High-throughput Phenotyping Imager Assay

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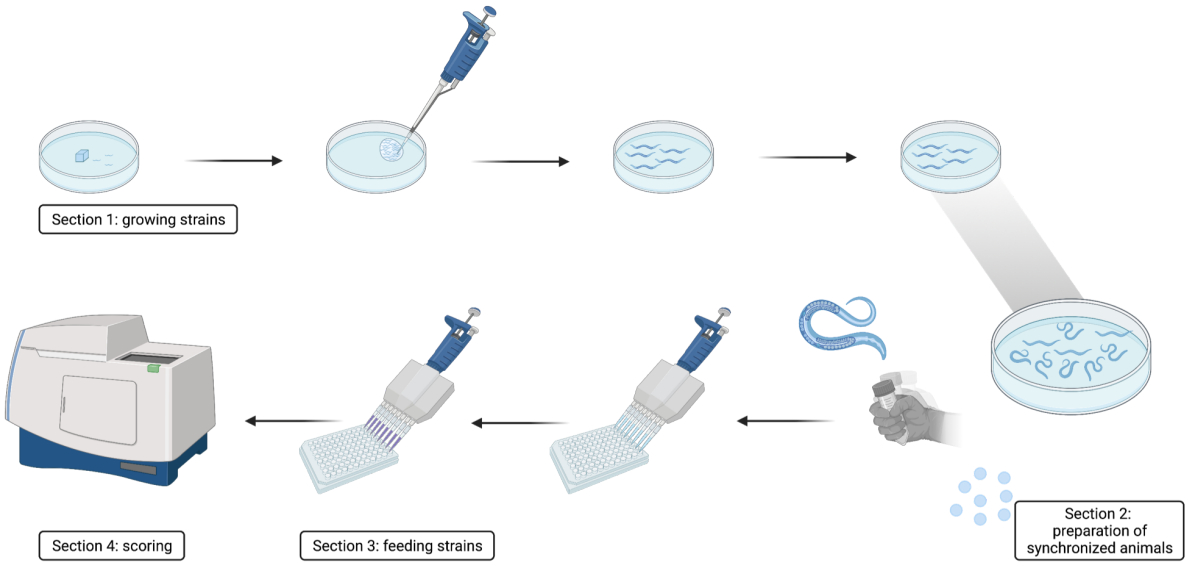
Last Updated: September 28, 2021



General imager schedule (detailed schedule on final page of this document):

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

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
			Chunk		Drop Bleach	Pick G1 L1s
	Pick G1 L4s			Pick G2 L4s		
	Conical Bleach	Feed		Image		

Section 1: Growing strains

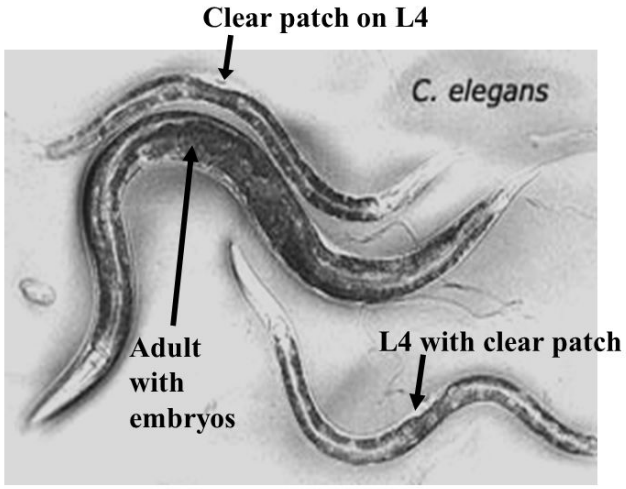
Notes on amplifying strains:

