The PA14 Non-Redundant Set of
*Pseudomonas aeruginosa* Transposon Insertion Mutants

**USERS MANUAL (version 1.0)**

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I. Overview

The PA14 Non-Redundant Set (PA14NR Set Version 1.0), a subset of the parental PA14 transposon insertion library (see Liberati et al. 2006 and http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi?section=LIBRARY_DESC), was created to expedite genome-scale screening of the *Pseudomonas aeruginosa* chromosome. The PA14NR Set is a collection of mutants in which each disrupted PA14 gene is represented by a single insertion mutant (or in some cases two mutants). A total of 5,459 mutants are included in the PA14NR Set Version 1.0, which correspond to 4,596 predicted PA14 genes (77% of all 5,962 predicted PA14 genes).

Selected mutants were colony-purified to insure that the PA14NR Set is free of cross-contaminants and to keep phenotypic variant sub-population sizes to a minimum. When handling several thousand mutants, well-to-well cross-contamination becomes a major problem in both screening and in making copies of the PA14NR Set. Contaminating sub-populations, which increase in number with each round of liquid subculture, further dilute the expected mutant clone in a particular well and are often very difficult to detect. Prevention of cross-contamination is essential for maintaining the integrity of the PA14NR Set. We have incorporated several preventative measures in the protocol developed for copying the PA14NR Set (see below).

Another major issue when handling PA14NR Set mutants is the tendency of PA14 to form small colony variants (SCVs) (see Drenkard and Ausubel, 2002). We have observed that the frequency of the appearance of PA14 small colony variants increases dramatically when cultures are grown statically, grown with agitation but with a low surface-to-volume ratio, grown for longer than 16 hours, or if the amount of culture used to inoculate a culture is diluted too extensively (see General Guidelines below). Based on these observations, great care has been taken to minimize the size of phenotypic variant subpopulations while creating the PA14NR Set.
II. PA14NR Set Production Schematic

Distributed copies of the library are made by culturing inoculum from frozen Storage Plates. For a complete description of PA14 Transposon mutant library and PA14NR Set production see [http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi](http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi).
III. PA14NR Set Care and Maintenance

A. Copy the PA14NR Set sent to you immediately using the protocol described in Section V. Do not begin working with the PA14NR Set until you have copied it. Manipulation of frozen cultures can cause cross-contamination. Use your original set as a master copy for long-term storage.

B. Work in a sterile environment.

C. Removing and replacing aluminum seals.
   Warm seal with your hand before peeling seal off, being careful not to let any part of the seal retouch the plate. Avoid reusing seals and instead replace with new seals, being careful not to touch the part of the seals that will be in contact with the wells. When picking out of a well with a tip or metal pin, let the plate warm slightly for 15 seconds on the bench. This will make the frozen culture soft enough to avoid chipping the frozen culture that can cause pieces of frozen culture to pop out of source wells into adjacent wells.

D. Periodically re-colony purify the PA14NR Set.
   Frozen copies of the PA14NR Set used as sources for inoculation will most likely become contaminated with frequent use. Because it is impossible to detect minimal amounts of contamination it is difficult to monitor the extent of cross-contamination. Therefore, we recommend periodic colony-purification of the entire PA14NR Set. We have streaked 24 PA14NR Set cultures per 234 mm² Nunc Bioassay Tray (VWR cat. #73520-774) filled with 200 mL of LB agar containing appropriate antibiotics. Single colonies selected for propagation are first confirmed to be free of SCVs by examination under a dissecting microscope. Selected colonies are hand picked into a 2.0 mL Deep 96-well Titerblock (see Section V) containing 600 µL LB and the appropriate antibiotics, grown and transferred as described below.
IV. General Guidelines for PA14NR Set Propagation

1) Glycerol (to final concentration of 15%) must be added and mixed into PA14NR Set cultures before transfer. Transferring PA14 cultures grown in LB or LB + 15 µg/mL gentamycin for various lengths of time, with or without agitation, to either 96 or 384-well plates, resulted in a high frequency of cross-contamination of both adjacent and non-adjacent wells. This occurred whether the transfer was performed by the Biomek FX robot or by hand using a multi-channel pipetor. We assume this cross-contamination is the result of aerosols from the tips as they are held over destination plates. We found that the addition of glycerol (final concentration of 15%) prior to transfer eliminated cross-contamination of wells. Thus, when creating the NR Set, glycerol was added and mixed into master plate cultures before transfer. Whatever the cause of cross-contamination, it is essential that 15% glycerol be added to cultures to be transferred when making copies of the library.

