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Antibiotic Dereplication Using the Antibiotic Resistance Platform

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Dear editor,

We are pleased to present our manuscript for “Antibiotic dereplication using the Antibiotic Resistance Platform”.

Our paper describes the need for a more comprehensive method for dereplicating natural product antibiotics derived from Actinobacteria and other microbes that does not require the use of expensive analytical equipment or sophisticated data interpretation. To provide a solution to this problem, we introduce the Antibiotic Resistance Platform (ARP) and Minimal Antibiotic Resistance Platform (MARP). These platforms utilize a library of isogenic *Escherichia coli* strains expressing individual resistance genes that are effective against the most commonly re-discovered antibiotics. When this library is grown in the presence of an antibiotic-producing organism, the identity of the compound can be deduced by the growth of the *E. coli* indicator strain expressing the associated resistance gene. Our *E. coli* library is currently comprised of over >90 resistance genes that confer resistance to 18 antibiotic classes.

We believe that the Journal of Visualized Experiments (JoVE) is the most appropriate journal for us to submit this manuscript to because interest for the ARP and/or MARP library has been expressed worldwide, and in response it is important to provide users with a detailed protocol. Although this dereplication method does not require extensive equipment or data interpretation, there are a number of techniques that are important to be followed in order for the method to be carried out successfully. With the help of the detailed instruction videos prepared by JoVE, our protocol can be appropriately demonstrated and distributed to those interested.

Sincerely,

Haley L. Zubyk

Georgina Cox

Gerard D. Wright

TITLE:

Antibiotic Dereplication Using the Antibiotic Resistance Platform

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KEYWORDS:

antibiotic, dereplication, drug discovery, actinomycetes, *Escherichia coli*, secondary metabolite, resistance enzymes, natural product

SUMMARY:

We describe a platform that utilizes a library of isogenic antibiotic resistant *Escherichia coli* for the dereplication of antibiotics. The identity of an antibiotic produced by bacteria or fungi can be deduced by the growth of *E. coli* expressing its respective resistance gene. This platform is economically effective and time-efficient.

ABSTRACT:

One of the main challenges in the search for new antibiotics from natural product extracts is the re-discovery of common compounds. To address this challenge, dereplication, which is the process of identifying known compounds, is performed on samples of interest. Methods for dereplication such as analytical separation followed by mass spectrometry are time-consuming and resource-intensive. To improve the dereplication process, we have developed the antibiotic resistance platform (ARP). The ARP is a library of approximately 100 antibiotic resistance genes that have been individually cloned into *Escherichia coli*. This strain collection has many applications, including a cost-effective and facile method for antibiotic dereplication. The process involves the fermentation of antibiotic-producing microbes on the surface of rectangular Petri dishes containing solid medium, thereby allowing for the secretion and diffusion of secondary metabolites through the medium. After a 6-day fermentation period, the microbial biomass is removed, and a thin agar-overlay is added to the Petri dish to create a smooth surface and enable the growth of the *E. coli* indicator strains. Our collection of ARP strains is then pinned onto the surface of the antibiotic-containing Petri dish. The plate is next incubated overnight to allow for *E. coli* growth on the surface of the overlay. Only strains containing resistance to a specific antibiotic (or class) grow on this surface enabling rapid identification of the produced compound.

This method has been successfully used for the identification of producers of known antibiotics and as a means to identify those producing novel compounds.

INTRODUCTION:

Since the discovery of penicillin in 1928, natural products derived from environmental microorganisms have proven to be a rich source of antimicrobial compounds[1]. Approximately 80% of natural product antibiotics are derived from bacteria of the genus *Streptomyces* and other actinomycetes, while the remaining 20% is produced by fungal species[1]. Some of the most common antibiotic scaffolds used in the clinic such as the β-lactams, tetracyclines, rifamycins, and aminoglycosides, were originally isolated from microbes[2]. However, due to the rise of multidrug resistant (MDR) bacteria, our current panel of antibiotics has become less effective in treatment[3,4]. These include the "ESKAPE" pathogens (i.e., vancomycin-resistant enterococci and β-lactam-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacter* sp.), which are a subset of bacteria deemed to be associated with the highest risk by a number of major public health authorities such as the World Health Organization[3–5]. The emergence and global spread of these MDR pathogens results in a constant need for novel antibiotics[3–5]. Regrettably, the past two decades have demonstrated that the discovery of novel antibiotics from microbial sources is increasingly difficult[6]. Current approaches to drug discovery include the high-throughput screening of bioactive compounds, including natural product extract libraries, allowing for thousands of extracts to be tested at a given time[2]. However, once antimicrobial activity is detected, the next step is to analyze the contents of the crude extract to identify the active component and eliminate those containing known or redundant compounds[7,8]. This process, referred to as dereplication, is vital to prevent and/or significantly reduce the time spent on the re-discovery of known antibiotics[7,9]. Although a necessary step in natural product drug discovery, dereplication is notoriously laborious and resource-intensive[10].

Ever since Beutler et al. first coined the term "dereplication", extensive efforts have been made to develop innovative strategies for the rapid identification of known antibiotics[11,12]. Today the most common tools used for dereplication include analytical chromatographic systems such as high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance-based detection methods[11,13]. Unfortunately, each of these methods requires the use of expensive analytical equipment and sophisticated data interpretation.

In an attempt to develop a dereplication method that can be rapidly performed without specialized equipment, we established the antibiotic resistance platform (ARP)[10]. The ARP can be used for the discovery of antibiotic adjuvants, the profiling of new antibiotic compounds against known resistance mechanisms, and the dereplication of known antibiotics in extracts derived from actinobacteria and other microbes. Here, we focus on its application in antibiotic dereplication. The ARP utilizes a library of isogenic *Escherichia coli* strains expressing individual resistance genes that are effective against the most commonly re-discovered antibiotics[14,15]. When the *E. coli* library is grown in the presence of a secondary metabolite-producing organism, the identity of the compound can be deduced by the growth of *E. coli* strains that express its associated resistance gene[10]. When the ARP was first reported, the library consisted of >40 genes

conferring resistance to 16 antibiotic classes. The original dereplication template was designed to encompass a subset of resistance genes per antibiotic class to provide information regarding antibiotic subclass during the dereplication process. Today, the ARP is comprised of >90 genes that confer resistance to 18 antibiotic classes. Using our extensive collection of resistance genes, a secondary dereplication template has been developed and is known as the minimal antibiotic resistance platform (MARP). This template was created to eliminate gene redundancy and to simply provide information regarding the general antibiotic class that a dereplicated metabolite is related to. Additionally, the MARP template possesses both wildtype and a hyperpermeable/efflux deficient strain of *E. coli* BW25113 (*E. coli* BW25113 Δ*bamB*Δ*tolC)*, compared to the original incarnation of the ARP, which only utilizes the hyperpermeable strain. This unique aspect creates additional phenotypes during dereplication, indicating a compounds ability to cross the outer membrane of Gram-negative bacteria. Here, we describe a robust protocol to be followed when dereplicating with either the ARP and/or MARP, highlight the most critical steps to be followed, and discuss the various possible outcomes.

PROTOCOL:

1. Preparation of *E. coli* library glycerol stocks (from agar slants)

1.1. Streak the ARP/MARP *E. coli* strains from lysogeny broth (LB) agar slants onto Petri dishes containing LB agar and the appropriate selectable marker (**Table 1**).

1.2. Prepare cultures for each of the *E. coli* strains by inoculating 3 mL of LB containing the appropriate selectable marker with a single colony. Grow overnight at 37 °C with aeration (250 rpm).

