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Microscale Adaptation of *In Vitro* Transcription/Translation for High-Throughput Screening of Natural Product Extract Libraries

Andrew N. Lowell¹, Nicholas Santoro^{1,2}, Steven M. Swaney^{1,2}, Thomas J. McQuade^{1,2}, Pamela J. Schultz¹, Martha J. Larsen^{1,2}, and David H. Sherman^{1,3,4,5,*}

¹Life Sciences Institute, University of Michigan, 210 Washtenaw Ave., Ann Arbor, MI 48109-2216, USA

²Center for Chemical Genomics, University of Michigan, 210 Washtenaw Ave., Ann Arbor, MI 48109-2216, USA

³Department of Chemistry and Medicinal Chemistry, University of Michigan, 210 Washtenaw Ave., Ann Arbor, MI 48109-2216, USA

⁴Department of Microbiology and Immunology, University of Michigan, 210 Washtenaw Ave., Ann Arbor, MI 48109-2216, USA

⁵Department of Chemistry, University of Michigan, 210 Washtenaw Ave., Ann Arbor, MI 48109-2216, USA

Abstract

Novel antimicrobials that effectively inhibit bacterial growth are essential to fight the growing threat of antibiotic resistance. A promising target is the bacterial ribosome, a 2.5 MDa organelle susceptible to several biorthogonal modes of action used by different classes of antibiotics. To promote the discovery of unique inhibitors, we have miniaturized a coupled transcription/translation assay using *E. coli* and applied it to screen a natural product library of ~30 000 extracts. We significantly reduced the scale of the assay to 2 μ L in a 1536-well plate format and decreased the effective concentration of costly reagents. The improved assay returned 1327 hits (4.6% hit rate) with %CV and Z' values of 8.5% and 0.74, respectively. This assay represents a significant advance in molecular screening, both in miniaturization and its application to a natural product extract library, and we intend to apply it to a broad array of pathogenic microbes in the search for novel anti-infective agents.

Keywords

antimicrobial screening; microscale high-throughput screening; natural products extract library; transcription/translation

Antibiotic resistance is a growing international threat, with 23 000 deaths, 2 million patients, and \$20 billion expended in the clinical setting annually in the United States alone (¹). The

*Corresponding author: David H. Sherman, ; Email: davidhs@umich.edu

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pharmaceutical industry's approach to this problem, combinatorial libraries, has not proven as promising as once hoped⁽²⁾. Combinatorial synthesis has produced millions of compounds, but the consistent production of potent leads and eventual therapies has remained elusive⁽³⁾. In an effort to identify new, promising antimicrobials, we sought to develop a high-throughput screening assay for inhibitors of prokaryotic ribosomes that we could apply to natural product extracts. Over the past decade, our laboratory has isolated phylogenetically unique pure-culture microbial strains that we have grown and extracted to create a prefractionation natural products extract library (NPEL) of more than 30 000 samples, a collection housed in the Center for Chemical Genomics (CCG) at the University of Michigan⁽⁴⁾. The NPEL has already proven to be a potent resource in high-throughput screening, furnishing promising leads toward antivirals⁽⁴⁾, effective inhibitors of siderophore virulence-factor biosynthesis for MRSA and *Bacillus anthracis*⁽⁵⁾, and potent aggregation inhibitors for neurodegenerative disorders⁽⁶⁾. Our intention was to develop a high-throughput screen for the identification of new antibacterial agents and apply it to the NPEL.

We envisioned adapting a coupled transcription/translation assay used previously for screening industrial chemistry libraries to the NPEL^(7,8). A transcription/translation screen is desirable because it enables rapid screening of libraries against the bacterial ribosome, an established target for several classes of antibiotics including macrolides, aminoglycosides, tetracyclines, and oxazolidinones⁽⁹⁾. Because different classes of ribosome inhibitors have orthogonal molecular modes of action, resistance by a pathogen to one class does enable it to counter other classes^(10,11). Hence, new chemical scaffolds uncovered by a high-throughput screen are likely to be effective against drug resistant bacterial pathogens.

