# High-throughput Phenotyping Assay - SORTER

## General sorter schedule:

*Remember to "sign up" for the days you will be sorting/scoring on the Andersen Lab COPAS Biosort signup Google Calendar. If you do not have access, ask Erik.*

*It is also good practice to sort/score during normal business hours (Mon-Fri) because the sorter can be a tricky machine and it is often useful to have others around to help troubleshoot issues.*

| Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|
| | | Chunk A1 | Chunk A2 | Bleach clean A1 | Pick A1G0 (L1s)<br>Bleach clean A2 | Pick A2G0 (L1s) |
| Pick A1G1 (L4s) | Pick A2G1 (L4s) | | Pick A1G2 (L4s - multiple plates) | Pick A2G2 (L4s - multiple plates) | | |
| | Bleach A2 | V2: Feed A2 | V2: Sort A1 (with drug) | V2: Sort A2 (with drug) | | |
| Bleach A1 | V2: Feed A1 | V2: Clean sorter | V3: Score A1 | V3: Score A2 | | |
| | V3: Feed A1 (with drug) | V3: Feed A2 (with drug) | | | | |
| V2: Score A1 | V2: Score A2 | | | | | |

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### Step 1: Growing strains

*<u>A note on amplifying your populations:</u>*

- *Make sure your source plate is no more than one month old before chunking. If it is, you should chunk to a fresh plate, allow the plate to starve, and then chunk from the new source plate for the assay. Proper strain management involves chunking your entire 15˚ C strain stock every **3 months** and asking Robyn for a fresh stock every year.*
- *For each generation picked, use plates from the same batch of NGMA and OP50, make sure there will be enough 6 cm plates for each generation.*
- *Pull out plates to room temperature several hours (or the night) before picking to ensure you will not be picking to "wet" plates. If you will use more than 50% of a box of plates, pull out another box for the other lab members*
- *It is **NECESSARY** to 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 affected by shifting temperatures.*

- *These steps ensure that trans-generational effects of starvation and high levels of crowding are minimized.*

1. Using sterile, autoclaved spatulas, chunk a starved plate onto a fresh OP50-spotted plate. This step is usually done on Wednesday/Thursday for normal sorter assays (**see schedule at the beginning of this protocol**). Store your plates in the 20˚ incubator between generations, unless worms are growing slowly, in which case store your worms at room temperature on your bench.

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2. After 48 hours, pipette 15 µL bleach solution away from the lawn of a new plate. Pick ten to twenty gravid worms into the bleach. Do this for each strain. This will help prevent the spread of contamination from the chunk plate.

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3. After 24 hours, pick 20-30 L1s to a new plate for each strain. If for some reason the L1s didn't survive the bleach, make a note of it and pick from the chunk plate for generation one. This pick is generation zero.

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4. After 48 hours, pick five late-stage L4s to one 6 cm plate from each strain. This pick is generation one. **NOTE:** Worms may have grown into young adults by this time. Pick the youngest worms you can find.
    a. Depending on the scale of your assay, you might want to pick more than one 6 cm plate at this stage, the next pick will be to amplify the population.
        i. **Low number of strains and high replication** (i.e. 4 strain dose response, NILs) - pick two to four plates
        ii. **Medium number of strains** (i.e. 12) - pick two plates
        iii. **High number of strains and low replication** (i.e. mapping) - pick one plate

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5. Three days later, pick five late-stage L4s of each strain to multiple plates. This is generation two (aka the "big pick"). 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. If strains are growing slowly or have a low population number, pick six or seven worms per strain. **Try to pick as fast as possible. You don't want one strain to be four hours older than the last strain.**

   a. It is helpful to pick in the morning to ensure plump, gravid adults for bleaching in four days
   b. Depending on the scale of your assay, you will pick a different number of plates at this step. It is important to think about the layout of your 96-well plate and determine the number of replicates for each strain you desire. Ideally, one 6 cm plate of N2 yields approximately 3-5,000 embryos after the bleach. However, wild isolates (like CB4856) yield MUCH less (1-3,000). A general estimate is found below:
      i. **Low number of strains and high replication** (i.e. 4 strain dose response, NILs) - pick 20 plates
      ii. **Medium number of strains** (i.e. 12) - pick 12-15 plates
      iii. **High number of strains and low replication** (i.e. mapping) - pick two plates

