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

USERS MANUAL (version 2.2)

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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 5,850 mutants are included in the PA14NR Set Version 1.0, which correspond to 4,596 predicted PA14 genes.

Background	Transposon	Antibiotic resistance	No. of Mutants	No. of Plates	Plate Name
PA14 (wild type)	*MAR2xT7*	Gentamicin	5,593	59	PAMR_nr_XX_X_dXX
ExoU (2 backgrounds)	*MAR2xT7*	Gentamicin	210	3	EXMR_nr_XX_X_dXX
PA14 (wild type)	*PhoA*	Kanamycin	47	1	PAPHO_nr_XX_X_dXX
PA14 (wild type)	none			1	PAMR_nr_Control_dXX

PA14NR Set Background/Transposon Breakdown. During pilot production of the parental PA14 transposon insertion library, different transposons and backgrounds were used. Some PA14 genes were only represented by mutants in these other backgrounds or with Tn*phoA* insertions. We have included these mutants to maximize coverage of the genome.

As described in Liberati *et al.* mutants were created in two different *exoU* backgrounds. In addition, several mutants included in the PA14NR Set are Tn*phoA* insertions. Use 15 µg/ml gentamicin to select for *MAR2xT7* and 200 µg/mL kanamycin to select for Tn*phoA*.

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). For example, mutants selected for the PA14NR Set were colony-purified before assembly into the PA14NR Set to minimize cross-contamination.

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 18 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. Streaked colonies were checked for SCV phenotypes microscopically. While great effort was taken to avoid selection of SCVs, we have seen a handful of colony-purified PA14NR Set mutants with SCV phenotypes. These mutants will be noted in future versions of our website.

Colonies were picked and used to inoculate source blocks that were incubated at 37°C for 14-16 hours shaking in a HiGro (Gene Machines). Empty wells and low growth wells were noted.

Glycerol was added to Source block cultures to a final concentration of 15%. Glycerol/culture mix was then transferred to several Storage Plates that are currently stored in our lab. One set of Storage plates was used to inoculate another source block for Distribution Plate production. Using a method similar to that outlined in Section V, glycerol was added to the source block for a final concentration of 15%. Glycerol/culture mix was transferred to several Distribution Plates. PA14NR Set Distribution Plates are shipped to requestors.

II. What to do when you first receive the PA14NR Set.

a. Be sure the plates are frozen upon arrival. As you remove the plates out of the biosafety bags place them directly into a –80°C freezer rack. There is a total of 64 plates – be sure you have enough –80°C space.

b. Prepare to replicate the PA14NR Set. Repeated use of the PA14NR Set WILL lead to well-well cross-contamination and degradation of the library over time – regardless of any safety measures taken. Sealing and unsealing plates, slightly warming plates to pick colonies and condensation buildup all contribute to library degradation. Therefore, we recommend that upon receipt of the PA14NR Set, users first work out conditions to make copies of the PA14NR Set that minimizes cross-contamination of copied plates, make the copies and use the original as a master copy. As explained in other sections of this manual, a Control Plate is included that contains wild type PA14 in wells surrounded by uninoculated wells. Use the Control Plate to define replication methods where no cross-contaminants observed in the uninoculated wells. You may want to create your own control plates to further test your method.

c. Replicate the PA14NR Set. Using the method below, up to 20 copies can be made from 1 inoculated block. Decide how much –80°C space you want to dedicate to PA14NR Set copies. Use the original copy of the PA14NR Set as a master copy.

III. Plate Numbering

Originally we planned to format the PA14NR Set in a 384 well format. Because quality control experiments showed that 384-well plate formatting was prone to well-well cross-contamination (see Guideline V), we abandoned this approach, however, the current plate numbering system is a based on a 384-well format. PAMR plate numbers start at 1_1, 1_2, 1_3, 1_4, 2_1, 2_2, 2_3, 2_4, etc and proceed to 15_3. EXMR plates start at 1_1 and proceed to 1_3. There is just one PAPHO plate numbered 1_1. Note that several wells in plates PAMR_nr_14_3, PAMR 15_3, EXMR_nr_3_3, PAPHO_nr_1_1 and PAMR_nr_Control were never inoculated. A full list of PA14 NR Set plate and well locations, whether they contain mutants and the identity of a mutant in a particular location will be available on our website as a downloadable file in the coming days.