2) Avoid carry over of culture mix on transfer tips. Culture aspiration and dispensing is prone to drops of culture hanging from tip ends that can easily cross-contaminate wells as the robotic head moves over the plates laid out on the deck. To avoid this, we have set the Biomek FX to touch the tips to the side of the wells with each aspiration and dispensing step (see below).

3) Library Propagation should be performed in a 96-well format. Even with glycerol addition to cultures prior to transfer, we were unable to inoculate 384-well plates without cross-contaminating adjacent wells either by hand or robotically. This is due to the necessity of touching the tips to the side of the wells both after aspiration of culture in source plates and after dispensing culture in destination plates. Because 384-well plate well walls are shared between wells culture on the well walls can cross-contaminate other wells more easily. In contrast, well walls of the 96-well plates used for the library, are not shared between different wells and we have found that when using the methods described in Section V of this manual, are free of cross-contaminants. Therefore, we do NOT recommend formatting the NR Library in a 384-well format. Instead, all propagation should be performed in 96-well plates. If copying the library using a robot like the Biomek FX liquid handler, we highly recommend using the method outlined in Section V of this manual.

4) Keep culture times to a minimum. We do not grow cultures more than 16 hours. Generally we allow for 14 hours of growth – although individual mutants may take longer to get to saturation, they are almost always at a high enough density to be used for transfer to copy plates.

5) Grow cultures with agitation in deep-well blocks. We have found that low levels of aeration of PA14 cultures greatly increases the incidence of SCV formation. We have tested cultures grown in a Gene Machines HiGro with supplemental O\textsubscript{2} and cultures grown in a regular shaker with no extra O\textsubscript{2} added. Both conditions prevented the appearance of variants.

6) Keep the surface to volume ratio high, insuring proper aeration of the culture. We found that growing cultures larger than 750 µL in 2.0 mL Deep-96-well Titerblocks (USA Scientific cat. #7556-9600) resulted in the appearance of variants. We grow 600 µL cultures in deep-well
polypropylene Titerblocks (USA Scientific cat. #7556-9600) resulting in a Surface Area to Volume ratio of 0.92) and use these cultures to inoculate copy plates.

7) **Avoid extensive dilution of inoculum.** We have found that the larger the degree dilution, the higher the frequency of variants. We assume this is because the increased amount of cell division required to saturate the culture promotes the appearance of variants in the culture. Preliminary tests have shown that inoculating 600 µL media with an average size wild type colony picked with a pipet tip is sufficient to prevent the appearance of variants in the saturated culture. When using thawed liquid stocks to inoculate media, we generally aim for dilutions of 1:50.

8) **Keep surface area of tip coated with culture/glycerol mix to a minimum.** We have found that even when source cultures contain 15% glycerol, transfer of cultures will result in well-to-well cross-contamination of source and destination plates if the majority of the surface area of the transfer tip is coated with culture/glycerol mix. To avoid this issue we discard the tips that are used to mix glycerol into the culture and use fresh tips to transfer the mixed cultures. Furthermore, the Biomek FX is programmed to have transfer tips aspirate culture mix 11 mm below the liquid surface rather than submerging them any further into the culture mix.
V. Copying the PA14NR Set

We strongly recommend that investigators who receive copies of the PA14NR Set make a copy or copies as soon as possible and keep the original set as a master that is not used for general screening purposes. Copy the PA14NR Set using the protocol described in this manual (PA14NR Set Propagation), the same protocol that was used to make the copies of the library distributed from the Ausubel laboratory. Be sure to run quality control tests of the copying protocol using the PA14 Control plate prior to copying PA14NR Set plates. The PA14 Control Plate contains several cultures of wild type PA14 separated by uninoculated wells. If inappropriate wells become contaminated, the protocol must be reworked and retested. Check test plates several days after inoculation since it can take several days for cross-contaminated wells to develop visible cultures. If you do not have access to a liquid handling robot, you must develop a manual transfer protocol and test it using the PA14 Control Plate.

A. The PA14 Control Plate

![The PA14 Control Plate](image)

The PA14 Control Plate. Green wells represent wells containing wild type PA14 culture. Cultures were picked from single colonies, grown and transferred as described for the PA14NR Set below. White wells represent uninoculated wells.