1.3. Combine 800 µL of culture and 200 µL of sterile 80% glycerol in a 1.8 mL cryovial. Mix by inverting the tubes 3−4 times, and store at -80 °C.

2. ARP/MARP frozen stock library plate preparation

2.1. Streak the ARP/MARP strains from the glycerol stocks prepared in section 1 onto a new set of Petri dishes containing LB agar and the appropriate selectable marker. Grow overnight at 37 °C.

2.2. Using aseptic technique, pipette 500 µL of cation adjusted Mueller Hinton broth (MHB) from a sterile reservoir into each well of a sterile 96 deep well plate.

2.3. With the plates prepared in step 2.1, use an applicator stick to inoculate the 96 deep well plate in accordance with the ARP/MARP map (**Supplemental Figure 1** and **Supplemental Figure 2**). Ensure that the appropriate selectable marker is added to each well. Incubate the deep well plate overnight at 37 °C with aeration (250 rpm).

2.4. Ensure that there are no contaminated wells by referring to the ARP/MARP map. Repeat if

contaminated. Using a multi-channel pipettor, transfer 100 µL from each well of the deep well plate to a sterile 96-well round bottom plate. Repeat this step to create multiple frozen stock library plates.

NOTE: It is best to prepare at least 5 library plates at a time to keep from repeating steps 2.1−2.4 in the event of frozen stock library plate contamination.

2.5. Finish making the ARP/MARP frozen stock library plates by pipetting 100 µL of sterile 50% glycerol into each well and mix by gently pipetting up and down.

2.6. Cover the plates with sterile aluminum seals and ensure that each well is individually sealed.

2.7. Number the plates and dedicate only one frozen stock library plate for inoculating new templates at a given time. Keep the remainder as back-ups in the event of frozen stock library plate contamination.

2.8. Place the plate lid on top of the aluminum seal and store at -80 °C.

3. Seed culture and dereplication plate preparation

3.1. Using an applicator stick, inoculate 3 mL of *Streptomyces* antibiotic medium (SAM) (or other appropriate medium for the organism being tested) in a test tube containing one sterile glass bead (to break-up the mycelium) with the producing strain that is to be dereplicated. For *Streptomyces,* gently scrape spores from the surface of a colony.

3.2. Using the same wooden applicator stick, streak a sterility control on a Petri dish containing Bennett's agar.

3.3. Incubate the seed culture at 30 °C with aeration for 6 days (250 rpm) and incubate the sterility control plate at 30 °C for 6 days.

NOTE: Refer to **Table 2** for SAM and Bennett's media recipes. The above instructions are suitable for most actinomycetes. Alter growth media as necessary for other bacteria and fungi.

3.4. Prepare dereplication plates by aspirating 23 mL of Bennett's agar into a serological pipette and dispense 20 mL evenly across the surface of a rectangular Petri dish (**Table of Materials**), leaving the remainder of the medium in the pipette to prevent air bubble formation.

NOTE: Ensure that the surface being used to pour plates is level and perform this step before the agar has cooled too much; a flat surface is imperative for library pinning in the next stages.

3.5. Gently rotate the plate until the medium covers all areas of the plate and do not disturb it until the agar has set completely.

3.6. Prepare nitrocellulose membrane sheets (**Table of Materials**) by using a rectangular Petri dish lid as a tracing template so that the sheets fit the surface of the dereplication plate. Cut the sheets and autoclave them in a sterile pouch.

NOTE: This membrane allows for organisms to sporulate on its surface, while secondary metabolites may be excreted into the medium below. Once grown, the membrane is removed to provide a clean surface for dereplication. The closer fit that the membrane paper has on the surface of the Bennett's agar, the cleaner the dereplication result.

3.7. Check the sterility control plate to ensure that no contaminants are present after 6 days of incubation. If contamination-free, remove the lid of the rectangular Petri dish and pipette 200 µL of seed culture onto the surface of the Bennett's agar.

3.8. Evenly spread the culture across the surface of the entire plate using a sterile cotton swab.

3.9. Place the nitrocellulose membrane prepared in step 3.6 over top of the culture on the surface of the Petri dish. Begin by aligning the bottom edge of the membrane to the bottom edge of the Petri dish, and slowly apply the membrane from the bottom edge to the top edge of the plate.

3.10. Use a sterile cotton swab to smooth out any air bubbles that may have formed between the membrane-agar interface, ensuring that the membrane is flush to the agar.

3.11. Put the lid back on the rectangular Petri dish and place it upside down in a sealed plastic bag. Incubate at 30 °C for 6 days.

4. Dereplication plate MHB overlay and ARP/MARP library plate preparation

4.1. After 6 days, remove the dereplication plate from the 30 °C incubator. Using sterile tweezers (autoclaved or sprayed thoroughly with 70% ethanol), carefully remove the nitrocellulose membrane from the surface of the Bennett's agar.

NOTE: This step will remove the hydrophobic spores and mycelia grown on the surface of the membrane to provide a clean surface for dereplication, facilitating step 4.2.

4.2. As described for step 3.4, ensure the work surface is level and use a serological pipette to aspirate 23 mL of cation adjusted MHB agar. Create an overlay by dispensing 20 mL evenly across the surface of the dereplication plate, leaving the remainder of the medium in the pipette to prevent air bubble formation.

4.3. Gently rotate the plate until the medium covers all areas and do not disturb it until the agar has set completely. Once cooled and solidified, return the dereplication plate to the sealed plastic bag and store it upside at 4 °C overnight.

NOTE: This step allows for diffusion of secondary metabolites from the fermented Bennett's medium into the MHB agar overlay. If the nitrocellulose membrane was not prepared properly there will be spore growth around the edges of the plate, which have hydrophobic properties that repel the MHB agar. Do not pour the overlay on top of these spores because it can result in contamination of the overlay.

4.4. On the same day that the overlay is poured, inoculate a fresh ARP/MARP template by pipetting 100 µL of cation adjusted MHB into each well of a 96-well plate.

NOTE: To reduce the chance of spreading contamination during dereplication, use a single ARP/MARP library plate to only dereplicate 2−3 dereplication plates. Therefore, inoculate enough 96-well ARP/MARP plates based on the number of strains that will be dereplicated.

4.5. Take the frozen stock ARP/MARP library plate out of the -80 °C freezer. Remove the aluminum seal before condensation begins to form on its underside, thereby decreasing the chance of contaminating neighboring wells in the library plate.

4.6. Using 96-well pinning tools (or other forms of inoculation equipment), carefully pin from the frozen stock ARP/MARP library plate and inoculate the fresh MHB-containing 96-well plates.

4.7. Put a new sterile aluminum seal on the frozen template once complete and return it to the -80 °C freezer. Place the inoculated 96-well plates inside of a sealed plastic bag and incubate at 37 °C with aeration (250 rpm) for 18 h.

NOTE: New frozen stock library plates can be prepared from this step after ensuring that no contamination is present. Add glycerol to the plate before storing at -80 °C as described in step 2.5.

5. Dereplicating using the ARP/MARP

5.1. Remove the ARP/MARP template from the incubator and ensure that no contaminants are present. Always dereplicate using a template that is freshly prepared and not directly from the frozen stock.

5.2. Remove the dereplication plates from 4 °C and allow to equilibrate to room temperature. If there is condensation, open the lids and allow to dry in a sterile environment.

5.3. Using pinning tools (or other inoculation equipment), pin from the ARP/MARP library plate onto the surface of the MHB agar overlay of the dereplication plates. Be careful not to pierce the agar.