6. In the afternoon, four days before bleaching (~84-90 hours, the same day as your gen. 2 pick), make enough fresh bleach solution for your assay (often ~200 mL) (**see recipes page**). Store in the dark at 4°C until needed.
7. The same afternoon, make enough 500 mL batches of fresh K Medium for your assay (often 1 or 2 500 mL batches are needed) (**see recipes page**). <u>At any point in the assay if you notice your K medium is contaminated, make new.</u>
8. If you are doing a 96 well bleach, check that there are enough autoclaved deep 96 well plates and autoclaved pasteur pipettes before your bleach day. They must be cleaned at least a day in advance so they have time to dry before autoclaving. Check deep 96 well plates for any white residue at the bottom of the wells, as these may contaminate your assay.
9. 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. **Usually, we grow worms for the last two to three generations at 21.5ºC. Do not treat different strains differently from each other.**

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**Step 2: Prepping (bleaching) strains**

10. Four days after picking generation four, check the strains to ensure your worms are staged properly for your bleach. We want a plate with many gravid animals but not a huge population size and no starvation.
11. Take your bleach solution out to room temperature (keep dark) before you start washing your plates into the deep 96 well plate (or 30-45 minutes before you are ready to bleach). This allows the bleach to warm up a little bit, allowing for more effective bleaching.
12. Follow instructions for **CONICAL BLEACHING** or **96-WELL BLEACHING** depending on the scale of your assay (***protocols found at the end***).
    a. Due to the variation with 96-well bleaching, it is only recommended for large-scale mapping assays (48+ strains)
    b. Due to the variation between bleaches, it is **highly recommended** to perform multiple (three) bleaches per day. Make sure to pick enough plates in the second generation and make sure your independent bleaches are performed independently (not at the same time). If possible, bleach all strains for a bleach-assay together, if not possible, bleach the same set of strains together for each bleach-assay (i.e. all N2-background NILs in one set and all CB-background NILs in a second set)

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**Step 3: Feeding strains**

*A note on feeding:*

- *We feed plates one day after bleaching, as long as the L1 larvae hatched*
- *We feed each plate at the same time of day we plan on sorting/scoring the worms 48 hours later*
- ***For V2 assay (sorting/scoring)**: follow V2 feeding procedure (feed just food before sorting and make up drug plates to sort into)*
- ***For V3 assay (no sorting):** follow V3 feeding procedure (feed L1s with food + drug combination)*

13. The next morning after bleaching, 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.
    a. Also check for leftover chunks/contamination from the bleach and crystals. Wells with crystals or contamination **CANNOT** be run through the sorter.
14. Make food from bacterial lysate (recipe at bottom of this protocol) or frozen HB101 previously prepped (see protocol). You must feed worms around the

same time you will run the plates on the sorter two days later. If you're running many plates, start making food early in the morning.

a. **V2 assay:** Make bacterial lysate at a concentration of 50 mg/mL or frozen HB101 at the stock concentration of OD 100
b. **V3 assay:** Make bacterial lysate at a concentration of 15 mg/mL or frozen HB101 at a concentration of OD 30.
    i. Add kanamycin to the food mix. Kanamycin final concentration in the well should be 50 µM (final concentration in the tube will be 150 µM). That is 18.75 µL of our 80 mM stock per 10 mL of solution.
    ii. Prepare drug dilutions and add to bacterial lysate. It might be helpful to use this shiny app to calculate drug dilutions and amount of food needed.
        1. Get fresh dd$H_2O$ and/or DMSO for standardizing solvent conditions. Use double distilled water fresh from the millipore near the sink. 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.