B. PA14NR Set Propagation

The PA14NR Sets distributed from the Ausubel laboratory were copied from original Storage Plates (PA14NR Set Production schematic above). Cultures from slightly thawed Storage Plates were picked using a metal 96-well pin head and used to inoculate 600 µL LB containing either gentamicin (15 µg/mL) or kanamycin (200 µg/mL) in 2.0 mL Deep 96-well Polypropylene Titerblocks (USA Scientific cat. #7556-9600) (See http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/productionmethods.cgi). Cultures were handled essentially as original colony-purified
cultures (see Biomek FX Transfer Protocol below) and grown overnight at 37°C with agitation (250 rpm) in a Gene Machines HiGro shaker incubator for approximately 15 hours in which supplemental O₂ was injected into the growth chamber at regular time intervals. But as mentioned in the Guidelines, supplemental O₂ is not necessary to prevent the selection of SCVs. 200 µL 60% glycerol was added to each culture and mixed. Tips used to add and mix glycerol were discarded. 40 µL of each culture/glycerol mix was transferred to 20 Greiner Bio-One CellStar 96-well flat-bottom plates (Greiner Bio-One cat. 655180) containing 160 µL LB containing the appropriate antibiotics as described in the transfer protocol below. Plates were sealed with aluminum seals (Diversified Biotech AluminaSeal #ALUM-1000) and stored at –80°C.

**Biomek FX Transfer Protocol**

Screen shots of the Biomek FX Software setup are shown for each step.

**Step 1: Biomek FX Instrument Setup**

a) Deep-Well Titerblocks (USA Scientific cat. #7556-9600) containing 600 µL of grown PA14 cultures per well

b) 96-well Flat bottom plates (Greiner Bio-One cat. 655180) containing 160 µL LB liquid media and appropriate antibiotics per well set up on the Biomek FX platform deck

c) Reservoir containing 160 mL 60% glycerol

d) Robotic Tips: (2 boxes of tips per Deep-Well Titerblock, one for glycerol addition and mixing, another for transfer of culture/glycerol mix) p250 µL Biomek FX tips (Abgene cat. # TN250R-AFX)
Step 1: Biomek FX Instrument Setup
Step 2: Glycerol Addition

200 µL sterile 60% glycerol is added to each well 10 mm below the top of the bacterial culture media in each well resulting in a total volume of 800 µL. The glycerol is mixed with the bacterial media in 150 µL increments three times followed by a tip touch on the side of each well. Tips are discarded.
Step 3: Aspiration of Culture/Glycerol Mix

Using a new set of tips, 40 µL of each culture/glycerol mix is aspirated from each well 11 mm below the top of the liquid in each well. Tips are touched on the side of the well following aspiration.
Step 4: Dispensing Culture/Glycerol Mixes into Destination Plates

40 µL of aspirated culture/glycerol mix is dispensed 1mm from the top of the 160 µL LB containing antibiotics in the destination plates. 1 µL is mixed one time 3 mm from the bottom of the well to remove the final amount of liquid from the tip. Tips are touched on the side of the well following the dispensing step. This step is repeated 20 times to make 20 copies of the PA14NR Set with the same tips. Because the deck can hold only 10 destination plates at a time, destination plates are inoculate 10 plates at a time. After the first 10 destination plates are inoculated, they are replaced with another 10 destination plates (containing LB and antibiotics) on the Biomek FX platform deck. Transfer from the original Titerblock containing the culture/glycerol mix is repeated 10 more times. Plates are sealed with AluminaSeals (Diversified Biotech) and moved to –80°C.
VI. Confirming the Identity of a PA14NR Mutant

The identity of any particular mutant should be confirmed. PCR of a culture derived from a purified colony using a transposon specific primer and a gene-specific primer and sequencing of PCR products is recommended (see Detailed Methods in Liberati et al. and http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/productionmethods.cgi). Sequences can then be subjected to BLAST of the PA14 genome (see http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/blast.cgi). If the BLASTX algorithm is used to search the PA14 protein database (described at http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/blast.cgi), individual PA14 GeneID numbers will indicate which gene has been interrupted. A GeneID search will show where a particular mutant is located in the PA14NR Set (highlighted in red) and link you to the Mutant Report and the Transposon Insertion Map. Conversely, the identity of mutants at particular PA14NR Set plate locations can be found at http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/search.cgi?searchType=SEARCH_PLATE_POSITIONS.

VII. References