5.4. After pinning the template onto the surface of the dereplication plates, allow the template inoculum to dry for 3−5 min. Place the inoculated dereplication plates upside down in a sealed plastic bag and incubate overnight at 37 °C.

5.5. Analyze dereplication results the following day by comparing growth on the dereplication plate to wells that correspond to the ARP/MARP map (**Table 3** and **Table 4**).

REPRESENTATIVE RESULTS:

The following results were obtained when a collection of antibiotic-producing strains of interest were dereplicated using the ARP and/or MARP.

A diagram of the ARP/MARP dereplication workflow is depicted in **Figure 1**, and library plate maps are shown in **Supplemental Figure 1** and **Supplemental Figure 2**. **Figure 2** demonstrates a positive dereplication result wherein the environmental extract WAC 8921 is identified as a chloramphenicol producer. **Figure 3** shows a lack of ARP growth entirely, which indicates the presence of either an unknown antibiotic or a less commonly found antibiotic that is not accounted for in the ARP/MARP library plate. **Figure 4** demonstrates a growth pattern that is unique to the MARP because of its utilization of both wildtype *E. coli* BW25113 and a hyperpermeable and efflux deficient mutant *E. coli* BW25113 Δ*bamB*Δ*tolC.* This result suggests the presence of a compound with antimicrobial activity that is unable to surpass an intact outer membrane. **Figure 5** shows an *E. coli* growth pattern that suggests the improper sterilization of pinning tools and **Figure 6** shows an example of ARP/MARP frozen stock library plate contamination. **Figure 7** demonstrates what happens if the agar overlay is pierced during dereplication. Lastly, **Figure 8** shows MHB overlay related contamination that can occur during the dereplication process.

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic of the dereplication process. The producing strain to be dereplicated is streaked onto a rectangular Petri dish as a lawn and a nitrocellulose membrane is placed on top. The plate is then incubated for 6 days wherein the producing-strain related biomass grows on the surface of the membrane while and secondary metabolites produced are secreted into the Petri dish media. After a 6-day fermentation period, the membrane is removed and an MHB overlay is added to the surface of the antibiotic-containing media to provide a smooth surface for pinning. The ARP/MARP *E. coli* library, which is arranged in a 96-well plate format according to the ARP/MARP Maps, is then pinned onto the surface of the overlay. After incubating the tray overnight at 37 °C, the growth of *E. coli* strains expressing specific resistance genes indicates the identity of the compound produced.

Figure 2: Dereplication of a known antibiotic. The producing strain WAC 8921 was dereplicated using the ARP template. Growth of *E. coli* BW25113 Δ*bamB*Δ*tolC* pGDP1:*CAT* on the surface of the MHB agar overlay indicates that WAC 8921 is a chloramphenicol producer.

Figure 3: Dereplication of an unknown antibiotic. The producing strain WAC 9941 was dereplicated using the ARP template. A lack of *E. coli* library growth was seen on the surface of the rectangular Petri dish, indicating that either WAC 9441 is producing an unknown antimicrobial compound or a rare antibiotic that is not accounted for in the ARP.

Figure 4: Identification of an antimicrobial compound that cannot traverse an intact outer membrane. The producing strain WAC 4178 was dereplicated using the MARP template. Strains of *E. coli* BW25113 are capable of growing on the surface of the secondary metabolite-containing media, whereas all strains of *E. coli* BW25113 Δ*bamB*Δ*tolC* cannot grow. This suggests that WAC 4178 is producing an antimicrobial compound that cannot traverse an intact outer membrane.

Figure 5: Contamination due to non-sterile pinning tools. The producing strain WAC 7094 was dereplicated using the ARP template. The presence of *E. coli* library growth in areas that did not have an assigned *E. coli* strain suggests that the pinning tools used to inoculate the MHB agar overlay of the rectangular Petri dish were not properly sterilized. This results in the transfer of unknown *E. coli* strains across the overlay.

Figure 6: Contamination due to a contaminated frozen stock ARP/MARP template. The producing strain WAC 3683 was dereplicated using the ARP template. Three distinct *E. coli* colonies grew on the rectangular Petri dish surface: two correspond with *E. coli* BW25113 Δ*bamB*Δ*tolC* expressing STAT, a streptothricin resistance enzyme, and the other corresponds to *E. coli* BW25113 Δ*bamB*Δ*tolC* expressing VIM-2_{ss}, a β-lactam resistance enzyme. Due to a lack of replicating bla_{VIM2ss} colony growth, in addition to the lack of cross-resistance known to occur between these two antibiotic classes, it can be assumed that a strain other than *E. coli* Δ*bamB*Δ*tolC* pGDP1: bla_{VIM2ss} is growing in the respective frozen library plate well.

Figure 7: Pierced MHB agar overlay. The producing strain WAC 5106 was dereplicated using the ARP template. This strain was found to be a streptomycin producer, as indicated by the growth of *E. coli* BW25113 Δ*bamB*Δ*tolC* pGDP3:*aph(6)-Ia*. Puncture holes can be seen on the surface of the MHB agar overlay along the perimeter of the plate. While this does not affect the dereplication results, it can make the data difficult to interpret at first glance.

Figure 8: Contamination of the MHB agar overlay. Contaminated MHB agar produces an irregular growth pattern on the surface of the overlay that becomes visible after incubating the plate overnight at 37 °C. Although *E. coli* growth may still be visible through the contamination, it is advised to repeat the experiment before extrapolating data from the plate.

Table 1: Selectable markers used in the pGDP plasmid series. Streak the ARP/MARP *E. coli* strains onto LB agar Petri dishes containing the appropriate selectable marker at the right concentration for each plasmid.

Table 2: Recipes for SAM and Bennett's media, and Czapek mineral mix. Adjust SAM and Bennett's to pH 6.8 before autoclaving, and filter sterilize the Czapek mineral mix.

Table 3: Well designation table for the minimal ARP strains. This table indicates which well of a 96-well plate that each of the minimal ARP strains can be found in according to the minimal ARP library plate map. The table also lists which antibiotic class each gene confers resistance to. Please note that some genes may confer resistance to more than one antibiotic within the given

antibiotic class.

Table 4: Well designation table for the ARP strains. This table indicates which well of a 96-well plate that each of the ARP strains can be found in according to the ARP library plate map. The table also lists which antibiotic class each gene confers resistance to. Please note that some genes may confer resistance to more than one antibiotic within the given antibiotic class.

Supplemental Figure 1: Library plate map used for the original antibiotic resistance platform (ARP) template. Organize the respective *E. coli* strains in a 96-well plate using this format to ensure that all necessary controls and duplicates are included. This figure has been modified from Cox et al.[10].

Supplemental Figure 2: Library plate map used for the minimal antibiotic resistance platform (MARP) template. Organize the respective *E. coli* strains in a 96-well plate using this format to ensure that all necessary controls and duplicates are included.

DISCUSSION:

The protocol described above can be applied to both the discovery of novel antimicrobial compounds and adjuvants that can be used in conjunction with existing antibiotics to rescue their activity. The platform takes advantage of the high substrate specificity of resistance mechanisms and their cognate antibiotics, to dereplicate compounds within crude natural product extracts. Although the time required for dereplication plates to be prepared is lengthy (~2 weeks), the dereplication process itself is complete after a single overnight incubation period, which is rapid in comparison to the time it can take to isolate and characterize a compound from crude extracts. Additionally, no expensive or highly specialized equipment is required, making this platform accessible and cost-effective.

