**High-throughput phenotyping assay v2.0**
**Andersen laboratory, Winter 2016**

1. Using sterile, autoclaved spatulas, chunk a starved plate onto a fresh OP50-spotted plate. Use plates all from the same batch of NGMA and same OP50. **NOTE**: If the plates you are chunking from are several months old, you should chunk a larger area of agar to the new plate to ensure that enough worms come off of the chunk.

2. After 48 hours, pick five late-stage L4s to one 6 cm plate from each strain. This pick is generation one. Make sure all of the plates are from the same batch of NGMA and same OP50

3. Three days later, pick five late-stage L4s to a fresh plate for each strain. Make sure all of the plates are from the same batch of NGMA and same OP50. This pick is generation two.

4. Three days later, pick five late-stage L4s to fresh plates for each strain. Make sure all of the plates are from the same batch of NGMA and same OP50. This pick is generation three.
    - If you are setting up **dose responses** pick to **four plates**.
    - If you are setting up **heritability assays** pick to **three plates**.
    - If you are setting up **mappings** pick to **one plate**.

5. Four days later, pick five L4s of each strain to two plates for every well that will be setup in the high-throughput assay. Make sure all of the plates are from the same batch of NGMA and same OP50. This is generation four.
    - To set up dose responses, you will have five wells for each of four strains in quadruplicate. Therefore, you will have 20 wells and require a total of 40 plates from each strain. Write down the order that you picked each strain from one to 40. In that way, you can mix older plates with newer plates to even out the age of the worms picked.
    - To set up heritability assays, you will have seven wells for each of 12 strains. Therefore, you will need 14 plates from every strain.
    - To set up mappings, you will have one well for each strain. Therefore, you will only need to pick two plates of every strain.

    - These previous steps ensure that transgenerational effects of starvation and high levels of crowding are minimized.
    - Pay attention to the timing for each strain. Some strains grow more slowly. It is best to group strains together with similar growth speeds. We've found that it is often good practice to note the time and date that the plate is setup. In that way, you can see how long it takes to get to a plate of gravid worms without starvation.
    - Again, make sure the NGMA plates and OP50 for all strains picked on this day come from the same batch.
    - Try to pick as fast as possible. You don't want one strain to be four hours older than the last strain.
    - It is **necessary** monitor your strains through this whole process. If they are growing slowly you can leave the worms at room temperature the day/night before you pick them. Start monitoring at the first generation to make subsequent picking as easy as possible. Be careful of temperature sensitive strains or mutants, as they are most to shifting temperatures.

7. Before the bleaching and synchronization process, do the following:
    a. In the afternoon, four days before bleaching (~84-90 hours), make 200mL of fresh bleach solution. Follow the recipe at the bottom of this protocol as closely as possible. Store at 4°C until needed.
    b. Empty the vacuum flask attached to the 96-well aspirator. It works more reliably and reproducibly when empty.
    c. Set up the 96-well aspirator (if necessary) following the Setup 96-well Aspirator protocol (i.e. clean the aspirator with ethanol if required).

8. Pay close attention to your strains as they grow during this last generation. On the day of the bleach, you want to have a healthy robust population of gravid animals. Check the plates two and three days after picking to make sure that they will be staged for the bleach and staged close to each other. Oftentimes, we take out a few plates (or the entire) batch for 12 to 24 hours to speed up slow strains.

9. Take the bleach solution out of the 4°C fridge about 30-45 minutes before you will finish filling the wells of the deep-well plate. This allows the bleach to warm up a little bit, allowing for more effective bleaching.