15. Start with one or two plates. 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, you can feed two plates every fifty minutes. 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.
    a. **V2 assay:** Add 5 µL of food to every well that has worms in it (It might be helpful to use the repeat pipette)
    b. **V3 assay:** add 25 µL of food + drug to every well that has worms in it
16. Seal with Rayon gas permeable strip, and label the plate top with the plate number and time that you added the food solution. Put the plates back in the humidity chamber once they are fed. When done for the day, make sure your paper towels are still damp and parafilm the box shut.
    a. **V3 assay**: also label your plate with the drug condition
17. Shake for 48 hours at 180 rpm at 20ºC on the Excella E24R shaker.

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## (Optional Step 4: Sorting - V2 assay ONLY)

*A note on sorting:*

- *To prevent contamination, change pipette tips often when setting up plates*
- *Set up your plates next to the HEPA air filter on the sorter setup bench (to the right of the imager)*
- ***You must sterilize the sorter before use. This is usually done the Wednesday before sorting. Follow the separate sterilization protocol in the red binder by the sorter starting at step four.***

18. For the V2 assay, two days after feeding you will sort your worms. 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.
19. Next, spray the pipettes you will use with 70% ethanol on the bench and wipe them down.
20. Then, spray the entire bench with 70% ethanol and wipe away ethanol with paper towels in one direction.
21. Repeat these steps on the sorter bench area too.
22. Make up 10 mg/mL bacterial lysate (**described below in recipes**) or frozen HB101 bacteria at a concentration of OD 20 in a 50 mL or 150 mL conical, depending on how much you need.
    a. Add kanamycin to the 10 mg/mL bacterial lysate mix. Kanamycin final concentration should be 50 µM. That is a 1:1600 dilution of our 80 mM stock or 6.25 µL per 10 mL of solution.
    b. It might be helpful to use this shiny app to calculate drug dilutions and amount of food needed.
23. Get fresh dd$H_2O$ and/or DMSO for standardizing solvent conditions. Use double distilled water fresh from the millipore near the sink. 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.
24. 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
25. 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. For dose responses, you will use 1.7 mL posi-click tubes, one per concentration/five tubes per plate.
    a. It might be helpful to use this shiny app to calculate drug dilutions and amount of food needed.
26. Pipet the 10 mg/mL lysate into the 15 mL conical or 1.7 mL tubes, as dictated by the set up sheet.
27. Add DMSO or water solvent, as dictated by the set up sheet.
28. Add drug, as dictated by the set up sheet.
29. Cap conical and vortex for five seconds to mix.
30. Decant into a one-time-use trough (USA Scientific, #1346-1010). For dose responses, pipette 600 µL into 12-channel reservoirs. Each tube should be pipetted into two separate troughs.
31. Using a 12-channel pipette, pipet 50 µL of drug solution into each well of the 96-well plate needed for the assay.
32. Pipet 50 µL of wash solution (**M9 with 0.1 mg/mL FUdR**) into each wash well. For dose responses, pipette 600 µL FUdR into the wash well channel of the reservoir before setting up plates.
33. Label the plate with the plate number, drug name, and solvent name.
34. 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 until they are very damp.
35. 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.
36. Before sorting add M9 (**no azide!!!**) to bring the final volume up to 250 µL. Typical assays have 50 µL of L4 worms, lysate, and K medium, so add 200 µL of M9 before sorting.
    a. **If you forget, the sorter will fill with bubbles and won't sort accurately. In which case. Run two full rows of 300 µL water in each well.**
37. Once all target plates are setup, turn on the sorter (follow this protocol). 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 with strains in rows. 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.

a. **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.

38. 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.
39. 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.
40. After counting L4s for each strain, 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.
41. 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.
42. 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.
43. 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.

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**Step 5: Scoring - V2 and V3 assay**

44. Score plates!
    a. 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.

b. **V2 assay:** Add 200 µL of M9 with 50 mM sodium azide to each well about 10 minutes before scoring.
    i. M9 with 50mM sodium azide can be made by adding 3.25 grams of sodium azide per liter of M9.
c. **V3 assay**: Add 175 µL of 5 mM sodium azide in $dH_2O$ to each well about 10 minutes before scoring
    i. 5 mM sodium azide in $dH_2O$ can be made by diluting 50 mM sodium azide (**see Recipes**).
d. ***NOTE:*** *If you are testing pharyngeal pumping, 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 µM microspheres (Polysciences, cat. # 19507-5). The beads are stored in the undercounter refrigerator. Add 5 µL 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 µL of M9 with 50 mM sodium azide to stop feeding and straighten the worms.*
e. 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.