Another major benefit of this platform is its flexibility. The ARP can be expanded to contain resistance genes that encompass more antibiotic classes. This is achieved by monitoring literature for the emergence of novel resistance enzymes and using basic molecular cloning techniques to add the genes to the *E. coli* library. Furthermore, the dereplication template is customizable based on the desired level of broad or narrow range substrate specificity that an individual wants to use when dereplicating. Any combination of genes in the *E. coli* library can be used to make novel library plates with the ability to detect compounds with different profiles. For example, a β-lactamase expressing *E. coli* template could be developed to allow for the highly specific dereplication of β-lactams and their different subclasses.

While this platform was initially designed to dereplicate compounds on solid media, it also been works in liquid media. This is useful when working with compounds that have already been purified wherein only a limited amount is available for testing, or when working with compounds that do not diffuse easily or consistently in solid media. Lastly, while this protocol was described using the *E. coli* strains BW25113 and BW25113 Δ*bamB*Δ*tolC,* the platform can be used with the resistance gene library expressed in different strains of *E. coli* (dereplication phenotypes may vary). Ultimately, the Antibiotic Resistance Platform is flexible, has many applications, and is

advantageous over other dereplication methods.

To ensure that reproducible and non-contaminated results are obtained, it is critical to follow appropriate sterilization and aseptic techniques. Failure to do so will result in contamination of the pinning tools, library plate, or the dereplication plate itself. While selectable markers are present in the *E. coli* library, which can help prevent contamination caused by bacteria missing the marker, it does not prevent the cross-contamination of strains using the same marker. To reduce the risk of this happening it is essential to carefully sterilize the bacterial pinning tools before pinning from the library plate. Each library plate should only be pinned from 3−4 times maximum before discarding for a new template. This prevents the spread of contamination across all dereplication plates if a library plate becomes contaminated during the pinning process. Additionally, no antibiotics are used when inoculating the dereplication plate and so great care must be taken to prevent contamination before it is left to ferment for six days. Another possible source of contamination is in the MHB agar overlay of the dereplication plate. If the overlay media is contaminated, growth will only appear after the overnight incubation at 37 °C post-pinning. Overlay contamination can make it extremely difficult to analyze the growth of the *E. coli* library on the overlay surface. To reduce the chances of overlay contamination, prepare MHB agar fresh before pouring the overlay. It is recommended that until an individual is comfortable with this method, dereplication plates should always be prepared in duplicate or triplicate such that in the event of contamination of one plate, data can still be extracted from the hopefully non-contaminated plates.

Lastly, it is important to note that the ARP/MARP has limitations. This protocol is not suitable for the de-replication of producing-strains that produce more than one bioactive compound. Each strain in the *E. coli* library has been designed to express a single resistance gene. If two antibiotics are being produced by an organism, neither resistance gene will confer resistance to the second antibiotic, thereby resulting in cell death of both strains. Thus, this possibility must be considered when dereplication results suggest the presence of a novel antibiotic because the production of multiple antibiotics cannot be detected by the current single construct *E. coli* library. One approach that can be taken to combat the challenge of dereplicating strains that produce more than one antibiotic involves using the agar-plug procedure described in the original ARP paper by Cox et al.[10]. In this method, a portion of a fermented solid medium is removed from an antibiotic-producer containing plate and placed onto a lawn of indicator ARP strain. The indicator strain can be any of the resistant *E. coli* strains in the ARP library. Zones of inhibition are then used to compare the bioactivity of a producing-strain against the ARP strains and a wild-type strain. ARP strains that form an inhibitory zone of decreased size compared to the wild-type strain can resist the compound being produced. This method has proven to be effective at identifying strains capable of producing multiple antibiotics[10].

In summary, for obtaining the best results when dereplicating with the ARP/MARP it is recommended that dereplication plates are prepared in duplicate or triplicate. Other critical steps in the protocol include pinning from a fresh library plate (never frozen) and removing as much biomass as possible from the producing-strain during the membrane removal stage. If all necessary steps are followed, one should have successful dereplication results for a producing-

strain of interest within a two-week time frame.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1 - Schematic for Dereplication

Click here to access/download;Figure;Figure 1 - Schematic for ARP Dereplication Revised.ai

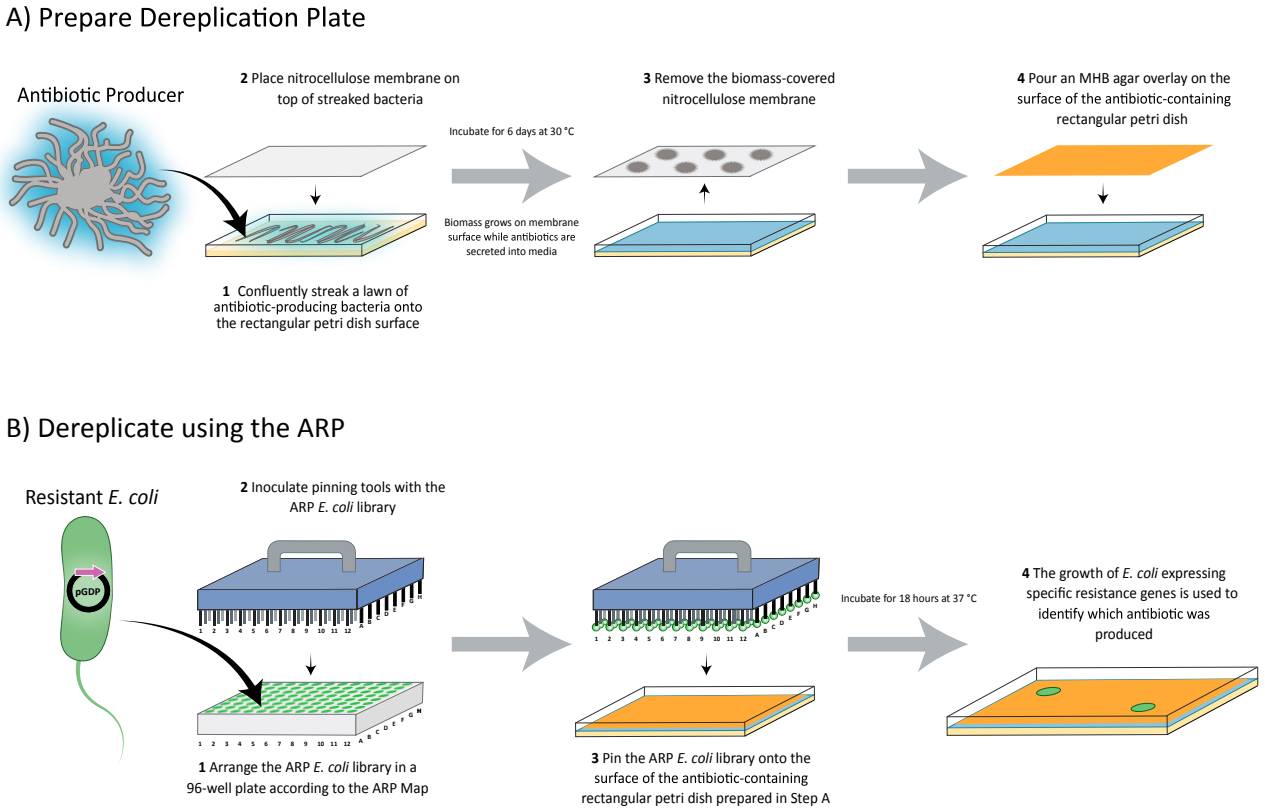

Figure 2 - Chloramphenicol Producer

Click here to access/download;Figure;Figure 2 - WAC 8921 Chloramphenicol Producer.ai

