4. Aliquot 15 μL of the appropriate antimicrobial (e.g., ampicillin (5 mg/mL) or chloramphenicol (1.25 mg/mL)) into wells E12–H12. If an antimicrobial is not available, aliquot neat DMSO to these wells.
5. If an appropriate antimicrobial agent (*see***Subheading 2.4.**, **step 5**) is available, add the working bacterial culture to the 300 mL reservoir. If not, add LB media to wells E12–G12 of the 2-mL high-profile plate. Add working bacterial culture to all other wells.
6. Aliquot compounds and bacteria to assay plates as in **Subheading 3.2.**, **steps 2–11**, ignoring the comment regarding the control block in **step 2**.
7. Plot percent growth/signal against compound concentration and fit to:

$$\%\text{Growth} = \frac{R}{1+\left(\frac{[I]}{EC_{50}}\right)^{s}} + B,$$

where R is the extrapolated percentage growth in the absence of inhibitor, $[I]$ is the concentration of tested compound (μM), s is the slope (or Hill factor), and B is background signal, i.e., the extrapolated percentage growth at which the inhibitor exerts its maximal effect. Define the MIC for each compound as the lowest concentration at which no visible growth of the strain is observed (*see* **Note 30**).

4. Notes

1. If inhibitors are sought in the screen, an adequate positive control would be to use bacteria-free media (i.e., exclude the target from the assay), or to include a known antimicrobial agent. If activators are sought, it is not strictly necessary to have a positive control.
2. It is preferable to use clear, black, or white plates for absorbance, fluorescence, and luminescence detection, respectively. It is possible to use black or white plates with clear bottoms to enable the detection of both absorbance and either fluorescence or luminescence.
3. If you include antimicrobials in the screening broth, it is not strictly necessary to use sterile tips.
4. It is preferable to use an incubator that maintains humidity above 80%; this helps in preventing evaporation from assay plates during incubation.
5. Cryoprotectants such as glycerol or DMSO are used for freezing and long-term storage of cells to prevent any damaging effects caused by the formation of ice crystals. Frozen glycerol stocks are made by mixing an aliquot of the overnight culture with an equal volume of sterile 30% glycerol in LB media, and storing in cryovials at –80°C. Alternatively, frozen DMSO stocks are made by mixing 1 mL of overnight culture with 90 μL of freezing solution (DMSO and 10% ethanol) and storing in cryovials at –80°C.
6. Using a single colony guarantees cell homogeneity. For a more stringent condition of homogeneity, a single colony is picked from the plate and streaked on a second plate from which the liquid culture would finally be inoculated with a single colony.

7. The conditions describe requirements for wild-type *E. coli* to reach stationary phase when grown in rich LB media; the length of incubation needed for a culture to reach stationary phase is dependent on the strain of interest and growth media used. A growth profile of the strain of interest should performed in order to establish these conditions (*see* **Subheading 3.2**).
8. Subculturing serves to bring the cells to a phase of exponential growth.
9. If the 3 mL subculture is found to reach mid-log phase too slowly, the subculture can be set up in a flask with a better surface area-to-volume ratio (e.g., 500 μL overnight culture into 50 mL LB media in an Erlenmeyer flask) to increase the growth rate.
10. If the screen is being run all day, then several subcultures should be set up from the overnight culture at regular time intervals. This ensures that the later assay plates are not set up with an overgrown screening broth. For this purpose, the subcultures need to be staggered a few hours apart (**Table 1**).
11. Generally, when adding microbes to assay plates with test compounds, it is desirable to use a culture concentration in which the organism is in lag phase or early log phase. At the time of final detection, after an incubation period, the microbes should be in log phase; if the stationary phase is reached, the assay will be much less sensitive to subtle effects of small molecules tested.
12. Z' is a simple, dimensionless statistical measure of the quality of a small-molecule screening campaign. It is defined as:

$$Z' = 1 - \left(\frac{3\sigma_{+c} + 3\sigma_{-c}}{|\mu_{+c} - \mu_{-c}|} \right),$$

where σ_{c+}, σ_{c-}, μ_{c+}, and μ_{c-} are the standard deviations (σ) and means (μ) of the positive (c+) and negative (c−) controls *(7)*. A Z' value of 0.5 or greater represents a set of data with a good

Table 1
Establishment of working bacterial cultures for primary screening throughout the day

Procedure	1	2	3
Subculture set up at	8 a.m.	10 a.m.	12 noon
Working bacterial culture set up at	11 a.m.	1 p.m.	3 p.m.

screening window and clear separation between the positive and the negative controls.

13. *See* **Fig. 1** for an example of a growth curve for *E. coli*. Twenty hours was chosen as the incubation time for the primary screen, as the bacteria were still in log phase at this time and the calculated Z' was greater than 0.5.
14. It is good practice to spin compound plates in a centrifuge to prevent compound loss when unsealing the plates.
15. Make the label short, mark whichever side is still legible when on the liquid handler, and, preferably, keep the legible side facing outwards when in the incubator for easy identification; it is not necessary to label lids.
16. It is suggested that a 0.5 mL block be used, as this volume should be sufficient to hold the total volume of DMSO needed for a day's screening.
17. By alternating positive and negative controls in this manner, it is easier to spot well-specific variations in each assay plate.
18. *See* **Fig. 2** for an example of how to organize the working surface of the Biomek FX for this example.
19. Spray the bench surface in front of the liquid handler with 95% ethanol and wipe down. Remove the lids from the sterile 96-well plates by beginning with the row farthest from the operator, and working towards the operator; this minimizes hand movement over open plates, a potential source of contamination.
20. Recall that the control block contains reagent in columns 1 and 12 only, whereas the same columns in the compound storage plates are empty.