10. Four days after picking generation four, use two mL of M9 to wash worms off from plates and add to the appropriate well. Add the M9 to the first plate, gently shake the M9 back and forth in the plate, dump the M9 onto the next plate and shake the M9 back and forth. Continue this for each plate needed for one well (normally two plates per well). After the final plate, transfer the worms using a Pasteur pipette to a single well of a deep 96-well plate (Arctic White, AWLS-X10005).
    - We want a plate with many gravid animals but not a huge population size and no starvation.
    - It is necessary to add the same volume to each well so that the 96-well aspirator functions properly. This is why you should only add M9 to a set of plates directly before you wash the worms from those plates into a well.
        - If liquid remains on the plates for too long, it will absorb into the plate, giving different final volumes.
    - It helps to array the plates you will wash off in the spacing and structure of the 96-well plate too. It is important to make sure this step goes efficiently and quickly. The longer the adults and embryos stay in the hypoxic condition, the more a stress response might be triggered. This step goes more smoothly and quickly when two people work together - one washes plates, the other pipets into wells.
    - The deep-well plates can be reused. After completing the entire bleach process/aliquoting the embryos, dump liquid down sink, add some 50% Clorox bleach to each well, shake on the microplane vortex for 2-3 minutes, then wash 3-5 times with water. Dry them overnight, then wrap with tin foil and autoclave. We reuse them 5 times, so put a tick mark on the side after each use.
    - The sealing mats can also be reused. Wash them simultaneously with the deep-well plates. Mark with a tick. Wrap in tin foil and autoclave. The mats can be reused five times.
    - NOTE: Write down the strain organization in the deep-well plate. You will use this information later to make a CSV file which will be used in data analysis.

11. Carefully remove the Pasteur pipette bulb and leave the pipet into the well to mark the well that has been filled.

12. After washing all worm strains into the deep-well plate, remove all Pasteur pipettes. Be careful to avoid cross contamination, by slowly removing them one at a time and allowing excess liquid to drain

into the well. Put the pipettes into 50% bleach solution until the setup is complete. Then rinse the pipettes 3-5 times with water, blot on paper towel, and autoclave to use again.

13. Make sure all wells, including the blanks have an equal volume of liquid. It is easier to fill them and make even now, then when you are adding bleach to mostly empty wells. This step is crucial to allow the 96-well aspirator to remove liquid from each well evenly. Add 1.4 mL to wash wells to ensure that they are not the problematic during aspiration.

14. Securely place the sealing mat on the deep-well plate (check to make sure the sealing mat is flat across the whole plate), and centrifuge at 1100 rpm for one minute using the 5810R clinical centrifuge at room temperature. Use an appropriately weighed balance plate.
    - The volume of the liquid per well should not exceed 1.7 mL to prevent contamination during centrifugation.

15. Take off the sealing mat, and remove the M9 using the 96-well aspirator (VP 177A-1, V&P Scientific). Check to make sure that every well has been aspirated. If not, then remove the M9 by hand.
    - ***When using the aspirator be aware that the aspirator may leak after aspiration! After you finish <u>any</u> aspirating, gently push or slide the plate out of the manifold base. Once the plate is clear of the aspirator, hold a paper towel gently against the aspirator pins and tilt the aspirator towards the side with the tubing to clear any liquid remaining in the manifold. Gently blot the pins to remove any remaining moisture.***
    - Be careful not to disturb the worm pellet. To avoid disturbing the pellet, it is best to aspirate the M9 as soon after the centrifuge finishes as possible.

16. Add 700 µl of bleach solution by manually using the 12-channel 300 µL pipette. Seal with the sealing mat after all bleach has been added.
    - Use fresh bleach solution. Bleach solution should have been prepared four days ahead of the bleaching process.

17. Take the sealed plate over to the balance and tare for that weight. Hurry! Wasted time leads to over bleaching.

18. Shake on the microplate vortex at 1450 rpm for 2 minutes then rotate the plate 180º and shake for 2 more minutes.
    - Use a rubber band to hold the deep-well plate onto the vortex.
    - Much of the variability in the number of L1s and survival of L1s comes from this step. If you note that all adults are dissolved after four minutes, then add 50% more embryos per well when titering. Survival is compromised whenever the animals are over-bleached.
    - You want the adult worms to be 90% dissolved by the bleach but not completely dissolved. In other words, there should be occasional adult carcasses floating around. However, when the options are to have more carcasses and more healthy embryos, or fewer carcasses and fewer healthy embryos, it is better to err on the side of the former.

19. While the plate is shaking, put M9 into a disposable reagent trough. Get new tips on the 12-channel pipette to prevent putting any extra bleach into the deep-well plate.