45. 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 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 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 an experienced sorter warrier work with it to see how it works.

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# ~ RECIPES ~

## **Bleach Solution** (Per 200 mL):

40 mL NaOCl (in the cold room, from Fisher, cat #SS290-1), add ~ 100 mL 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 (undercounter fridge #1) 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. Throw out old bottles of bleach in the fridge as necessary.

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## **K medium** (Per 500 mL):

51 mM NaCl (**5.1 mL of 5 M NaCl**)
32 mM KCl (**16 mL of 1 M KCl**)
3 mM $CaCl_2$ (**1.5 mL of 1 M CaCl2**)
3 mM $MgSO_4$ (**1.5 mL of 1 M MgSO4**)

We keep a separate set of salts to only use for sorter experiments. Pour yourself as much as you need into 50 mL conicals, then pipette into a graduated cylinder. Mix the four salts with increasing water, then fill to 500 mL of d$H_2O$.

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

Add 1.25 µg/mL unfiltered cholesterol (125 µL of 5 mg/mL cholesterol) 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. Record which batch you used of each reagent (date/initials) in your lab notebook; this way we can quickly figure out if a batch of something has gone bad.

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## Bacterial Lysate:

1. Wearing gloves, weigh out 100 mg bacterial lysate into autoclaved tubes using a clean spatula (wipe with ethanol before use).
    a. V2 assay: For feeding L1 larvae, each tube will make ~2 mLs lysate solution. For setting up assay plates, each tube will make ~10 mLs of lysate solution.
    b. V3 assay: For feeding L1 larvae, each tube will make ~6.6 mLs of lysate solution
2. Shortly vortex tubes of 100 mg bacterial lysate and add 650 µL of K medium to each tube
3. 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.
4. Add 300 µL K medium.
5. Vortex 30 seconds. Like above, if you have many tubes to prepare, put the completed tubes shaking on the plate vortexer.
6. Spin down in the microfuge for 30 seconds at 5000 rpm.
7. Pipette ~980 µL 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.
8. Add K media for final concentrations:
    a. V2 assay - feeding L1s before sorting: For every tube of lysate used initially, add 1 mL of K medium (to get to a 50 mg/mL concentration).
    b. V2 assay - making drug plates: 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.
    c. V3 assay - feeding L1s before scoring: add 5.67 mL K medium for every 1 mL of 100 mg/mL (1 tube) of lysate. The final concentration is 15 mg/mL
9. Add kanamycin.
    a. V2 assay - feeding L1s before sorting: Add 62.5 µL of 80 mM kan per 10 mLs of solution.
    b. V2 assay - making drug plates: Add 6.25 µL of 80 mM kan per 10 mLs of solution.
    c. V3 assay - feeding L1s before scoring: Add 18.75 µL of 80 mM kan per 10 mLs of solution.
10. Vortex to resuspend before pipetting into wells.
11. Record which bottle of lysate you used, as well as which batch of Kanamycin in your lab notebook.

## Frozen HB101 bacteria:

1. Prepare enough HB101 for all planned experiments for an entire project at one time. See HB101 growth protocol HERE.
    a. HB101 will be frozen at OD 100 in aliquots of appropriate sizes (1, 5, 10, 15 mL, etc.)
2. Thaw enough HB101 for the day, depending on your need
3. Dilute with K media to the appropriate concentration
    a. V2 assay - feeding L1s before sorting: Do not dilute, feed directly from this stock
    b. V2 assay - making drug plates: Dilute 1:5 to OD 20
    c. V3 assay - feeding L1s before scoring: Dilute 1:3.333 to OD 30
4. Add kanamycin
    a. V2 assay - feeding L1s before sorting: Add 62.5 µL of 80 mM kan per 10 mLs of solution.
    b. V2 assay - making drug plates: Add 6.25 µL of 80 mM kan per 10 mLs of solution.
    c. V3 assay - feeding L1s before scoring: Add 18.75 µL of 80 mM kan per 10 mLs of solution.
5. Vortex to resuspend before pipetting into wells