This study was motivated by the potential cost and throughput advantages of reducing the scale of a direct ribosome inhibitor screen using natural product extracts as the source of chemical diversity. Previous studies successfully optimized a coupled transcription/translation screen in 384-well plates at a total scale of 16 μ L using a luminescence-based reporter on a small-molecule library, which resulted in the discovery of a potential lead for a new, orthogonal class of ribosome inhibitor^(7,8). This type of assay is amenable for use in 1536-well plates⁽¹²⁾, and a number of secondary and counter screens are available to enable the decoupling of transcription from translation^(7,8,13), remove intercalative inhibitors⁽¹⁴⁾, discriminate compounds that interfere with luminescence^(7,8), and identify general (eukaryotic) ribosome inhibitors^(7,8,14). Our objectives were to create an improved method by significantly increasing efficiency through a reduction in reaction scale and to apply this screening technology directly to natural product extracts.

Methods and Materials

General procedure for the preparation of natural product extracts

The strains for the NPEL were derived predominately from collections of marine actinomycetes, and their isolation as pure cultures has been described elsewhere⁽⁴⁾. For the preparation of library samples, pure strains were started as seed cultures in 17 mL polystyrene dual-position-cap tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) charged with International *Streptomyces* Project Medium 2 (ISP2, 3 mL) and incubated

(28 °C) on a rotary shaker (200 rpm) until aggregation of the suspended mycelium was apparent (2–10 days). A 250-mL baffled Erlenmeyer flask containing ISP2 media (100 mL) was inoculated with the seed culture (3 mL) and incubated on a rotary shaker (28 °C, 200 rpm) for 5 days. After centrifugation, the cell-free broth was transferred to a new sterile flask with a polypropylene mesh bag (Midwest Filtration, Cincinnati, OH, USA) containing 1.5 g of washed Amberlite XAD16N resin (Sigma-Aldrich Co., St. Louis, MO, USA) and incubated on a rotary shaker (28 °C, 200 rpm) for 16 h. Residual broth was pressed out of the resin bag, and the resin was extracted with 50% methanol/ethyl acetate (20 mL). After evaporation, this crude extract was diluted to a final concentration of 15 mg/mL in dimethyl sulfoxide, placed into bar coded tubes, and reformatted into 384-well parent plates.

The pure microbial isolates were also co-cultured with either *Corynebacterium glutamicum* (ATCC 13869) or *Rhodococcus erythropolis* (ATCC B-16025) as previously described⁽¹⁵⁾. Extraction was accomplished by including a polypropylene mesh bag containing 1.5 g of washed Amberlite XAD16N resin in the 250-mL baffled Erlenmeyer flask when the seed culture (3 mL) and co-culture (2 mL) were introduced. After incubation on a rotary shaker (28 °C, 200 rpm) for 5 days, the resin bag was processed into crude extract as described above.

Reagents

DNase- and RNase-free water and erythromycin were purchased from Sigma-Aldrich Co. Kanamycin was purchased from Gold Biotechnology (St. Louis, MO, USA). Bacterial culture media and extracts were purchased from EMD Millipore (Billerica, MA, USA). S30 extract, circular; amino acid mixture, complete; S30 premix without amino acids; Wizard SV Minipreps DNA Purification Systems; and Nano-Glo Luciferase Assay System were purchased from Promega Corp. (Madison, WI, USA). XL2-Blue Ultracompetent Cells were purchased from Agilent Technologies (Santa Clara, CA, USA). Luciferase containing plasmid, pT7-NLuc; *E. coli* T7 S30 extract system for circular DNA; and purified Nano-Glo Luciferase were complementary samples from Promega Corp.

Luciferase plasmid preparation

The pT7-NLuc plasmid was transformed into XL2-Blue Ultracompetent Cells according to the published protocol. After overnight growth on a rotary shaker (37 °C, 200 rpm), the resulting culture was harvested and purified using a Wizard SV Minipreps DNA Purification System. Plasmid DNA concentrations were assessed by spectrophotometry (NanoDrop ND-1000 Spectrophotometer; ThermoFisher Scientific, Waltham, MA, USA).

Primary *E. coli* transcription/translation assay

Primary screening experiments were carried out in 1536-well black 10 μ L round-bottom microplates (Corning no. 3936; Corning Incorporated Life Sciences, Tewksbury, MA, USA) at a final volume of 2 μ L. Centrifugation was performed using a swinging-bucket rotor (SX4750; Beckman Coulter, Inc., Palo Alto, CA, USA) in a bench top centrifuge (Allegra X-12R; Beckman Coulter, Inc.).