20. Also while the plate is shaking, fill the blank deep-well plate until the weight is within 10 g of the tared bleach plate weight.

21. After four minutes total shake time, spin down the worms at 1100 rpm for one minute using the 5810R clinical centrifuge. Always make sure to remove the sealing mat carefully after the spin to avoid cross contamination.

22. Aspirate out the bleach solution using the 96-well plate aspirator. Move quickly!

23. Use 12-channel 300 µL pipette to add 700 µl of M9 to every well and shake on the microplate vortex for 15 seconds. Move quickly!

24. Spin down the worms at 1100 rpm for one min. using the 5810R clinical centrifuge.

25. Remove the M9 using the 96-well aspirator. Move quickly!

26. Repeat steps 21-23 two more times for a total of three M9 washes. Move quickly! After the bleach, the wells look yellowish. After washing, the wells should look much less yellow. You can vortex for the washes longer if you have lots of worms and need extra bleach time. If you are over-bleaching (by accident) or have very few worms, then perform the bleach step for 15-30 seconds less in each two-minute shake.

27. During your last spin, fill a disposable reagent trough with K medium (recipe at end of this protocol) and change out the pipette tips.

28. Using the 12-channel 300 µL pipette, add 400 µL of K medium to all wells. If you are concerned that your titers will be too low (due to over bleaching or poorly grown animals), resuspend in a final volume of 300 µL K medium.

29. Calculate the titer of embryos by resuspending the embryos using the Eppendorf Xplorer 12-channel repeat pipetter. Use the mix function to mix 100 µl x5 three times. Then pipette 10 µl onto the bottom of a 96-well plate to count the number of embryos.
    - For dose responses, count the embryos from each strain (N2, CB4856, DL238, JU258) twice.
    - For heritabilities, count the embryos from one row of the plate that is not row H (the wash row).
    - For GWAS or RIAILs mappings, count the embryos from two or three rows.

30. After calculating the titer, dilute so that there are approximately 20-60 embryos per 10 µl. There will certainly be variation across the strains. Try to maximize the number that will fall in the ideal range while minimizing the number that would fall outside of that range.

31. Pipette the amount of embryo solution to get to about 25-50 animals from each well of the deep-well plate into the number of tissue-culture treated 96-well flat-bottom plates that are needed for your assay. Usually, 10 $\mu$L of embryo solution works well. Use the Eppendorf Xplorer 12-channel repeat pipetter to speed up the process and decrease repetitive strain. Remember to mix each row three times before aspirating and dispensing to tissue-culture plates. Use the 12-channel pipet set to 100 $\mu$L to mix.
    - Transfer the embryos from the well of the deep 96-well plate to the corresponding well of the flat-bottom 96-well plate (e.g. embryos from well A1 of the deep-well plate should **only** be pipetted into well A1 of the flat-bottom plates)

- Use a piece of tape across the deep-well plate to keep track of where you have pipetted. Remove distractions to limit losing your place and to decrease confusion.

32. Add K medium to bring the volume of each well up to 50 $\mu$L. When making K medium, make 500 mL batches a few days in advance of preparing plates to aliquot embryos or to make assay plates. Also when making K medium, add all water and salts, then filter with Thermo Filter Unit (Cat #566-0020). After filtering, add cholesterol that has been filtered (Cat. # PN 4192, Pall Life Sciences). Decrease any opportunities for the filtered K medium to become contaminated. If your K medium is contaminated, make new.

33. Seal all 96-well plates with Rayon gas permeable strip (Fisher cat #14-222-043). Try to avoid wrinkles in the Rayon strip.

34. Shake overnight at 180 rpm at 20ºC on the Excella E24R shaker in a freshly made humidity chamber.
    - To make a humidity chamber, place damp paper towels in a clean (bleached and rinsed) plastic box, close the box, and seal all around the top lid edge with parafilm.