- *Make sure your source plate is no more than one month old before chunking. If it is, you should ask Robyn for a fresh chunk, allow the plate to starve, and then chunk from the new source plate for the assay. We store all source plates at 15ºC. Each strain is chunked every three months and then discarded after one year.*
- *For picking each generation, use plates from the same batch of NGMA and OP50. Before starting, make sure that you have enough 6 cm plates for each generation.*
- *Put plates at room temperature the night before picking to ensure you will not be picking to cold plates that have condensation.*
- *Label plates using printed labels, avoiding handwritten strain names.*
- *Label all plates and tubes before the steps that require them (most notably the bleach preparation).*
- *Organize 6 cm plates containing the same strain into the same box when possible, stacking no higher than five high. Whenever possible, organize groups of strains into boxes such that easily confusable strain names (i.e., JU3291 vs. JU2391) are not in the same box. Also, mark plates with confusing names so that people will more easily notice the differences.*
- *It is **NECESSARY** to monitor your strains through this whole process. If they are growing slowly, you may leave the worms at 21.5ºC (or higher) the day/night before you pick them. Worms are reared at 20ºC under standard conditions, including generation zero of the high-throughput assay strain growth. However, growing each strain population at 21.5ºC from step 4 (first L4 pick) to the time of plate washing causes greater embryo yields from the bleach preparation. Start monitoring at the first generation to make subsequent picking as easy as possible. If worms are growing faster or slower, they can be moved to 20ºC or to a higher temperature, respectively, for generation two. Be careful of temperature-sensitive strains or mutants, as they are most affected by shifting temperatures.*
- *Monitor the temperature in all incubators to make sure that they match the temperature in the protocol.*

1. Using a sterile, autoclaved spatula, chunk from a starved plate onto two fresh OP50-spotted 6 cm plates. Store these 6 cm plates in the 20ºC incubator (Andersen lab: use the top shelf of the incubator named **RAVENCLAW**). Throughout the experiment, make sure that temperature in all incubators is carefully monitored. Aim to chunk a plate that yields about 50 adult worms, assessed by the number of arrested larvae on the source plate.
2. After 48 hours, pipette 15 µL of bleach solution away from the lawn of a new plate and pick ten to twenty gravid hermaphrodites into the bleach spot. Swirl gently with the pick to ensure all worms are removed from the pick. Store these 6 cm plates upside down (lid up) in the 20ºC incubator (Andersen lab: **RAVENCLAW on the second from the top shelf)**.

3. After 24 hours, pick 20-30 L1s to a new labeled 6 cm plate for each strain. If the L1s didn't survive the bleach, make a note of it so that larger chunks or fresher source plates may be used for future assays. This pick is generation zero. Store these 6 cm plates in the 20ºC incubator (Andersen lab: **RAVENCLAW on the third from the top shelf)**.
4. After 48 hours, pick five late-stage L4 hermaphrodites to labeled 6 cm plates from each strain. These animals are distinguished by a distinct white half-moon shaped patch near the midsection of the worm, containing what looks like a classic Christmas tree where the vulva is developing (*see the image below*).

Identifying hermaphrodites



This pick is generation one. **NOTE:** Worms may have grown into young adults by this time, in which case pick the youngest adult worms you can find. If you find males on source plates, avoid picking young adults from these plates. Check the plates one and two days after picking to make sure that they will be properly staged for the generation two pick. Grow this generation of animals at 21.5°C (Andersen lab: in **LUNA**, divided into a single layer of boxes on the top two shelves).

   a. Pick at least three 6 cm plates. Depending on how many generation two plates you need to pick (*see step 5*), you may need to pick more. Based on calculations for the Andersen Lab 2021 GWA mappings, each 6 cm plate from this L4 pick provides enough animals for approximately seven generation-two plates.Two examples:
      i. If one needs to pick ***five plates*** per strain for their big pick, pick *at least three* 6 cm plates in this step. Even though it may be possible to obtain five plates worth of animals from one 6 cm plate in step 5, picking three 6 cm plates here safeguards against your single plate becoming contaminated or containing males at the next step, ending your assay.

ii. If one needs to pick ***36 plates*** per strain for their big pick, pick *at least* five 6 cm plates in this step.