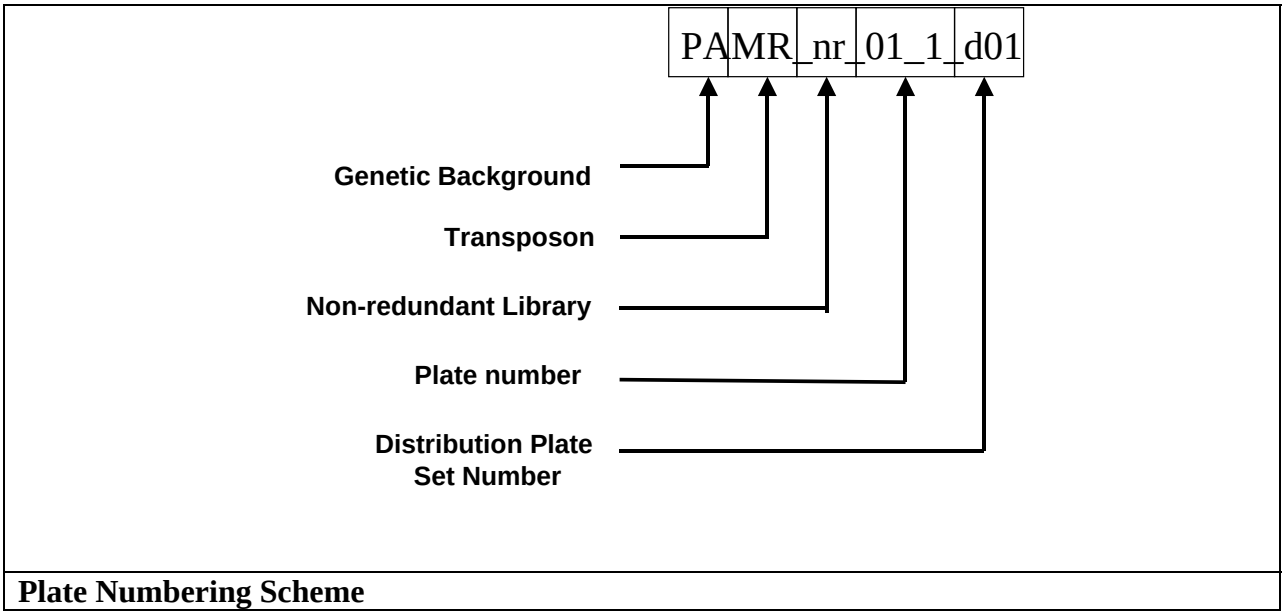
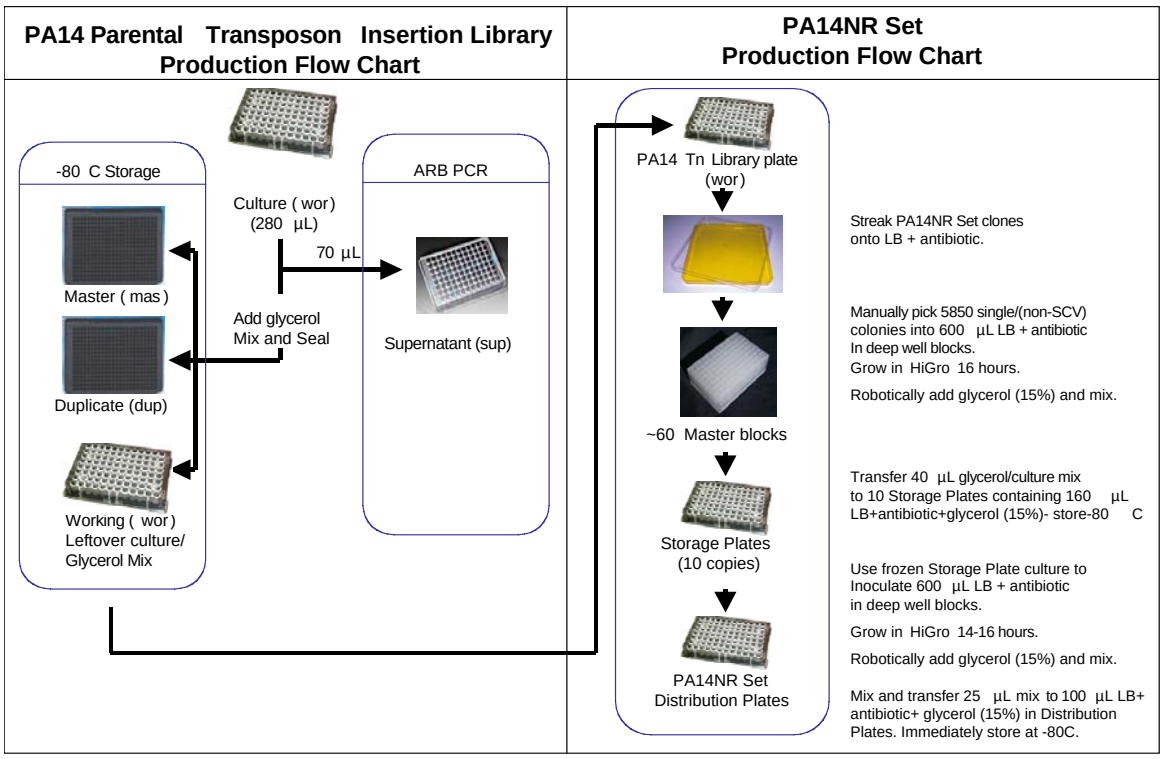