35. At the end of the day, remember to: (1) charge the automatic pipette dispenser, (2) wash out the deep-well plate and sealing mat as described above, (3) check that the deep-well plate and sealing mat are still within five uses and throw them out if old, (4) clean out the 96-well aspirator by aspirating a tip box lid filled with distilled water two times and 95% ethanol one time, (5) and make sure the vacuum line is off, (6) clean out the pasteur pipettes as described above, and (7) aliquot using an autoclaved spatula 100 mg of powdered lysate to as many autoclaved microfuge tubes as you will need for the entire feeding of L1s and set up of drug plates. Typical assays require 10-20 tubes of 100 mg of aliquoted lysate. Please wear gloves and be very careful with the lysate in the stock bottle. Contamination affects everyone!

36. Before starting step 37, check one or two plates that were aliquoted the previous day. Check for successful hatching of L1 larvae. If there was successful hatching, continue with protocol. If not, contact Erik.

37. Starting early in the day (8 AM), make food from bacterial lysate (final concentration 50 mg/mL). The recipe is located at the bottom of this protocol. Add 5 $\mu$L of lysate food to every well that has worms in it. Make sure to resuspend the bacteria before you add it to the plates! Feed a plate every 25 minutes through the day. Use a timer to make sure you don't forget and lose time. If your day is busy and you miss a feeding, you can feed two plates at once at the next feed.
    - Ensure that the bacterial food is mixed into each well. Every well should look cloudy. It is easiest to see when you look at the wells above a black background. To ensure the food droplet fully gets into the well, spin in the clinical centrifuge (use a blank balance) for two seconds using a short spin.

38. Seal with Rayon gas permeable strip, and label the plate top with the plate number and time that you added the food solution.

39. Shake for 48 hours at 180 rpm at 20ºC on the Excella E24R shaker in a humidity chamber.

40. Before making target plates, clean the bench to reduce contamination of wells. Wear a lab coat and start by spraying 10% bleach across the bench. Wipe away the bleach in one direction and then toss the towels. Use the cheap paper towels by the sink for these cleaning steps.

41. Next, spray the pipets you will use with 70% ethanol on the bench and wipe them down.

42. Then, spray the entire bench with 70% ethanol and wipe away ethanol with paper towels in one direction.

43. Repeat these steps on the sorter bench area too.

44. Make up 10 mg/mL bacterial lysate (as described below) in a 50 mL or 150 mL conical, depending on how much you need.

45. Add kanamycin to the 10 mg/mL bacterial lysate mix. Kanamycin final concentration should be 50 $\mu$M. That is a 1:1600 dilution of our 80 mM stock or 6.25 $\mu$L per 10 mL of solution.

46. Get fresh dd$H_2O$ and/or DMSO for standardizing solvent conditions. Use double distilled water fresh from the distiller in the Lamb autoclave room. Decant into a 2 mL tube. Pipet from the DMSO stock bottle into a new 2 mL tube. Label the tubes for the solvent inside.

47. Organize the area. Get 96-well plates from the consumables bench for the assay. Get troughs for pipetting solution to the wells. Make sure you have M9 with 0.1 mg/mL FUdR for wash wells. Take out tubes of drugs from -20ºC or 4ºC storage (cold room by Morimoto lab).

48. Look at the drug preparation sheets to figure out what volume conical or tube you need to set up the plates for the assay. For mappings, NIL tests, heritabilities, etc., we use a single drug for an entire plate.15 mL conicals are good for this volume.

49. Pipet the 10 mg/mL lysate into the 15 mL conical, as dictated by the set up sheet.

50. Add DMSO or water solvent, as dictated by the set up sheet.

51. Add drug, as dictated by the set up sheet.

52. Cap conical and vortex for five seconds to mix.

53. Decant into a one-time-use trough (USA Scientific, #1346-1010).

54. Using a 12-channel pipet, pipet 50 $\mu$L of drug solution into each well of the 96-well plate needed for the assay.

55. Using the same pipette and the same tips, pipet 50 $\mu$L of wash solution (M9 with 0.1 mg/mL FUdR) into each wash well.

56. Label the plate with the plate number, drug name, and solvent name.

57. Put the plate into a temporary humidity chamber box. Temporary humidity chambers are plastic shoe boxes bleached and ethanol cleaned before use. Add about 10 paper towels from near the sink to the box and put in distilled water from the tap.