## Sodium Azide

V2 assay: 50 mM sodium azide in 1x M9
6.5 g sodium azide in 2 L 1x M9

V3 assay: 5 mM sodium azide in $dH_2O$

1. Make a stock of 50 mM sodium azide in $dH_2O$:
    a. 3.25 g sodium azide in 1 L $dH_2O$
    b. Store in cabinets under sorter
2. Dilute stock:
    a. Remove 100 mL of $dH_2O$ from a 1 L bottle
    b. Add 100 mL of 50 mM sodium azide from step 1

## M9 + FUdR

0.1 mg/mL FUdR in 1x M9
50 mg FUdR *(-20C small bottles)* in 500 mL 1x M9
Aliquot into 50 mL conicals and store above sorter setup bench

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# ~ BLEACH SYNCHRONIZING ~

## CONICAL BLEACHING (Please see HTA imaging protocol for additional details)

1. Wash worms off plates into a 15 ml conical tube. You can decant M9 onto one plate of Strain A, transfer the liquid to another plate of strain A, etc. Wash all plates from a single strain into one 15 ml conical tube. It is easy to pour from the final 6 cm plate into the 15 mL conical with a little bit of practice. We typically pour two mLs of M9 onto one plate, and transfer the same solution to four other plates before pouring it into the conical. Repeat for all strains.
2. Spin down worms at 1100 rpm for 1 minute using the table-top clinical centrifuge.
3. Aspirate off the M9, being careful to not suck up any of your worms.
4. Look at the worm pellet size. A small pellet (less than 500 µL) should use less bleach than a large pellet (greater than 1000 µL).
5. Add 7 mL of bleaching solution to each 15 mL conical, dependent on your pellet volume. Only process strains that have similar pellet sizes together. Otherwise, you will be separating bleaches from the same time. The freshness of the bleach solution matters. Ineffective bleaching solution (kept at 4ºC longer than one month or kept at room temp for longer than eight hours) will require more time to bleach worms and will not dissolve the worms uniformly. Shake the tubes manually and vigorously at a rate of 120 bpm. You can bleach multiple strains at the same time, but depending on experience you may want to start with only doing 4-5 strains at once.
6. After four minutes of shaking, check to see if the adult worms are dissolved every 30 seconds until complete. **Be very careful not to over bleach the worms**. Once you see that the carcasses are nearly dissolved, move to the next step. ***If there are only embryos left, then you have gone too long***.
7. Once there are just a few adults remaining, **move quickly** to spin down the embryos at 1100 rpm for 30 seconds. It is best to have the reagents next to the sink and prepared for the next few steps. Some table-top centrifuges do not have an option of 30 seconds, so you must stop the centrifuge manually after 30 seconds. If you feel you have under-bleached your worms, you may let them spin down for a full minute.
8. **Immediately** decant off the bleach into the sink carefully and quickly without disturbing the worm pellet.
9. Add 10 ml of M9 using a sterile glass pipette or pouring from a 50 mL conical to each 15 mL conical tube.
10. Invert three times as you walk back to the clinical centrifuge.
11. Spin down the embryos at 1100 rpm for 30 seconds.

12. Decant off the M9 into the sink carefully and quickly without disturbing the worm pellet.
13. Repeat steps 10-13 twice with M9 and twice with K medium.
    a. The K medium washes are essential to eliminate crystal formation.
14. After the fourth wash, resuspend the embryos in 1-2 mL of K medium.
15. Determine the titer of the embryos by counting the number of embryos in 5 µl. Adjust the resuspension liquid (K media) to obtain the desired concentration (*e.g.* 0.5 - 1 embryo per µl).
16. Vortex each 15 mL conical of embryos for at least five seconds before pouring into a single channel reservoir. Either pipette immediately or use the mix function before pipetting into assay plates. Pipette 50 µl of embryo solution to get to about 25-50 animals into the number of tissue-culture treated 96-well flat-bottom plates that are needed for your assay. 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 µL to mix.
17. Seal all 96-well plates with Rayon gas permeable strip (Fisher cat #14-222-043). Try to avoid wrinkles in the Rayon strip.
18. Shake overnight at 180 rpm at 20ºC on the Excella E24R shaker in a freshly made humidity chamber.
    a. 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.
19. At the end of the day, remember to charge the automatic pipette dispenser
20. Return to step 13 in the HTA protocol.