Plate Numbering Scheme

IV. 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.

PA14NR Set Distribution Plate Production

The PA14NR Set Distribution Plates 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 12-channel pipette with sterile tips. Frozen cultures on the tips were 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 grown overnight at 37°C with agitation (250 rpm) in a *Gene Machines* HiGro shaker incubator for approximately 14-16 hours in which supplemental O_2 was injected into the growth chamber at regular time intervals. But as mentioned in the Guidelines, supplemental O_2 is not necessary to prevent the selection of SCVs. 200 µL 60% glycerol was added to each culture and mixed robotically. Tips used to add and mix glycerol were discarded. 25 µL of each culture/glycerol mix was transferred to 22 96-well V-bottom plates (Abgene #MP-2000) containing 100 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.

V. General Guidelines for PA14NR Set Replication

I) 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. We have not found it necessary to quick spin frozen plates to remove condensation from the seal prior to opening, but because Distribution Plates undergo more handling than Storage plates (packing, shipping, unpacking etc.) we recommend quick spinning prior to unsealing to insure material on the seals won't cross-contaminate nearby wells.

II) Inoculating from frozen cultures. When picking out of a well with a tip or metal pin, let the plate warm slightly for 5-10 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. You need very little inoculum to grow cultures that will reach saturation, excessive digging into the frozen stock will increase the likelihood of cross-contamination. When inoculating using a 96-pin replicator, cool the pins after flaming by placing them in a 96-well plate of room temperature media prior to moving them to the frozen PA14NR Set plate.

III) 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 gentamicin 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 in this way. 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.

IV) 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).

V) 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, 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 IV of this manual.

VI) Keep culture times to a minimum. We generally do not grow cultures more than 16 hours. However, in tests where culture from different Distribution Plates were used to inoculate media in deep well blocks, we have found that it can take as long as 18 hours for cultures to clearly reach saturation (bright green cultures) presumably because Distribution Plate cultures are diluted. Testing 10 mutants grown for 18 hours from Distribution Plates showed no SCVs suggesting that under the conditions outlined here, growth for 18 hours should not result in increase the appearance of SCVs. Although individual mutants may take longer to get to saturation, they are almost always at a high enough density to be used to copy plates.

VII) Grow cultures with agitation in deep-well blocks. We have found that low levels of aeration during culture of PA14 increases the incidence of SCV formation. We have tested cultures grown in a *Gene Machines* HiGro with supplemental O_2 and cultures grown in a regular shaker with no extra O_2 added. Both conditions prevented the appearance of variants.

VIII) 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 Distribution plates.

IX) 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 or frozen glycerol stock 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.