Diluted *E. coli* S30 extract (1 μL), consisting of S30 extract, circular (0.3 μL) and water (0.7 μL) was dispensed into each well using an automated dispenser (Multidrop Combi; Thermo Fisher Scientific, Williston, VT, USA; equipped with a Small Tube Metal Tip Dispensing Cassette, Cat. No. 24073295, Thermo Fisher Scientific), and the plates were centrifuged ($2000 \times g$, rt, 2 min). The natural product extracts (50 nL, 375 $\mu\text{g}/\text{mL}$ final concentration) and negative controls (50 nL DMSO, 2.5% final concentration) were transferred into each well of the 1536-well daughter plates from parent 384-well library plates using a pintool (FP1S40; V&P Scientific, Inc., San Diego, CA, USA) attached to an automated liquid handler (Sci-clone ALH 3000 Workstation; Caliper/PerkinElmer Co., Hopkinton, MA, USA). Kanamycin solution (100 nL, 1000 $\mu\text{g}/\text{mL}$ in water, 50 $\mu\text{g}/\text{mL}$ final concentration) was applied to wells designated as positive controls by automated dispenser (Multidrop Combi nL; Thermo Fisher Scientific). After centrifugation ($2000 \times g$, rt, 2 min), the plates were incubated (rt) for 10 min. Diluted premix reagent solution (1 μL), consisting of amino acid mixture (0.1 μL); S30 premix without amino acids (0.4 μL); water (0.5 μL); and pT7-NLuc plasmid DNA (0.005 μL of a 300 ng/ μL solution in water, 0.75 ng/ μL final concentration), was dispensed into each well using an automated dispenser (Multidrop Combi; Thermo Fisher Scientific). The plates were centrifuged ($2000 \times g$, rt, 2 min) and incubated (rt) for 2 h. Kanamycin solution (100 nL, 1000 $\mu\text{g}/\text{mL}$ in water) was added to the wells designated as negative controls and wells containing natural product extract via automated dispenser (Nanodrop Combi nL; Thermo Fisher Scientific) to stop translation, and the plates were centrifuged ($2000 \times g$, rt, 2 min).

Luminescence readout

Freshly prepared luciferin reagent (2 μL ; Nano-Glo Luciferase Assay System) was applied to each well using an automated dispenser (Multidrop Combi; Thermo Fisher Scientific). The plates were centrifuged ($2000 \times g$, rt, 2 min) and incubated (rt) for 10 min. Plates were sealed (TopSeal-A: 384-well Microplates; PerkinElmer Inc., Waltham, MA, USA) and analyzed on a plate reader (Wallac EnVision 2104 Multilabel Reader; PerkinElmer Inc.) using an ultra-sensitive luminescence aperture (1536-L1 aperture; PerkinElmer Inc.). Sample processing was performed using MScreen (Center for Chemical Genomics, University of Michigan, Ann Arbor, MI, USA) (¹⁶).

Confirmation screening and deconvolution of luciferase inhibitors

Selected natural product extracts (25 nL) were arrayed using a liquid handler (Mosquito X1; TTP Labtech, Melbourn, UK) into 384-well small volume, black, non-binding microplates (Greiner no. 784900; Greiner Bio-One, Frick-enhausen, Germany) in triplicate. Microplates were prepared in duplicate.

To one set of microplates was added diluted *E. coli* S30 extract (2 μL) via automated dispenser (Multidrop Combi; Thermo Fisher Scientific). Kanamycin solution (200 nL, 1000 $\mu\text{g}/\text{mL}$ in water, 50 $\mu\text{g}/\text{mL}$ final concentration) was applied to wells designated as positive controls by automated dispenser (Multidrop Combi nL; Thermo Fisher Scientific); wells designated as negative controls were unaltered. After centrifugation ($2000 \times g$, rt, 2 min), the plates were incubated (rt) for 10 min. Diluted premix reagent solution (2 μL) was dispensed into each well using an automated dispenser (Multidrop Combi; Thermo Fisher Scientific).