## 96-WELL BLEACHING

1. Type up how you would like your strains to be organized in each 96 well plate (see plate setup examples at the end of this protocol). You will later use this information to make a CSV file used in data analysis. Once your worms are staged properly, sterilize your bench with bleach and ethanol, then lay out your fourth generation plates in the same order you will have them arranged in your 96 well plates.
2. Working with a partner, use M9 to wash worms off from plates and add to the appropriate well. Add two mLs of M9 to the first plate using a filler dispenser pipette. 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, have your partner transfer the worms using a Pasteur pipette to the corresponding well of a deep 96-well plate (Arctic White, AWLS-X10005). You can use a piece of foil wrapped around the deep-well plate to help keep your place. 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.
    a. 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.
3. Carefully remove the Pasteur pipette bulb and leave the pipette into the well to mark the well that has been filled.
4. 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. You can again use aluminum foil to help keep wells covered once the pipettes are removed. Put the pipettes into 50% bleach solution until the setup is complete.
5. 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 with the $dH_2O$ squirt bottle to ensure that they are not problematic during aspiration. Securely place the sealing mat on the deep-well plate. Check to make sure the sealing mat is flat across the whole plate.
6. Set up the 96-well aspirator following the Setup 96-well Aspirator protocol (i.e. clean the aspirator with ethanol if required). Empty the vacuum flask attached to the 96-well aspirator. It works more reliably and reproducibly when empty.

7. Also add ~1.4 mL to every well in the balance plate by the centrifuge labeled “Step One”. Tare your deep well plate on the scale and make sure the balance plate is within +/- 10 grams of that weight. Take the other balance plate (labeled “Step two”) by the centrifuge and make sure it is filled with 700 µL of water per well.
8. Centrifuge the deep well plate at 1100 rpm for one minute using the 5810R clinical centrifuge at room temperature with the first balance plate. The volume of the liquid per well should not exceed 1.7 mL to prevent contamination during centrifugation
9. 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.
    a. ***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.***
    b. 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.
10. Add 700 µl of bleach solution by manually using the 12-channel 300 µL pipette. Pipetting 233 µL three times down the plate works fine. Seal with the sealing mat after all bleach has been added.
    a. Use the fresh bleach solution that you have already pulled out to room temperature a half hour prior to bleaching. Bleach solution should have been prepared four days ahead of the bleaching process.
11. Take the sealed plate over to the balance and tare for that weight. Hurry! Wasted time leads to over bleaching.
12. Shake on the microplate vortex at 1450 rpm for 2 minutes then rotate the plate 180º and shake for 2 more minutes. Use rubber bands to hold the deep-well plate onto the vortex.
    a. 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.
    b. 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.

13. 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.
14. Also while the plate is shaking, check that the other balance deep-well plate is within 10 g of the tared bleach plate weight. This should be fine if you've added 700 µL to each well. Use a $dH_2O$ squirt bottle to even it out if necessary. Put this balance in the centrifuge.
15. 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.
16. Aspirate out the bleach solution using the 96-well plate aspirator. Move quickly!
17. 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!
18. Spin down the worms at 1100 rpm for one min. using the 5810R clinical centrifuge.
19. Remove the M9 using the 96-well aspirator. Move quickly!
20. Repeat steps 30-32 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.
21. During your last spin, fill a disposable reagent trough with K medium (see recipes) and change out the pipette tips.
22. 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.
23. 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.
    a. For dose responses, count the embryos from each strain (N2, CB4856, DL238, JU258) twice.
    b. For heritabilities, count the embryos from one row of the plate that is not row H (the wash row).
    c. For GWAS or RIAILs mappings, count the embryos from two or three rows.