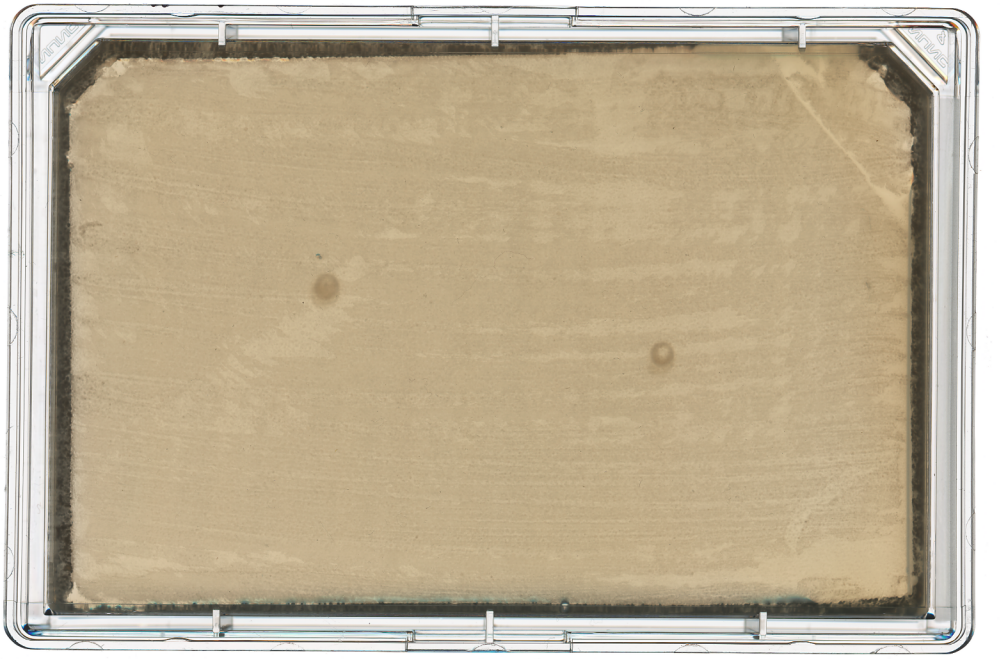

Figure 3 - No ARP Growth

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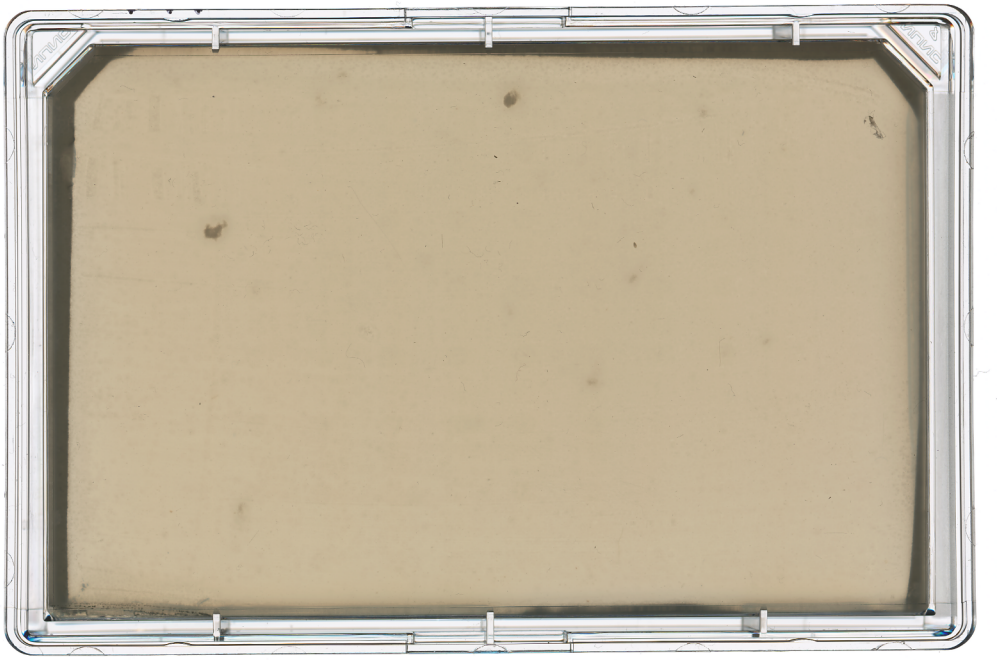

Figure 4 - Compound cannot pass outer membrane

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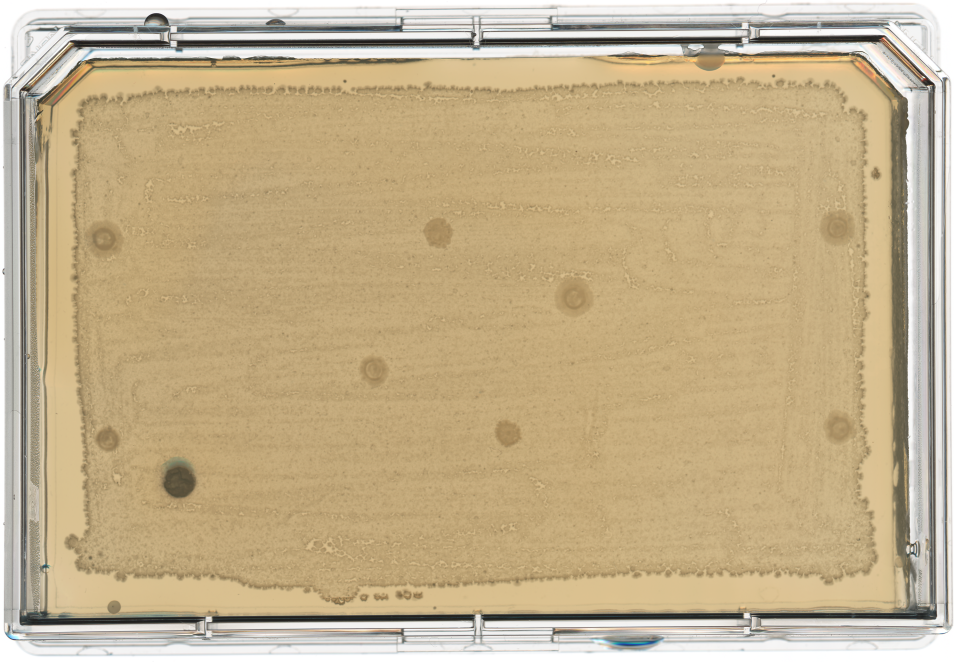

Figure 5 - Contaminated pinning tool

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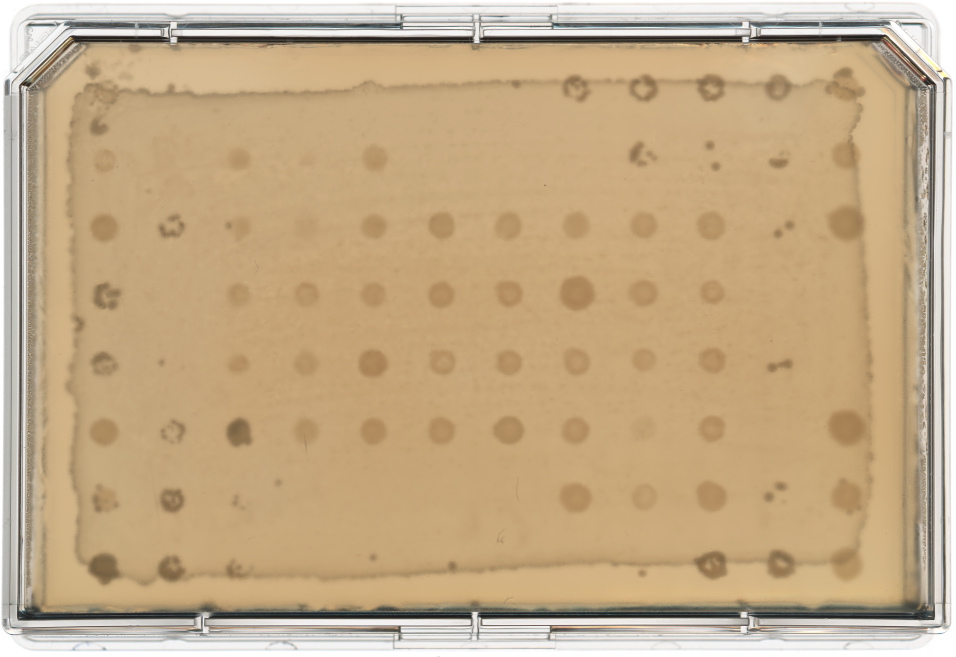