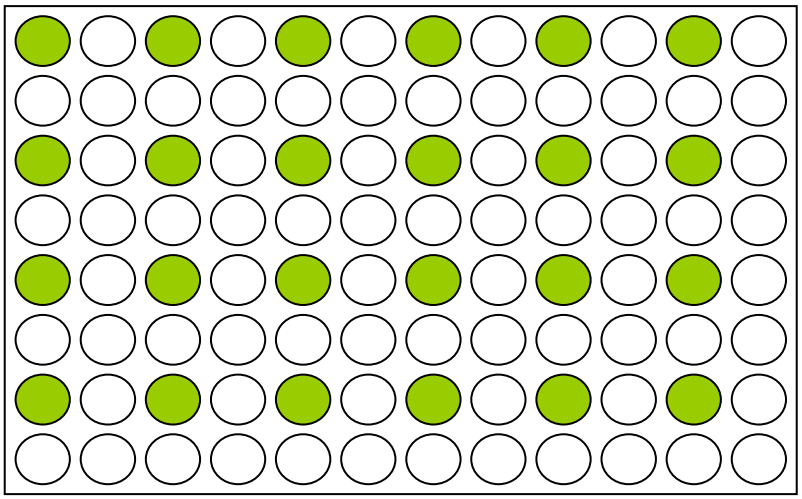
X) Keep surface area of tip coated with culture/glycerol mix to a minimum. We have found that even when source cultures contain 15% glycerol, transfer of cultures will result in well-to-well cross-contamination of source and destination plates if the majority of the surface area of the transfer tip is 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. Because our Biomek FX is not perfectly calibrated, setting the tips to aspirate at 11 mm translates into actual aspiration at approximately 5-6 mm below the liquid surface (setting aspiration at 9 mm below the liquid surface resulted in the tips aspirating above the actual liquid surface). Therefore, if attempting liquid transfer using a Biomek FX we recommend that several aspiration depths be tested before replication of the entire PA14NR Set.

VI. 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 a version of the protocol described below (PA14NR Set Replication Protocol: Copying the PA14 NR Set Distribution Plates for Use in Your Laboratory) tailored to tools available in your laboratory

A. Quality Control Testing of the PA14NR Set Replication Method using the PA14 Control Plate

Be sure to run quality control tests of the PA14NR Set Replication Protocol (see below) 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. Because glycerol inhibits growth of PA14, it is necessary to fill wells in test copy plates that will be receiving liquid from uninoculated wells with LB only. Conversely, because handling of PA14 cultures can produce aerosols, well receiving liquid from inoculated wells should contain LB+15% glycerol. Run the transfer method and check test plates several days after inoculation since it can take several days for cross-contaminated wells to develop visible cultures. If inappropriate wells become contaminated, the protocol must be reworked and retested. 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.



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 Replication Protocol: Copying the PA14 NR Set Distribution Plates for Use in Your Laboratory

Once you have confirmed that the replication method you are using (based on the method outlined below) is reproducibly free of cross-contamination using the Control Plate and in final tests, PA14NR Set plates with known empty wells (see excel file coming soon to the website listing all mutants in each plate and well position), replication of your copy of the PA14NR Set may commence. You should decide how many copies you want to make based on freezer space. The method shown here, is for making 10 copies. Tips follow liquid level in all aspiration and dispensing steps.

Inoculate Culture Blocks

1. Add 600 µL LB (+ antibiotics if appropriate) to a 2.0 mL Deep 96-well Polypropylene Titerblocks (USA Scientific cat. #7556-9600).

2. Quick spin media 1000 RPM for 2 seconds.

3. Remove PA14NR Set plate from freezer and place on dry ice while you prep the bench/hood: place block to be inoculated to next to the sterile tip box you will be using to inoculate block. Keep your tip discard box/Biohazard trash away from your culture block. Minimize the amount of time inoculated tips are held over the culture block.

4. Once prepped, you may want to quick spin the PA14NR Set plate (see Guideline I).

5. Warm the PA14NR Set plate seal with your hand for 3-5 seconds. Carefully remove the seal and set plate on benchtop. Note any dislodged culture/excessive condensation on seals.

6. Pick up a row of tips with a 12-channel pipette. Carefully, place tips in row A of plate, being sure not to touch tops of other wells. All tips should rest on top of the frozen material – gently rock the pipette back and forth insuring that all tips touch frozen culture. (See Guideline II).

7. Move tips to the bottom of wells in Row A of labeled culture block. Remove tips and discard.

8. Repeat steps 6 and 7 for all plate rows – using empty rows in the tip box as a guide as to which rows you have already inoculated.

9. Immediately seal the PA14NR Set plate with a fresh seal (see Guideline I). Insure that every well is sealed by making the impression of each well in the seal visible. A plate roller or even better, a square plastic plate sealer is useful here.