5. Three days later, pick five late-stage L4 hermaphrodites (look for the developing vulva in the crescent) of each strain to each plate for this generation. This pick is generation two (AKA the "big pick"). **Try to pick as fast as possible. You do not want one strain to be four hours older than the last strain. Do not pick males and avoid picking L4 hermaphrodites from plates with males if other plates have enough hermaphrodites.**
    a. Pick in the morning to ensure plump, gravid adults for bleaching four days later at 21.5ºC.
    b. Based on calculations for the Andersen Lab 2021 GWA mappings, each 6 cm plate provides approximately 1000 embryos for the average strain. Based on your experimental requirements, calculate the number of plates you need to pick. For example:
        i. An assay with eight strains and **twelve** conditions for each strain with **three** replicate bleaches and **four** biological replicates per condition would require
            1. 12 x 4 x 3 x 30 embryos = 4320 embryos
            2. 4320 embryos / 1000 embryos / plate = 5 plates (rounded up)
            3. Therefore, for this experiment, one should pick **five** 6 cm plates *per strain* to meet the experimental requirements.
    c. In subsequent experiments, if you find that you are producing a vast excess or dearth of embryos compared to your experimental requirements for your strain(s) of interest, you may adjust the number of picked plates accordingly. The above calculations provide an approximate starting point.
6. In the afternoon, four days before bleaching (~84-90 hours, the same day as your gen. two pick), make fresh bleach solution for your assay (**see recipes page**). Store in the dark at 4°C until needed.
7. The same afternoon, make 500 mL batches of fresh K medium for your assay (**see recipes page**). <u>At any point in the assay if you notice your K medium has gone bad (any solute crashing out and appearing as white flakes or granules floating), make a new batch.</u>
8. Grow this last generation of animals (generation two) at 21.5°C (Andersen lab: in **LUNA**, divided between the top three shelves of the incubator). On the day of the bleach, you want to have a healthy, robust population of gravid animals. Check the plates for L2 and L3 animals after two days and L4 and young adults after three days.

Section 2: Preparation of synchronized animals

9. Four days after picking generation two, check the strains to ensure your worms are staged properly for the bleach. Plates are staged properly for the bleach when the population on the plate is mostly composed of gravid adults. An appropriately staged plate contains adults that have begun to lay embryos throughout the plate and have begun migrating away from what remains of the bacterial lawn.

10. Take your bleach solution out to room temperature (keep it in the dark) 30-45 minutes before you start washing your plates, allowing the bleach to warm up a little bit and more effectively bleach worms.
11. Follow instructions for **CONICAL BLEACHING in steps 12-28.**
    a. Because of 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 that is not possible, bleach the same set of strains together for each bleach-assay (*e.g.*, all N2-background NILs in one set and all CB4856-background NILs in a second set).
12. Wash worms off plates into a labeled 15 ml conical tube. You can pour 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 2 mL of M9 onto one plate and transfer the same solution to four other plates before pouring it into the conical. Repeat for all strains.
13. Spin down worms at 254 g (Andersen lab: Eppendorf 5810R, 1100 rpm) for 1 minute using the table-top clinical centrifuge.
14. Aspirate off the M9, being careful to not suck up any of your worms.
15. Add 7 mL of bleaching solution to each 15 mL conical for pellet sizes of 100 µL and up. If pellet sizes are smaller, adjust volume of bleaching solution accordingly. We use 4 mL of bleach for all smaller pellets. 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. If multiple people are bleaching, try to ensure that each individual is shaking in approximately the same way to ensure greater consistency between strains bleached by multiple individuals. We have found it useful to shake to the beat of a song; use a playlist with songs that have the same beats per minute (bpm). We used 130 bpm. Shake by holding the tubes by the cap to avoid the transfer of warmth from the hands to the solution. 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. 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***. If you find a strain that is lagging, shake this strain more vigorously to catch it up to the other strains.
16. Once you find that only a few adults remain, **move quickly** to spin down the embryos at 254 g (Andersen lab: Eppendorf 5810R, 1100 rpm) for 30 seconds. It is best to have the reagents next to the waste/pour-off receptacle 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.