58. Before sorting add M9 (no azide!!!) to bring the final volume up to 250 $\mu$L. Typical assays have 50 $\mu$L of L4 worms, lysate, and K medium, so add 200 $\mu$L of M9 before sorting. If you forget, the sorter will fill with bubbles and won't sort accurately. In which case. Run two full rows of 300 $\mu$L water in each well.

59. Once all target plates are setup, turn on the sorter (follow other document on sorter use, found on Dropbox, called Andersen Sorter Protocols). Use program USE_for_L4 dispense.csv (or other appropriate .csv file) on the sorter to sort three L4s per well from each setup plate to each assay plate for scoring dose responses or mappings. Use the program USE_for_L4 dispense_columns.csv on the sorter to sort L4s from each setup plate to one assay plate for columnar formats. i recommend starting up the sorter while you are cleaning the bench and preparing the target plates. With practice, they can be done at the same time.
    - NOTE: You have to make a new save file for each plate sorting/scored. Please read the naming convention rules at the end of this document.

60. Throughout the day of using the sorter, keep a close eye on the level of the sheath fluid in the sheath bottle. Do not allow it to use up more than 10% of the total volume. This means that every 3-4 plates you need to depressurize the sheath bottle, remove the top, add in the amount of M9 that was used, put the top back on, and re-pressurize the bottle. Do this continually throughout the day to maintain a constant pressure within the sheath bottle, leading to constant flow rate through the flow cell.

61. Also throughout the day of using the sorter, count the number of sorted animals using a flashlight and write on the plate lid the number sorted. After that plate is completed, count the number of L4 animals in the well to confirm that the sorter is working correctly.

62. Also throughout the day of using the sorter, make sure that the Whirpool Whispure 510 HEPA air filter is running. This cleaning prevents contamination of the plates by the sorter.

63. After each plate is finished sorting, seal the target plate with gas-permeable Rayon strip (Axygen, cat#14-222-043 from Fisher). Make sure the edges are sealed and press hard onto all wells.

64. Put the finished and sealed plates in a long-term humidity chamber. Long-term humidity chambers are plastic shoe boxes bleached and ethanol cleaned before use. Add about eight C-fold paper towels from near the sorter to the box and put in distilled water from the tap. Make sure this box is very damp. Label the box with the assay name and score date.

65. After the last plate of the day, wrap the long-term humidity chamber with parafilm. Take care to wrap no more than 10 cm on the top of the box. Most of the parafilm should be on the bottom part of the box.

66. Shake for 96 hours at 180 rpm at 20ºC on the Excella E24R shaker in a humidity chamber. Make sure that during these four days, the boxes are completely sealed with parafilm and the paper towels inside are very damp. This will prevent excessive evaporation. Evaporation will decrease the volume in the wells of the plates, which can create problems with the sorter. Check the boxes during the four days if necessary.

67. When scoring plates, use program USE_for_analysis.csv (or other appropriate CSV file) on the sorter and without bubble trap. Be sure that the plate format is correct, depending on what assay you are running and the orientation of the strains.

68. Before scoring, be sure to make a solution of bacterial lysate and red fluorescent microspheres. This solution is 1 mg/mL lysate, and a 1:50 dilution of 0.5 $\mu$m microspheres (Polysciences, cat. # 19507-5). The beads are stored in the undercounter refrigerator by the set up bench. Add 5 $\mu$L of this solution to each well that has worms on the plate to be scored, and incubate while shaking at 20ºC, 180 rpm, for five minutes. After five minutes, add 200 $\mu$L of M9 with 50 mM sodium azide to stop feeding and straighten the worms. M9 with 50mM sodium azide can be made by adding 3.25 grams of sodium azide per liter of M9.

69. Make sure that any M9 used in the wells for sorting, scoring, or put in the sheath bottle have been made, filtered (Thermo Filter Unit, Cat #166-0045), autoclaved, and cooled to room temperature before using ahead of time.