The plates were centrifuged ($2000 \times g$, rt, 2 min) and incubated (rt) for 2 h. Kanamycin solution (200 nL, 1000 $\mu\text{g}/\text{mL}$ in water) was added to the wells designated as negative controls and wells containing natural product extract via automated dispenser (Nanodrop Combi nL; Thermo Fisher Scientific) to stop translation, and the plates were centrifuged ($2000 \times g$, rt, 2 min). Luminescence using freshly prepared luciferin reagent (4 μL ; Nano-Glo Luciferase Assay System) was analyzed as described above.

To the other set of plates was added purified Nano-Glo Luciferase (4 μL , 3.3 ng/mL in water) using an automated dispenser (Multidrop Combi; Thermo Fisher Scientific), both to the wells containing natural product extract and negative control wells. Wells designated as positive controls were charged with water (4 μL) via automated dispenser, and the plates were centrifuged ($2000 \times g$, rt, 2 min) and incubated (rt) for 10 min. Luminescence using freshly prepared luciferin reagent (4 μL ; Nano-Glo Luciferase Assay System) was analyzed as described above.

Results and Discussion

Preparation of natural product extracts

We have ongoing efforts to generate and maintain a large prefractionation extract library suitable for screening a variety of targeted assays without the upfront effort of purifying and identifying discrete compounds. In collaboration with the CCG, we have processed more than 30 000 extracts over 13 years from a variety of species, including bacteria from marine and terrestrial sediments, fungi, and lichens. Additionally, each species is preserved as a spore or glycerol stock so the microbes generating active compounds can be regrown for future bioassay-guided fractionation and structure elucidation.

After isolating the various species as pure cultures and preserving them⁽⁴⁾, small-scale (3 mL) liquid cultures were used to inoculate large-scale (100 mL) liquid cultures suitable for extraction. Co-cultures in which the production culture was challenged with a second live bacterial culture, such as *Rhodococcus* and *Corynebacterium*, to induce the production of cryptic secondary metabolite pathways were also prepared⁽¹⁵⁾. After extraction, the concentrated residues from the various species were resuspended in dimethyl sulfoxide at a concentration of 15 mg/mL. Samples of these extracts are routinely formatted by the CCG into 384-well plates suitable for use in high-throughput screens.

Development of a microscale *E. coli* transcription/translation screen

With the successful application of transcription/translation screens to combinatorial libraries^(7,8), we sought to apply this technology to natural product extracts. These samples provide an attractive avenue in the search for new antimicrobials⁽¹⁷⁾, because unlike combinatorial libraries, no synthetic chemistry efforts are required⁽²⁾ and secondary metabolites are a proven source of structurally unique antibiotics that have been developed for clinical use⁽³⁾. Using efficiently prepared extracts and bioassay-guided fractionation to separate the active components, the effort required to isolate secondary metabolites from a new bacterium is limited to only those strains that show promising biological activity.

Luciferase production from plasmid DNA via RNA polymerase and ribosome translation was the most straightforward method to probe the inhibition of protein synthesis (^{7,8}). To establish the effectiveness of this strategy when screening natural product extracts, we began optimization with a commercially available transcription/translation kit. Tests with firefly luciferase and NanoLuc luciferase both gave strong luminescence signals when mixed with their corresponding luciferin substrate. Total reaction volumes of the combined reagents (S30 fraction, amino acylated tRNA mix, nTPs, plasmid, and Premix) and luciferin were tested at volumes ranging from 0.25 to 4 μL (Figure 1A). While luminescence values linearized above 1 μL total imaging volume, application volumes in multiples of 1 μL were chosen to ensure the revolving dispenser robotics achieved maximum consistency between wells. NanoLuc was selected because it had a brighter luminescence signal and its smaller protein could be produced in higher yields. This trend was observed to be consistent among a variety of plate types. Black plates were selected to reduce signal dispersion into adjacent wells and maintain the luminescence output in a readable range.

Known ribosome inhibiting antibiotics (kanamycin and erythromycin) were used to determine the effective concentrations of antimicrobials that could be detected (Figure 1B). Kanamycin was effective over a range of 50–0.5 $\mu\text{g/mL}$, but a low concentration of erythromycin (0.2 $\mu\text{g/mL}$) was ineffective at reducing the luminescence to < 25% of negative control samples. By reducing the relative concentration of the luciferase plasmid from 3 to 0.75 ng/ μL , the corresponding decrease in transcript enabled effective inhibition by erythromycin at even the more dilute concentrations. Tests of negative and positive-control reactions with and without dimethyl sulfoxide (5% of the total reaction volume) showed no difference in luminescence.