Figure 6 - Contaminated frozen stock template

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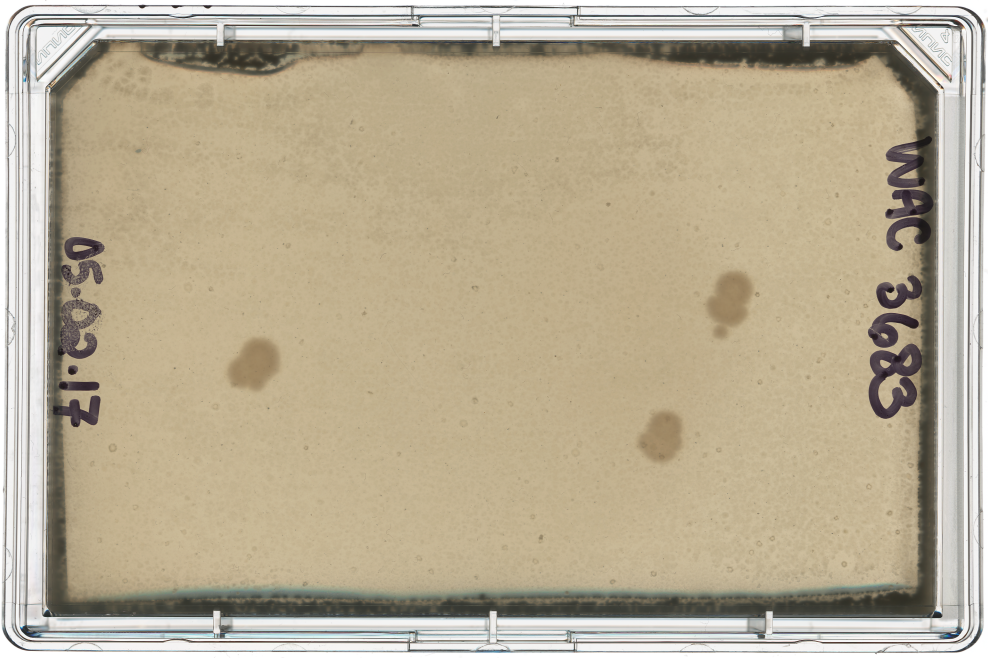

Figure 7 - Pierced agar overlay

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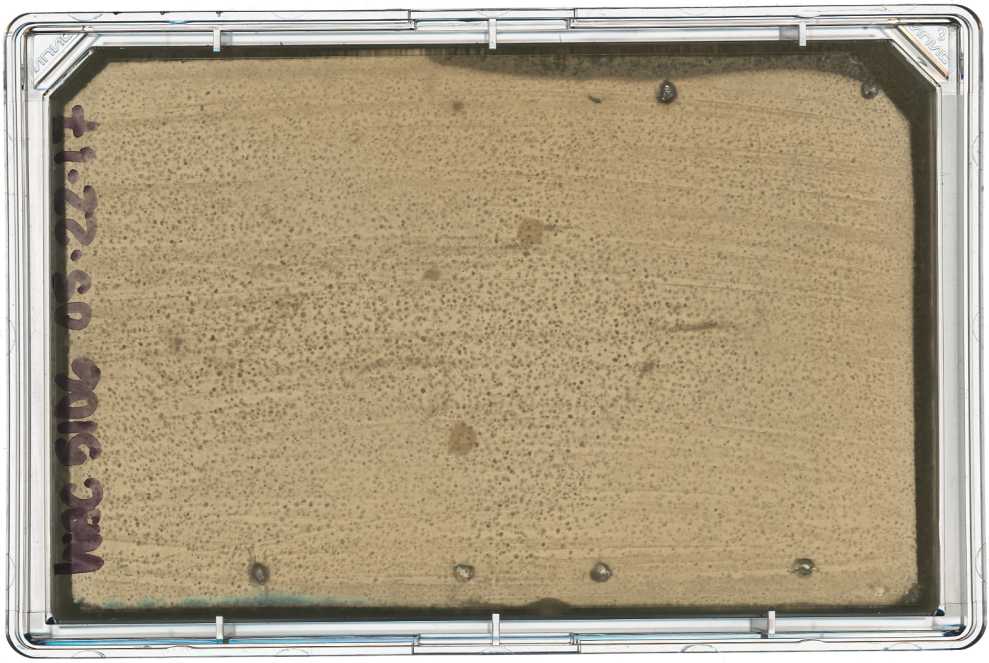

Figure 8 - Contaminated overlay

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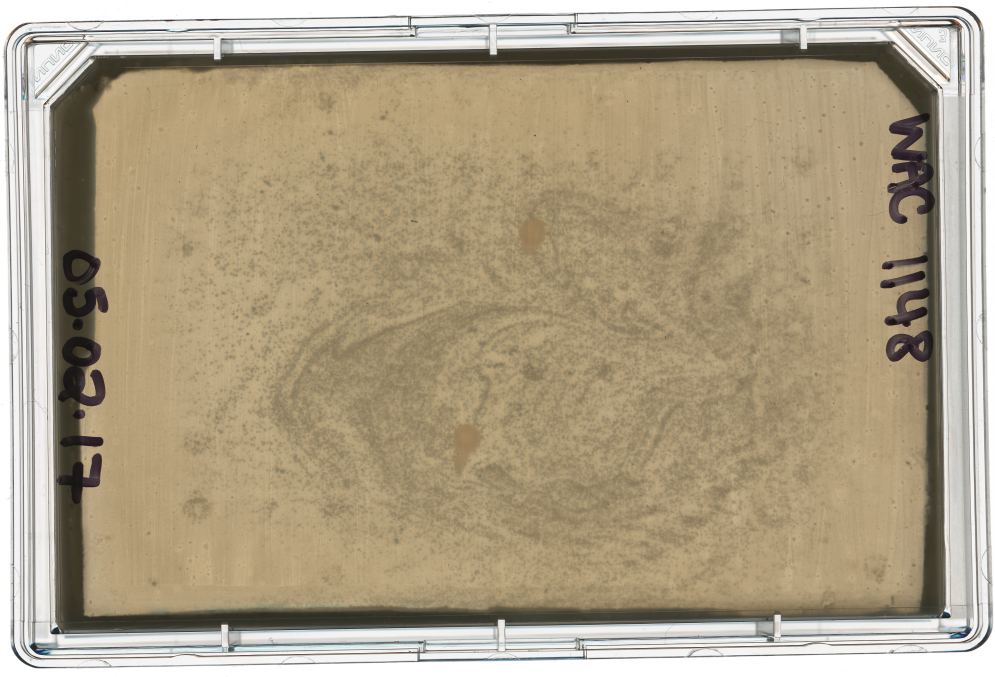