70. Throughout the day of using the sorter, keep a close eye on the level of the sheath fluid in the sheath bottle. Do not allow it to use up more than 10% of the total volume. This means that ever 3-4 plates you need to depressurize the sheath bottle, remove the top, add in the amount of M9 or water that was used, put the top back on, and pressurize the bottle. Do this continually throughout the day to maintain a constant pressure within the sheath bottle, leading to constant flow rate through the flow cell.

**NOTE**: When using the sorter, either for sorting or scoring, you MUST keep a close eye on it. It is a temperamental machine, and frequently has various problems. If you have to go to the bathroom, check email, go to lunch, etc., please find someone else to watch it. It CAN NOT be left alone. Read the documents the lab has in the Protocols folder in the AndersenLab DropBox about the sorter (AndersenSorterProtocols, SorterBestPractices) to familiarize yourself with the sorter, and its problems, and how to deal with them. Before taking on the sorter yourself, watch Erik or Robyn work with it to see how it works.

**K medium Recipe:**

Per 500mL:
51mM NaC (**5.1 mL of 5M NaCl**)
32 mM KCl (**16 mL of 1M KCl**)
3mM CaCl2 (**1.5 mL of 1M CaCl2**)
3mM MgSO4 (**1.5 mL of 1M MgSO4**)

Mix the four salts with increasing water, then fill to 500 mL of dH2O

Filter sterilize with Thermo Filter Unit (Cat #566-0020)

Add 1.25 ug/mL filtered cholesterol (125 µL of 5 mg/mL cholesterol) (Cat. # SLLG025SS, Millipore) after filtering

Mix, label, and store for up to two weeks. Be sure to check the K medium prior to use for any floating particulate, which is a sign of contamination. If K medium is contaminated, pour it down the drain and make new K medium.

**Bacterial Lysate Recipe:**

1. Add 650 $\mu$L of K medium to a tube of 100 mg bacterial lysate.
2. Vortex for 60 seconds, making sure that all lysate goes into suspension. If it doesn't go in easily, try running the microfuge tube along the length of a tube rack a few times and vortexing more. If you have many tubes to prepare, put the completed tubes shaking on the plate vortexer.
3. Add 300 $\mu$L K medium.
4. Vortex 30 seconds. Like above, if you have many tubes to prepare, put the completed tubes shaking on the plate vortexer.
5. Spin down in the microfuge for 30 seconds at 5000 rpm.
6. Pipette ~980 $\mu$L of the lysate solution into a 15 mL conical or 150 mL conical, depending on whether you are setting up feeds or assays. Be careful not to disturb the lysate pellet. Chunks of lysate will cause more lysate clumping.
7. For every tube of lysate used initially, add 1 mL of K medium (to get to a 50 mg/mL concentration) for days with feeding L1 larvae.
8. For assay set up, you will add 9 mL K medium for every 1 mL of 100 mg/mL lysate. The final concentration is 10 mg/mL lysate.

**Bleach Solution Recipe:**

Per 200 mL:

40 mL NaOCl (from Fisher, cat #SS290-1), add some dd$H_2O$
10 mL of freshly made 10 M NaOH (add 4g NaOH pellets to 10 mL of dd$H_2O$), add some dd$H_2O$
Add dd$H_2O$ up to 200 mL

Mix well, store at 4ºC until needed. This bleach should be made four days before use. If you make is earlier or later, you much adjust the bleaching time for stronger or weaker bleach, respectively.

**Sorter File Naming Conventions:**

There are four components to naming a file on the sorter for the current data analysis pipeline. The general outline looks like: **pXX_strains_condition_control**

**pXX** corresponds to the plate number. Plate 1 should be p01. Plate 21 should be p21.
**strains** corresponds to the strains that you set up in the deep-well plate (and subsequent 96-well plates). This area will be titled whatever this assay is called. Example: McGrathRILs1a, NILsTest4b, etc.
**condition** corresponds to the condition in each individual plate. abamectin, DMSO, copper, and tunicmycin would all be examples of conditions.
**control** is the appropriate control for the condition. DMSO, None, water, and DMSO (respectively) are the associated controls for the conditions listed above.