10. Set PA14NR Set plate on dry ice before returning to freezer.

11. Seal culture block with an Aeroseal (Diversified Biotech).

12. Place blocks in a HiGro (GenesMachines) set at 37°C, shaking at 380 rpm with O_2 injection for 2 seconds every 5 minutes. (See Guideline VII). We have found that shaking in a conventional shaker at 250 rpm without supplemental O_2 does not increase the incidence of SCVs.

13. Incubate blocks for no more than 16 –18 hours (See Guideline VI). Once the majority of cultures have reached saturation (become green in color) remove them from the incubator and set at room temperature while you prep for robotic liquid transfer from the source culture block to copy plates.

Biomek FX Transfer Protocol

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

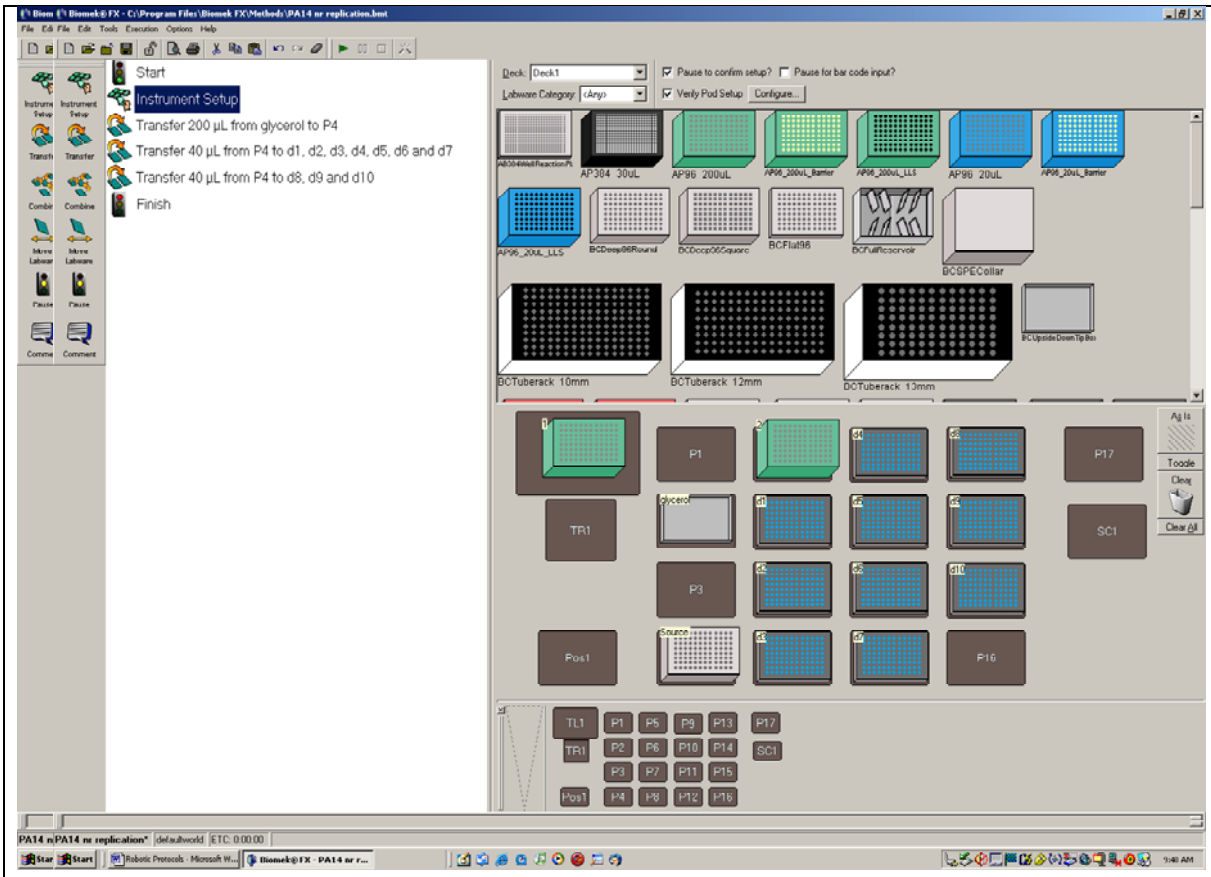
Step 1: Biomek FX Instrument Setup

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

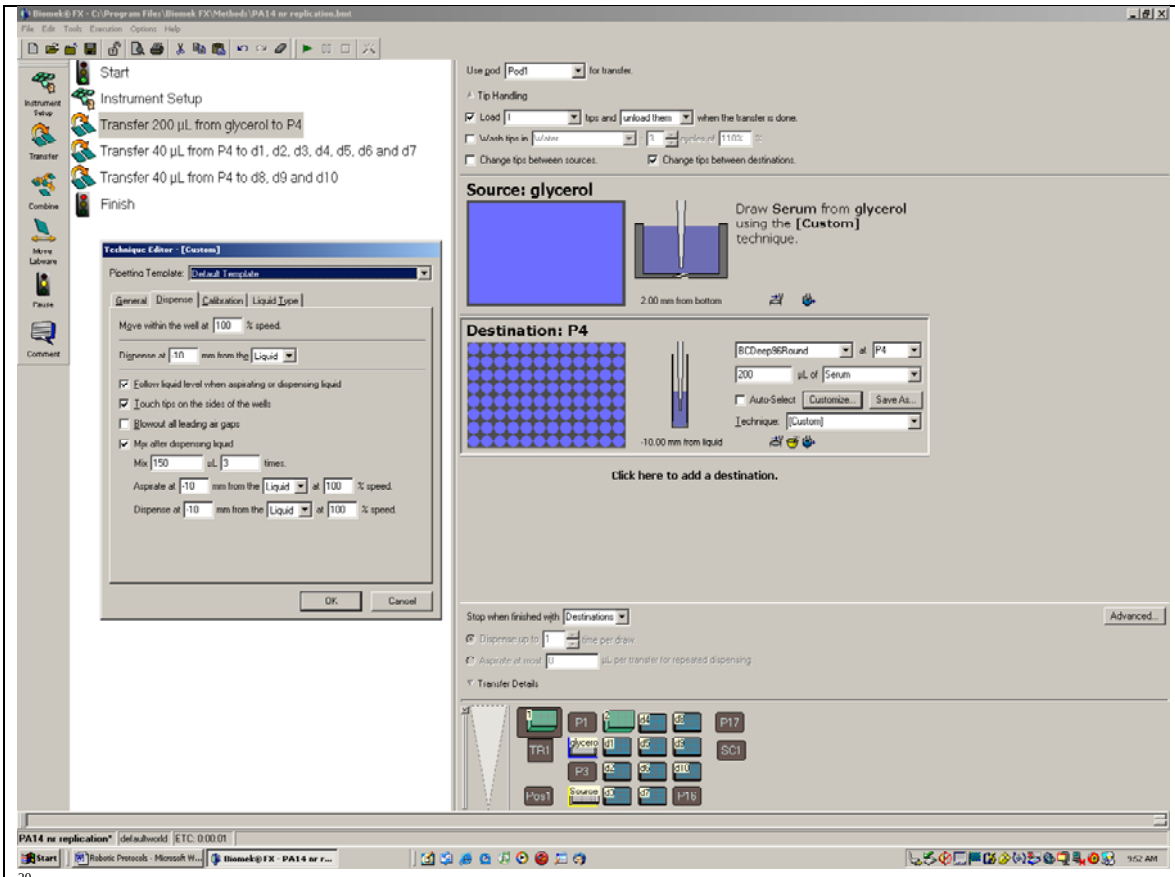
b) 96-well Flat bottom plates (Greiner Bio-One cat. 655180) containing 160 µL LB liquid media and appropriate antibiotics per well set up on the Biomek FX platform deck

c) Reservoir containing160 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 or Beckman Coulter #717253).