Plasmid	Selectable Marker
pGDP1	Kanamycin 50 µg/mL
pGDP2	
pGDP3	Ampicillin 100 µg/mL
pGDP4	
None	-

Table 2

Click here to access/download;Table;60536_Table 2 - Recipies for media.xlsx

Media	Ingredient	Amount
SAM	Glucose	15 g
	Soya peptone	15 g
	NaCl	5 g
	Yeast extract	1 g
	$CaCO_3$	1 g
	Glycerol	2.5 mL
	ddH_2O	To 1 L
Bennett's	Potato starch	10 g
	Casamino acids	2 g
	Yeast extract	1.8 g
	Czapek mineral mix	2 mL
	Agar (optional)	15 g
	ddH_2O	To 1 L
Czapek mineral mix	KCl	10 g
	$MgSO_4 \cdot 7H_2O$	10 g
	$NaNO_3$	12 g
	$FeSO_4 \cdot 7H_2O$	0.2 g
	Concentrated HCl	200 µL
	ddH_2O	To 100 mL

Table 3 - MARP Well Designation Table

Click here to access/download;Table;Table 3 - Minimal ARP Strain well designation table.xlsx

Antibiotic Class	Antibiotic	Resistance Gene	*E. coli* Strain	Well Position
Aminoglycosides	Streptomycin	*aph(3'')-Ia*	Δ*bamB* Δ*tolC* BW25113	B3, G10
	2- Deoxystreptamine	*rmtB*	Δ*bamB* Δ*tolC* BW25113	F3, C10
	Apramycin	*apmA*	Δ*bamB* Δ*tolC* BW25113	C5, F8
	Spectinomycin	*aph(9)-Ia*	Δ*bamB* Δ*tolC* BW25113	B5, G8
β-lactams	Penicillin	*NDM-1*	Δ*bamB* Δ*tolC* BW25113	B4, G9
	Cephalosporin			
	Carbapenam			
Lincosamides	Lincosamides	*ermC*	Δ*bamB* Δ*tolC* BW25113	D4, E9
Macrolides	Macrolides	*ermC*		
Type B Streptogramins	Type B Streptogramins	*ermC*		
Type A Streptogramins	Type A Streptogramins	*vatD*	Δ*bamB* Δ*tolC* BW25113	C3, F10
Streptothricin	Streptothricin	*STAT*	Δ*bamB* Δ*tolC* BW25113	D3, E10
Tetracyclines	Tetracycline	*tet* (A)	Δ*bamB* Δ*tolC* BW25113	D5, E8
Chloramphenicols	Chloramphenicols	*CAT*	Δ*bamB* Δ*tolC* BW25113	E4, D9
Fosfomycins	Fosfomycins	*fosA*	Δ*bamB* Δ*tolC* BW25113	F6, C7
Rifamycins	Rifamycins	*arr*	Δ*bamB* Δ*tolC* BW25113	E3, D10
Polymyxins	Polymyxins	*MCR-1*	wild-type BW25113	C6, F7
Echinomycins	Echinomycins	*uvrA*	Δ*bamB* Δ*tolC* BW25113	F4, C9
Sideromycins	Albomycin	*fhuB* mutant	Δ*bamB* Δ*tolC* BW25113	C4, F9
Tuberactinomycins	Viomycin	*vph*	Δ*bamB* Δ*tolC* BW25113	F5, C8
N/A	N/A	N/A	wild-type BW25113	C1, C12, F1, F12, E5, D8
N/A	N/A	N/A	Δ*bamB* Δ*tolC* BW25113	A1, A12, B1, B12, D6, D7, E6, E7, G1, G12, H1, H12

Table 4 -ARP Well Designation Table

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Antibiotic Class	Antibiotic	Resistance Gene	*E. coli* Strain	Well Position
Aminoglycosides	Streptomycin	*aph(3'')-Ia*	Δ*bamB* Δ*tolC* BW25113	B2, G11
		aph(6)-Ia	Δ*bamB* Δ*tolC* BW25113	C6,F7
	Spectinomycin	*aph(9)-Ia*	Δ*bamB* Δ*tolC* BW25113	A2, H11
	Gentamicin	*aac(3)-Ia*	Δ*bamB* Δ*tolC* BW25113	A3,H10
		ant(2'')-Ia	Δ*bamB* Δ*tolC* BW25113	A5, H8
		aph(2'')-Id	Δ*bamB* Δ*tolC* BW25113	A4, H9
		armA	Δ*bamB* Δ*tolC* BW25113	A6, H7
		aac(6')-aph(2'')-Ia	Δ*bamB* Δ*tolC* BW25113	B5,G8
	Kanamycin	*aph(3')-Ia*	Δ*bamB* Δ*tolC* BW25113	B4, G9
		aph(3')-IIIa	Δ*bamB* Δ*tolC* BW25113	B3, G10
	Hygromycin	*aph(4)-Ia*	Δ*bamB* Δ*tolC* BW25113	B6,G7
β-lactams	Amoxicillin	*TEM-1*	Δ*bamB* Δ*tolC* BW25113	F6, C7
	Ceftazidime	*CTX-M-15*	Δ*bamB* Δ*tolC* BW25113	F5, C8
	Oxacillin	*OXA-10*	Δ*bamB* Δ*tolC* BW25113	G5, B8
		OXA-48	Δ*bamB* Δ*tolC* BW25113	H5, A8
	Meropenem	*IMP-7ss*	Δ*bamB* Δ*tolC* BW25113	G4, B9
		KPC-2	Δ*bamB* Δ*tolC* BW25113	G6, B7
		NDM-1	Δ*bamB* Δ*tolC* BW25113	H6, A7
	Imipenem	*VIM-2*	Δ*bamB* Δ*tolC* BW25113	F4, C9
Lincosamides	Lincosamides	*ermC*	Δ*bamB* Δ*tolC* BW25113	C4, F9

Lincosamides	Lincosamides	*lnu(A)*	Δ*bamB* Δ*tolC* BW25113	C5, F8
Macrolides	Macrolides	*ermC*	Δ*bamB* Δ*tolC* BW25113	C4, F9
		mphA	Δ*bamB* Δ*tolC* BW25113	C3, F10
		mphB	Δ*bamB* Δ*tolC* BW25113	C2, F11
Type B Streptogramins	Type B Streptogramins	*ermC*	Δ*bamB* Δ*tolC* BW25113	C4, F9
		Vgb	Δ*bamB* Δ*tolC* BW25113	D4, E9
Type A Streptogramins	Type A Streptogramins	*vatD*	Δ*bamB* Δ*tolC* BW25113	D5, E8
Streptothricin	Streptothricin	*STAT*	Δ*bamB* Δ*tolC* BW25113	D3, E10
Tetracyclines	Tetracycline	*tet* (M)	Δ*bamB* Δ*tolC* BW25113	E5, D8
Chloramphenicols	Chloramphenicols	*CAT*	Δ*bamB* Δ*tolC* BW25113	E4, D9
Fosfomycins	Fosfomycins	*fosA*	Δ*bamB* Δ*tolC* BW25113	H4, A9
Rifamycins	Rifamycins	*arr*	Δ*bamB* Δ*tolC* BW25113	E3, D10
N/A	N/A	N/A	Δ*bamB* Δ*tolC* BW25113	A1, A12, B1, B12, D6, D7, E6, E7, G1, G12, H1, H12

Table of Materials

Click here to access/download;Table of Materials;60536_JoVE_Materials-ARP-Revised.xlsx