24. After calculating the titer, dilute so that there are approximately 25-50 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. Use the mix function again to check your titers before pipetting into your assay plates.
25. 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 µL 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 µL to mix.
    a. 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)
    b. Use a piece of foil across the deep-well plate to keep track of where you have pipetted. Remove distractions to limit losing your place and to decrease confusion.
26. Add K medium to bring the volume of each well up to 50 µL.
27. Seal all 96-well plates with Rayon gas permeable strip (Fisher cat #14-222-043). Try to avoid wrinkles in the Rayon strip.
28. Shake overnight at 180 rpm at 20ºC on the Excella E24R shaker in a freshly made humidity chamber.
    a. 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.
29. The deep-well plates can be reused. After completing the entire bleach process/aliquoting the embryos, dump liquid down the sink, add some 50% Clorox bleach to each well of the deep well plate, 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.
    a. **The deep well plates MUST be dumped down the sink immediately after aliquoting embryos. We have found that otherwise the embryos stick to the bottom of the plate and become impossible to clean out.**

30. Also after completing the aliquoting process, rinse the Pasteur pipettes 3-5 times with water, blot on a paper towel and let them dry before autoclaving again for reuse.
31. 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, and (6) clean out the pasteur pipettes as described above.
32. Return to step 13 in the HTA protocol.

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## PLATE SETUP EXAMPLES:

### Dose responses

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | A | A | A | A | Wash | B | B | B | B | B | Wash |
| C | C | C | C | C | Wash | D | D | D | D | D | Wash |
| C | C | C | C | C | Wash | A | A | A | A | A | Wash |
| D | D | D | D | D | Wash | B | B | B | B | B | Wash |
| D | D | D | D | D | Wash | C | C | C | C | C | Wash |
| B | B | B | B | B | Wash | A | A | A | A | A | Wash |
| B | B | B | B | B | Wash | D | D | D | D | D | Wash |
| A | A | A | A | A | Wash | C | C | C | C | C | Wash |

Where each column is a different, increasing concentration in each six-column block. One drug per plate, wash between each strain. Strains are rotated to decrease plate effect. (Each letter denotes a different strain, each color denotes a different treatment, with increasing font size corresponding to concentration)

### Heritability

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | B | C | D | E | F | G | H | I | J | K | L |
| A | B | C | D | E | F | G | H | I | J | K | L |
| A | B | C | D | E | F | G | H | I | J | K | L |
| A | B | C | D | E | F | G | H | I | J | K | L |
| A | B | C | D | E | F | G | H | I | J | K | L |
| A | B | C | D | E | F | G | H | I | J | K | L |
| A | B | C | D | E | F | G | H | I | J | K | L |
| Wash | Wash | Wash | Wash | Wash | Wash | Wash | Wash | Wash | Wash | Wash | Wash |

Where each column is a different strain, and the whole plate has the same concentration of a single drug.

**Mapping**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Wash | A | Wash | A | Wash | A | Wash | A | Wash | A | Wash |
| A | Wash | A | Wash | A | Wash | A | Wash | A | Wash | A | Wash |
| B | Wash | B | Wash | B | Wash | B | Wash | B | Wash | B | Wash |
| B | Wash | B | Wash | B | Wash | B | Wash | B | Wash | B | Wash |
| C | Wash | C | Wash | C | Wash | C | Wash | C | Wash | C | Wash |
| C | Wash | C | Wash | C | Wash | C | Wash | C | Wash | C | Wash |
| D | Wash | D | Wash | D | Wash | D | Wash | D | Wash | D | Wash |
| D | Wash | D | Wash | D | Wash | D | Wash | D | Wash | D | Wash |

Where every two rows is a separate strain and each position in every two row block contains a different drug. (Each letter denotes a different strain, each color denotes a different treatment)

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## 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.
    - In this example “McGrathRILs1a”, easysorter will name the round = 1, assay = a. Check out easysorter for more
    - Each bleach is a different assay. Each day is a different round. It is best practices to keep each assay/round in a new folder to designate this.
- **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.
    - It is important that “None” is written exactly as shown (i.e. not “none” or “NONE”)