Step 1: Biomek FX Instrument Setup



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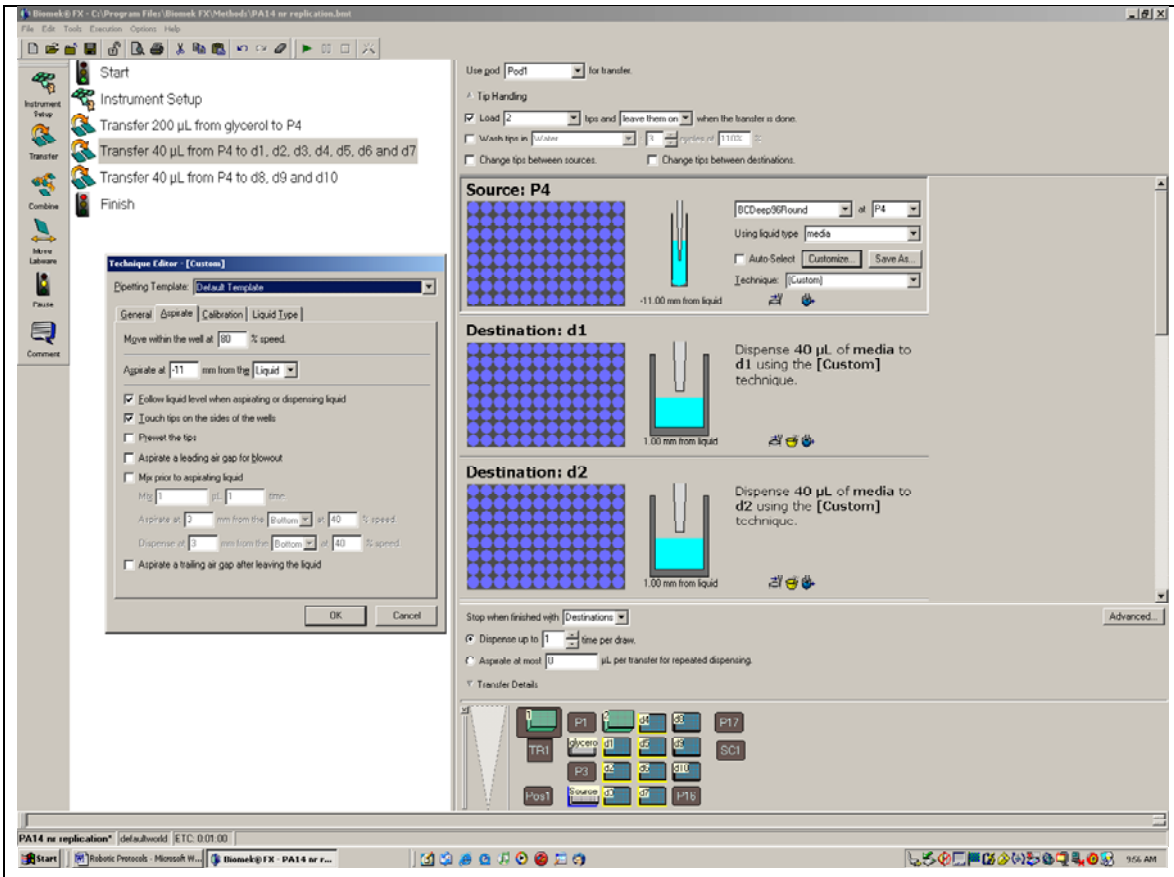
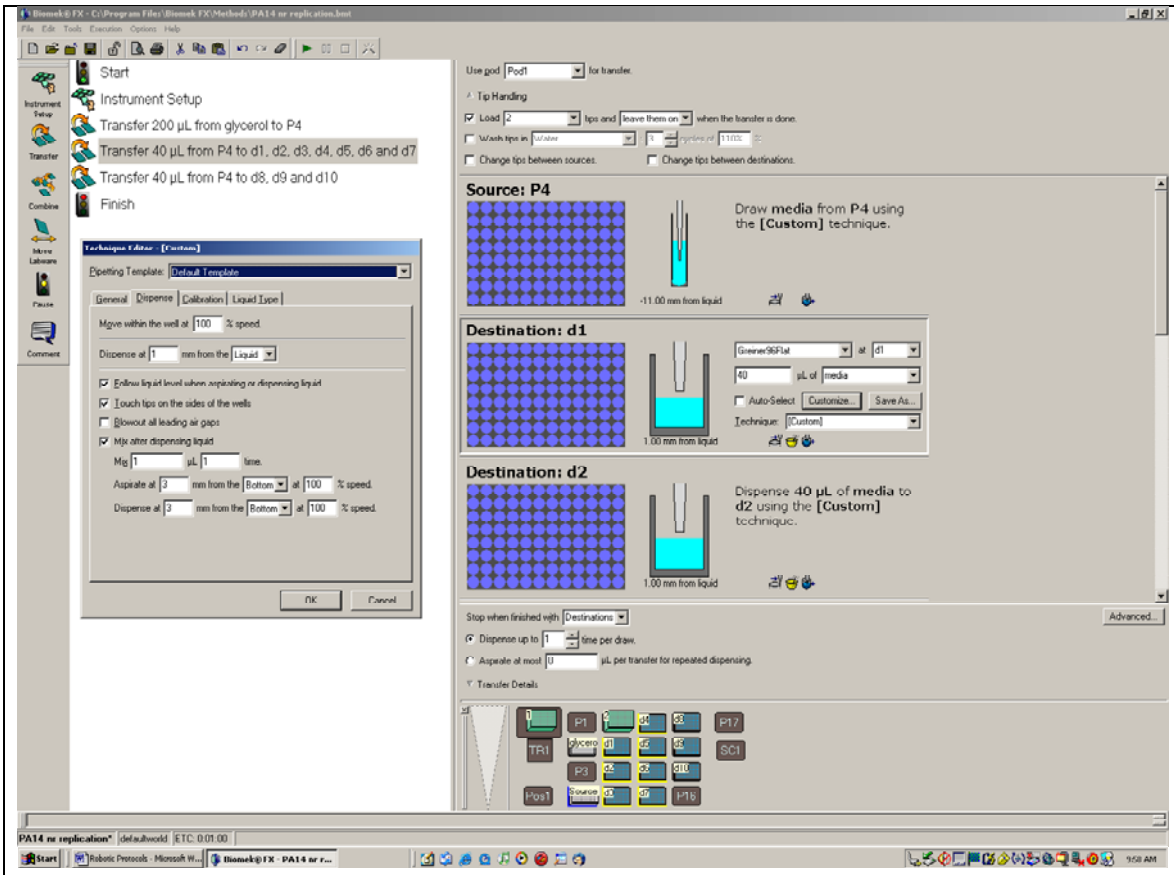
Step 2: Glycerol Addition