Name of Material/Equipment	Company	Catalog Number
Agar	Bio Shop	AGR003.5
AlumaSeal CS Films for cold storage	Sigma-Aldrich	Z722642-50EA
Ampicillin Sodium Salt	Bio Shop	AMP201.100
BBL Mueller Hinton II Broth (Cation-Adjusted)	Becton Dickinson	212322
BBL Phytone Peptone (Soytone)	Becton Dickinson	211906
Calcium Carbonate	Bio Shop	CAR303.500
Casamino acid	Bio Basic	3060
Cotton-Tipped Applicators	Fisher Scientific	23-400-101
CryoPure Tube 1.8ml mix.colour	Sarstedt	72.379.992
D-glucose	Bio Shop	GLU501.5
Disposable Culture Tube, 16x100mm	Fisher Scientific	14-961-29
Ethyl Alcohol Anhydrous	Commercial Alcohols	P016EAAN
Glass Beads, Solid	Fisher Scientific	11-312C
Glycerol	Bio Shop	GLY001.4
Hydrochloric Acid	Fisher Scientific	A144-212
Instant sealing sterilization pouch	Fisher Scientific	01-812-54
Iron (II) Sulfate Heptahydrate	Sigma-Aldrich	F7002-250G
Kanamycin Sulfate	Bio Shop	KAN201.50
LB Broth Lennox	Bio Shop	LBL405.500
Magnesium Sulfate Heptahydrate	Fisher Scientific	M63-500
MF-Millipore Membrane Filter, 0.45 µm pore size	Millipore-Sigma	HAWP00010
Microtest Plate 96 well, round base	Sarstedt	82.1582.001
New Brunswick Innova 44	Eppendorf	M1282-0000
Nunc OmniTray Single-Well Plate	Thermo Fisher Scientific	264728
Petri dish 92x16mm with cams	Sarstedt	82.1473.001
Pinning tools	ETH Zurich	-
Potassium Chloride	Fisher Scientific	P217-500
Potato starch	Bulk Barn	279
Sodium Chloride	Fisher Scientific	BP358-10
Sodium Nitrate	Fisher Scientific	S343-500
Wood Applicators	Dukal Corporation	9000
Yeast Extract	Fisher Scientific	BP1422-2

Comments/Description

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with lid, sterile, non treated

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4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
The appropriate edits have been made.

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
The appropriate edits have been made. NOTE is used for minor explanations for why a step is being performed and what is important to keep in consideration.

6. Please remove the embedded table from the manuscript and upload it separately to your Editorial Manager account in the form of an .xls or .xlsx file. Reference the table in the relevant step. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
The appropriate edits have been made.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.
The appropriate edits have been made. Paragraphs within the protocol have now been turned into sub-steps.

8. References: Please do not abbreviate journal titles; use full journal name.
The appropriate edits have been made.

9. Figure 1: Please replace “Omni Tray” with a generic term.
The appropriate edits have been made.

10. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.
The appropriate edits have been made.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript entitled "Antibiotic dereplication using the Antibiotic Resistance Platform" discusses a very interesting a current topic, as MDR pathogens are a serious concern for clinicians worldwide. The paper is extremely well-written and methodologically sound, and will be interesting to anyone working in the field on antimicrobial drug design and discovery. I have the following remarks:
Overall, the paper is extremely well-written (both from an organisational and language perspective), only minor changes are needed (which will be detailed below). The M&M section is impeccably written and the results are

presented/discussed in a similar fashion.
My main concern is the introduction of the paper, which may hinder the manuscript from reaching a very wide readership.
Thank you very much for your feedback. Regarding your concerns for the introduction restricting the readership of this article, we have added additional discussion on the need for new antibiotics and specific pathogens that we hope will add additional information to orient the non-expert reader.

Minor Concerns:
The authors should discuss the current problem pathogens in a few sentences (namely the ESKAPE pathogens, i.e. MRSA, VRE, ESBL- and carbapenemase-producers etc.), with the inclusion of the following references:
Molecules 2019, 24(5), 892; https://doi.org/10.3390/molecules24050892
MRSA: Antibiotics 2019, 8(2), 52; https://doi.org/10.3390/antibiotics8020052
Clin Infect Dis. 2009 Jan 1;48(1):1-12. doi: 10.1086/595011.

The appropriate edits have been made.

Reviewer #2:
Manuscript Summary:
This manuscript describes an E coli platform that has about 100 antibiotic resistance genes cloned into individual strains. These allow for rapid screening for new antibiotics with dereplication (identification of previously known antibiotics)

Major Concerns:
None - the manuscript is well-prepared, useful, easy to read and merits publication

Minor Concerns:
None

Thank you very much for your feedback.

!

	1	2	3	4	5	6	7	8	9	10	11	12
A	ΔTΔB	*aph(9)-Ia:*	*aac(3)-Ia*	*aph(2'')-Id*	*ant(2'')-Ia*	*armA*	*bla*$_{NDM-1}$	*bla*$_{OXA-48}$	*fosA*			ΔTΔB
B	ΔTΔB	*aph(3'')-Ia*:	*aph(3')-IIIa:*	*aph(3')-Ia*	*aac(6')-aph(2'')-Ia*	*aph(4)-Ia*	*bla*$_{KPC-2}$	*bla*$_{OXA-10}$	*bla*$_{IMP-7ss}$			ΔTΔB
C		*mphB*	*mphA*	*ermC*	*lnu(A)*	*aph(6)-Ia*	*bla*$_{TEM-1}$	*bla*$_{CTX-M-15}$	*bla*$_{VIM-2ss}$			
D			*STAT*	*vgb*	*vatD*	ΔTΔB	ΔTΔB	*tet*(M)	*CAT*	*arr*		
E			*arr*	*CAT*	*tet*(M)	ΔTΔB	ΔTΔB	*vatD*	*vgb*	*STAT*		
F				*bla*$_{VIM-2ss}$	*bla*$_{CTX-M-15}$	*bla*$_{TEM-1}$	*aph(6)-Ia*	*Lnu(A)*	*ermC*	*mphA*	*mphB*	
G	ΔTΔB			*bla*$_{IMP-7ss}$	*bla*$_{OXA-10}$	*bla*$_{KPC-2}$	*aph(4)-Ia*	*aac(6')-aph(2'')-Ia*	*aph(3')-Ia*	*aph(3')-IIIa*	*aph(3'')-Ia*	ΔTΔB
H	ΔTΔB			*fosA*	*bla*$_{OXA-48}$	*bla*$_{NDM-1}$	*armA*	*ant(2'')-Ia*	*aph(2'')-Id*	*aac(3')-Ia*	*aph(9)-Ia*	ΔTΔB

!

Supplemental Figure 2 - MARP Plate Map

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	1	2	3	4	5	6	7	8	9	10	11	12
A	ΔTΔB											ΔTΔB
B	ΔTΔB		*aph(3'')-Ia*	*bla*$_{NDM-1}$	*aph(9)-Ia*							ΔTΔB
C	WT		*vatD*	*fhuB* mutant	*apmA*	*MCR-1*	*fosA*	*vph*	*uvrA*	*rmtB*		WT
D			*STAT*	*ermC*	*tet*(A)	ΔTΔB	ΔTΔB	WT	*CAT*	*arr*		
E			*arr*	*CAT*	WT	ΔTΔB	ΔTΔB	*tet*(A)	*ermC*	*STAT*		
F	WT		*rmtB*	*uvrA*	*vph*	*fosA*	*MCR-1*	*apmA*	*fhuB* mutant	*vatD*		WT
G	ΔTΔB							*aph(9)-Ia*	*bla*$_{NDM-1}$	*aph(3'')-Ia*		ΔTΔB
H	ΔTΔB											ΔTΔB

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