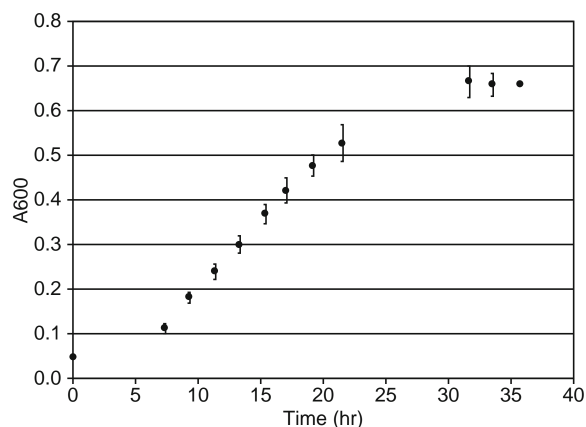

Fig. 1. *E. coli* growth over time in the presence of 5% DMSO. Data shown are the average of 96 replicates.

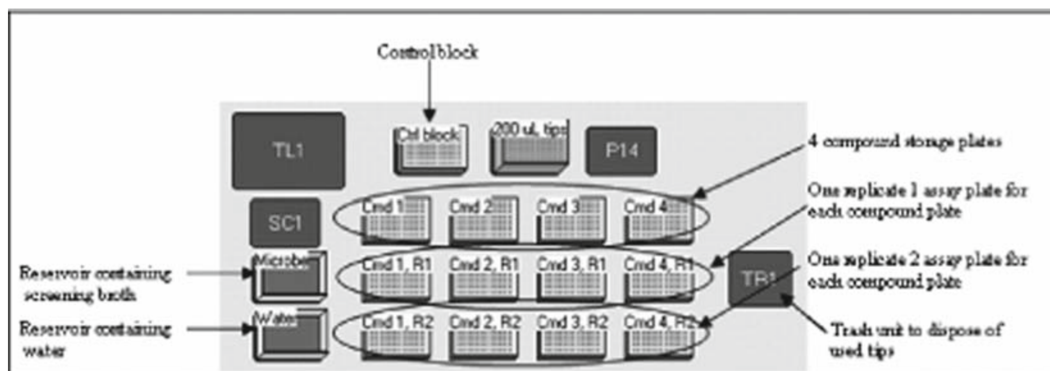

Fig. 2. One way in which to organize the working surface of the Biomek FX for a primary screen. Not shown is storage space for 20-μL tips for compound and control block transfer; these tips are introduced to the liquid handler by way of a shuttle (SC1) from the storage carousels. TL1 is the position where disposable tips are loaded onto the 96-channel pipetting head. One extra space on the liquid handler, P14, remains empty throughout each run.

21. Add compound to assay plates first so the same tips can be used for addition to both replicates; if the bacteria were in the plate first, a second box of tips would be required to avoid contaminating the compounds. If available, pin tools can be used to add compounds directly to microbes in assay plates.
22. It is recommended that the microbe is mixed in its storage reservoir at the start of each run by aspirating and dispensing 150 μL four times to prevent solution inhomogeneities from cell settling.
23. It is recommended that the screening broth be aspirated and dispensed several times after addition to each assay plate to ensure adequate mixing with compound.
24. Washing (i.e., aspirating and dispensing) with 150 μL water three times is sufficient to prevent compound carryover.
25. This step is particularly useful to flag compounds that directly interfere with the assay signal.
26. Directly stamping the time in detector output files is particularly useful for this step.
27. Increased humidity decreases media evaporation leading to well position-dependent data (i.e., edge effects).
28. There is no technical challenge to incubating assay plates with shaking (usually done at 150 rpm). However, we found that the standard deviation in the growth of microbes incubated without shaking was significantly lower than those grown with shaking; we therefore never shake during incubation time.
29. This dilution factor will result in 11 compound concentrations that span five logs at half-log intervals.

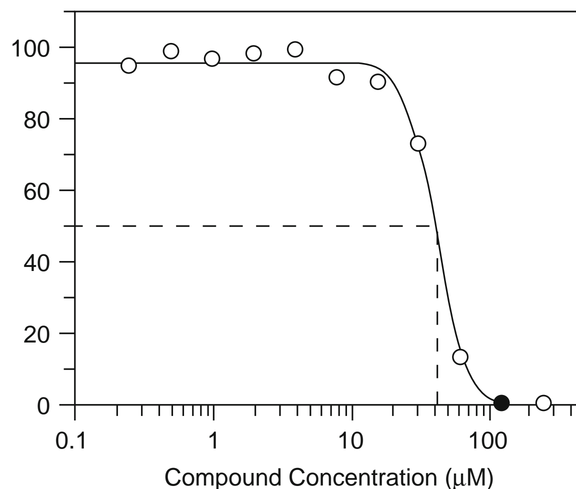

Fig. 3. Illustrative example of a dose–response relationship between *E. coli* and an active compound identified in a primary screen. The *solid line* shows the best fit of the data to a four-parameter equation to determine EC_{50} (*see* **Subheading 3.4.**, **step 7**). The calculated EC_{50} value (41.0 μM) is highlighted by the *dashed line*. The single solid data point was defined as the MIC (125 μM).

30. Although both the MIC and EC_{50} values of a compound are important measures of its bioactivity, the latter value is considered a more precise parameter by which to measure compound potency (**Fig. 3**). Values of EC_{50} are calculated from the entire dose–response data set and are not influenced by the selection of compound concentrations to determine the point of complete growth inhibition of the microbe, as with MIC values.

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