200 µL sterile 60% glycerol is aspirated from the reservoir (at 100% speed) and added to each well 10 mm below the top of the bacterial culture media resulting in a total volume of 800 µL. The glycerol is mixed with the bacterial media in 150 µL increments 20 times at 90% speed 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 from the culture block (see Guideline X). 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 10 times to make 10 copies of the PA14NR Set with the same tips. Quality control tests using this dispensing protocol showed no cross-contaminants. Plates are sealed on the Biomek FX deck with AluminaSeals (Diversified Biotech # ALUM-1000) insuring that each well is sealed. Moving plates off the deck before sealing showed increased incidences of cross-contamination in quality control tests. Plates are moved immediately to –80°C taking care not to disturb the liquid surface.

VII. PA14NR Set Long-term Care and Maintenance

A. Copy the PA14NR Set sent to you immediately using the protocol described above. 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. 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 mm^2 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 containing 600 µL LB and the appropriate antibiotics, grown and transferred as described above.

VIII. Confirming the Identity of a PA14NR Mutant

The identity of any particular mutant MUST be confirmed. We estimate that approximately 2.8% of the mutants in the PA14NR Set are incorrectly labeled in our database. If you determine that a particular PA14NR Set has been misidentified, we ask that you contact us so that we can update our database.

Arbitrary PCR of a culture derived from a purified colony and sequencing of PCR products is recommended (see 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.

Some mutants selected for the PA14NR Set were selected to represent genes from the initial PA14 gene prediction. Further annotation and analysis of the PA14 genome sequence has revealed that several of these predicted genes are not likely actual genes. They have recently been categorized as “inactive” genes. Therefore if BLAST of an ARB-PCR sequence derived from a PA14NR Set mutant against the PA14 protein database does not produce any hits, the mutant may belong to this class of mutants. The mutation carried by such mutants are most likely intragenic.

IX. References

Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., Villanueva, J., Wei, T., Ausubel, F. M. (2006). An Ordered, Non-Redundant Library of *Pseudomonas aeruginosa* Strain PA14 Transposon Insertion Mutants *Proceedings of the National Academy of Science USA.* **103 (8):**2833-8.

Nicole T. Liberati, Jonathan M. Urbach, Tara K. Holmes, Gang Wu, Frederick M. Ausubel (2006). Comparing Insertion Libraries in two *Pseudomonas aeruginosa* Strains to Assess Gene Essentiality. *Methods in Molecular Biology. In press.*

Drenkard, E. & Ausubel, F. M. (2002) *Nature* **416,** 740-3.