A schematic representation of the general throughput for this screen is shown in Figure 2. S30 extract (¹) is applied to each well, and then, the plates are centrifuged to ensure correct placement of the liquid in the well bottom. A natural product extract (or a control solution) is added to each well (²), the plates are centrifuged, and incubation allows interaction between potential inhibitors and the ribosome component of the S30 extract. Luciferase plasmid, a mixture of amino acylated tRNAs, and an nTP-containing premix (³) are then added to each well. The plates are centrifuged and incubated, enabling transcription and translation to produce luciferase: high amounts for uninhibited ribosomes and lower amounts when the components of the natural product extract function as inhibitors. Translation is terminated by the addition of kanamycin (⁴), luciferin reagent is added to the plates (⁵), and after centrifugation, individual wells are assessed by luminescence detection (⁶). Active extracts and kanamycin-containing positive control reactions will curtail luciferase production by inhibiting transcription/translation and give low luminescence values while negative controls and non-binding extracts will allow the formation of luciferase and hence show high luminescence levels.

The automated process was tested using a subset of the Prestwick Chemical Library (Prestwick Chemical, France) containing several known ribosome inhibiting antimicrobials. As per the schematic in Figure 2, S30 extract was applied via automated dispenser to 1536-well plates, which were subsequently centrifuged. Prestwick library compounds were robotically stamped into the 1536-well daughter plate from 384-well parent plates, while

wells for the positive controls were treated with kanamycin using an automated dispenser. The plates were centrifuged and incubated at room temperature for 10 min to enable the positive controls and inhibitors to interact with the ribosome. The reagent mixture, including amino acylated tRNA, NTPs, and luciferase plasmid, was introduced into each well using an automated dispenser. The plates were centrifuged and incubated at room temperature for 2 h, during which time luciferase protein was produced in various concentrations depending on the level of inhibition effected by the library compounds on the transcription/translation system. Protein production in the negative control and library wells was terminated by the addition of kanamycin solution, and the plates were centrifuged. Freshly prepared luciferin reagent was applied to all wells via dispenser. After centrifugation, the plates were incubated at room temperature for 10 min and assessed for luminescence using a plate reader equipped with an ultra-sensitive luminescence aperture.

Using a selection criterion of > 60% RLU inhibition for the 320 Prestwick compounds tested in duplicate on a 1536-well plate resulted in 11 hits, a 3.4% hit rate. Test plates screened on consecutive days generated the same results, validating the consistency of this method. Of these hits, seven were known ribosome inhibitors from the macrolide (josamycin, troleandomycin, erythromycin), aminoglycoside (dihydrostreptomycin, gentamicin), tetracycline (chlortetracycline), and chloramphenicol (thiamphenicol) classes of antibiotics. The other four (ethacrynic acid, bromocriptine, isoxicam, and tolfenamic acid) have unreported *in vitro* activity against *E. coli* as evidenced by their reproducible inhibition or were false positives due to contamination of the library plate. These encouraging results motivated us to move forward and screen the complete natural product extract library.

The luminescence signal in the negative control wells was so strong in these preliminary runs that dilution of all reagents with water was tested to further conserve reagents. Dilution of all mixtures by one-half with water showed an appropriate corresponding decrease in luminescence signal. Dilution to one-quarter of the original concentration with water, however, reduced the luminescence of the negative control wells to levels comparable to the kanamycin-containing positive control wells, an indication that effective protein synthesis had ceased. Additional dilution with an optimized buffer system to further conserve reagents may be possible.

Microscale evaluation and confirmation of the natural product extract library

The complete NPEL was screened using optimized conditions (see Methods and Materials section) in twenty-three 1536-well plates over a period of 3 days (Figure 2). Slight variations in the concentration of fresh luciferase plasmid from day to day resulted in different baseline luminescence levels for the negative control reactions, but these fluctuations did not affect the results of the individual plates and could be reduced using a large single batch of luciferase plasmid for the entire screen. Percent by plate analysis of the data successfully normalized the daily variations for both the controls (Figure 3A) and the natural product extracts (Figure 3B). The %CV and Z' values for the screen were excellent, ranging from 5% to 16% (average = 8.5%) and 0.53 to 0.84 (average = 0.74), respectively, per plate (Figure 3C) (¹⁶). Plates with higher numbers generally showed a higher percentage of hits (for example, plate 23) because they were more recently prepared and contained more

samples challenged with bacteria able to induce secondary metabolite gene cluster expression.

A recurring issue observed when using this screening protocol was the appearance of occasional large negative outliers. While most of the negative wells and negative control reactions were close to the average, a handful of wells were observed to generate double the average luminescence value. These aberrations were mostly eliminated by the use of fresh plasmid DNA, an observation that suggested freeze-thawed DNA was less homogeneous and introduced large, aggregated clumps of plasmid into a small fraction of wells. This over application of DNA resulted in increased levels of transcript, which in turn produced more protein, and hence a brighter luminescence signal. These outliers were controlled by applying an interquartile range adjustment to the negative control reactions to remove any spuriously high values (¹⁶). For the tested wells, it is possible that some compounds from the natural product extract library were themselves luminescent or otherwise enhanced the luminescence signal.

The hit rate for the screen was 4.6% (1316 extracts of 28 899 total) using a selectivity cutoff of 75% RLU inhibition or greater per plate (¹⁶). These active extracts were skewed, however, to the most recently prepared extracts and extracts derived from rich media (Table 1), which includes the *Corynebacterium* and *Rhodococcus* co-culture method (¹⁵). Parent plates for the older extracts have been used in a large number of screens, and the repeated freeze-thaw cycles may have degraded these extracts over time, resulting in a lower hit rate. In 2012, we improved our protocol to use a single ethyl acetate/methanol extraction for each strain instead of preparing three individual extracts of acetone, ethyl acetate, and methanol. We also began preparing co-cultures with mycolic acid producing bacteria (¹⁵) in rich media, an adaption that enables the expression of additional metabolic pathways and hence greater extract activity and chemical diversity. Selection criteria were updated accordingly to achieve a wider distribution of compounds by decreasing the cutoff for the oldest samples to 50% RLU inhibition (537 extracts from plates 1–12), lowering intermediate samples to a 60% threshold (717 extracts from plates 13–19), and raising the stringency for recent samples to 80% (632 extracts from plate 20–23). After removal of extracts that were no longer available for regrowth, we were left with a total of 1327 extracts (4.6%).

These selected hits were retested in triplicate to confirm their authenticity. Extracts were assayed at one-fourth the concentration of the primary screen to assess an overall concentration effect. Direct concentration assessment is not possible on the NPEL because the concentration of the active component of a particular extract is unknown. An extract was considered confirmed if it showed activity three of four times (between three confirmation tests and one primary test). Of the 1327 retested extracts, a 50% RLU inhibition threshold confirmed 647 extracts and a 90% RLU inhibition threshold confirmed 155 extracts as active (Table 1).

Purified luciferase protein was also tested in triplicate against the 1327 hits selected in the primary screen to remove samples that inhibited luciferase instead of targeting the desired transcription/translation pathway. Extracts were excluded if they inhibited luciferase at a

level > 50% even once (Table 1). Strenuous exclusion criteria (< 30%) were rejected because they began to encroach on the noise level inherent to the negative controls (Figure 3A).

Combining a 50% cutoff in the luciferase counterscreen with the 50% and 90% confirmation thresholds resulted in 421 and 110 extracts, respectively, with a hit distribution that follows the trends in Table 1. Source analysis of the 110 top extracts revealed that all were from actinomycetes species. This result mirrors well with the overall composition of the library, in which 99% of extracts are actinomycetes and the other 1% are composed of fungi, cyanobacteria, lichens, and myxobacteria. Likewise, breakdown by media type showed that richer media produced more active compounds. Of the top 110 extracts, 58 were from rich media (A3M and co-cultures), 49 were from ISP2 (which makes up over one-third of the library), and only three were from nutrient poor media. These extracts are being subjected to secondary screening to decouple transcription from translation, which along with regrowth, fractionation, and natural product identification of potential leads, will be reported in due course.

Conclusions and Future Directions

The two significant advances reported herein are the miniaturization of a coupled transcription/translation screen and its application to a natural product extract library. Previous studies using a coupled transcription/translation protocol focused only on discrete small-molecule libraries, leading to the identification of new antimicrobial leads at a 16 μL scale in 384-well format (^{7,8}), and demonstrating the feasibility of this method at a 4 μL scale in 1536-well plates (¹²). We reduced the screening scale to a total volume of 2 μL in low-volume 1536-well plates, a miniaturization that was further enhanced by the effective dilution of the working concentrations of the transcription/translation assay components. This method was effectively applied to a diverse collection of natural product extracts, materials that have regained interest in recent years because of their proven ability to provide effective therapeutics compared to combinatorial-based libraries (²). While the mixtures present in natural product extracts require additional purification and structure elucidation, focusing a concerted effort on select active extracts after the completion of a screen conserves the substantial resources needed to create a combinatorial library of pure compounds. As such, natural product extract libraries, especially with adaptations for the expression of silent and cryptic secondary metabolites, are a promising resource for the discovery of new antimicrobials. We intend to apply this technique to a variety of pathogenic bacteria to develop species specific therapeutics (¹¹).

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Figure 1.

(A) Comparison of NanoLuc (green diamonds) and firefly luciferase luminescence (yellow squares). Active luciferase is represented by solid lines, luciferase-free controls are dashed lines. (B) Comparison between various concentrations of NanoLuc plasmid DNA with various amounts of ribosome inhibiting antibiotics. Concentrations of kanamycin (blue) and erythromycin (red) are 50 or 20 $\mu\text{g/mL}$ (dark hue), 5 or 2 $\mu\text{g/mL}$ (medium hue), and 0.5 or 0.2 $\mu\text{g/mL}$ (light hue), respectively. The negative control is shown in gray.

Figure 2.
Schematic outline of the miniaturized screening process for the natural product (NP) extract library.

Figure 3.

(A) Scatter plot of the negative and positive control wells. Positive control wells are represented by red squares, and negative control wells are represented by blue squares. (B) Scatter plot of the natural product extract library wells (green squares). (C) Representative samples of assay plate results. Columns 1 and 2 of each plate contain the negative controls while 47 and 48 contain the positive controls. Columns 3, 4, 45, and 46 were left empty to prevent signal dispersion into adjacent wells.

Table 1
Natural product extract hits by plate

Plate numbers	1–8	9–15	16–23	All plates
Collection dates	2007–2009	2010–2011	2012–2014	2007–2014
ISP2 media				
No. of extracts	4663	3837	2371	10 871
>50% inhibition	1.5%	10.2%	37.1%	12.3%
>90% inhibition	0.4%	1.7%	3.5%	1.6%
Nutrient poor media				
No. of extracts	4625	3902	2281	10 808
>50% inhibition	0.5%	4.1%	13.8%	4.6%
>90% inhibition	0.2%	0.6%	0.9%	0.5%
A3M Media				
No. of extracts	–	–	1062	1062
>50% inhibition	–	–	49.1%	49.1%
>90% inhibition	–	–	8.9%	8.9%
<i>Corynebacterium</i> Co-culture				
No. of extracts	–	–	755	755
>50% inhibition	–	–	56.6%	56.6%
>90% inhibition	–	–	9.5%	9.5%
<i>Rhodococcus</i> Co-culture				
No. of extracts	–	–	1022	1022
>50% inhibition	–	–	46.3%	46.3%
>90% inhibition	–	–	8.0%	8.0%
Other media				
No. of extracts	689	958	2734	4381
>50% inhibition	34.0%	16.2%	21.0%	22.0%
>90% inhibition	9.6%	3.5%	3.3%	4.3%
All media types				
No. of extracts	9977	8697	10 225	28 899
>50% inhibition	3.3%	8.1%	31.2%	14.6%
>90% inhibition	0.9%	1.4%	4.3%	2.3%
Extract selection	96	336	895	1327
Confirmation screen				
>50% inhibition (triplicate)	20.8%	38.4%	55.6%	48.8%
>90% inhibition (triplicate)	8.3%	10.4%	12.5%	11.7%
Luciferase counterscreen				
<50% inhibition (triplicate)	89.6%	65.8%	71.2%	71.1%
<20% inhibition (triplicate)	40.6%	42.6%	28.4%	32.9%
Final extract selection	8	31	71	110