17. **Immediately** decant off the bleach into the waste/pour-off receptacle carefully and quickly without disturbing the worm pellet.
18. Pour 10 ml of M9 from a 50 mL conical to each 15 mL conical tube.
19. Invert three times as you walk back to the clinical centrifuge.
20. Spin down the embryos at 254 g (Andersen lab: Eppendorf 5810R, 1100 rpm) for 30 seconds.
21. Decant off the M9 into the sink/waste container carefully and quickly without disturbing the worm pellet.
22. Repeat steps 18-21 twice with M9. If you washed adults off of eight plates or fewer, do one final wash with 10 mL of K medium.
    a. This extra K medium wash is essential to eliminate crystal formation when the quantity of bleached animals is low. M9 and worms make crystals after 48 hours of incubation.
23. After the final wash, resuspend the embryos in 3 mL of K medium. Adjust the volume of K medium to the number of plates you bleached. For 15 plates per bleach, we find 3 mL is a good volume. For fewer plates, 2 mL might be more appropriate.
24. Determine the titer of the embryos by counting the number of embryos in five replicates of 3 µl. Pulse vortex, by starting and stopping vortexing every few seconds, each 15 mL conical of embryos for at least ten seconds before pouring into the reservoir. If embryos are too numerous to count, you may dilute the embryo suspension further in order to ensure accurate counts. If they are too dilute, spin down the embryos for 1 minute at 254 g (Andersen lab: Eppendorf 5810R, 1100 rpm), carefully pipet off some of the K medium and repeat the embryo counts. Adjust the resuspension liquid (K medium) to obtain the desired concentration (*e.g.*, 0.6 embryo per µl).
25. If embryos are also needed for archiving of samples for DNA or RNA isolation, embryos are initially concentrated at one embryo per µL. A second dilution of 0.6 embryos per µL is then made from this “stock”. This second dilution is then used for the HTA.
    a. For example: for 57 plates, a total volume of 50 µL * 4 replicate wells/strain * 57 plates = 11.4 mL of 0.6 embryo/µL is needed. To make some excess, 12.5 mL of 0.6 embryo/µL is needed. This dilution means adding 5 mL of K medium to 7.5 mL of 1 embryo/µL in K medium. In this example, an additional 2 mL of 1 embryo/µL is needed for plating L1 larvae for the collection of DNA and RNA from synchronized animals. Therefore, we make 9.5 mL of 1 embryo/µL for each strain, and take 7.5 mL of that dilution and add it to a new 15 mL conical already containing 5 mL of K medium. The 9.5 mL of 1 embryo/µL is made by pipetting 9.5 mL K medium in a conical, taking out the volume of concentrated embryos to get 1 embryo/µL, and adding that volume from the bleach conical after ten seconds of pulse vortexing. ***The volume to take out and add is determined by the following calculation: 9.5/(embryo/µL in your bleach conical)***.
26. Pulse vortex, by starting and stopping vortexing every few seconds, each 15 mL conical of 0.6/µL embryos for at least ten seconds before pouring into the reservoir. Pipette immediately and ensure proper mixing before pipetting into assay plates. For mixing, take 50 µl from the bottom of the trough and dispense it higher up on the side of the reservoir, repeat on the other side for a total of ten times (five per side). Pipette 50 µl of

embryo solution to distribute approximately 30 animals into each well of the tissue-culture treated 96-well flat-bottom plate (USA Scientific 96 well Cellstar Clear TC PLT*FLAT Cat #: 5665-5180) (RPI 96 Well Assay Plate, Sterile, Flat Bottom with Lid, Individually Wrapped, 100 per Case, Cat #: 141380)

27. Remember to **mix each row** five times before aspirating and dispensing to tissue-culture plates.
    a. We use 12-channel reservoirs (USA Scientific 12-well V-Bottom Reservoir Cat #: 3824*3412)
    b. With these 12-channel reservoirs, we prepare a row of the assay plate in each reservoir: Three strains per row, four wells each for a total of the 12 channels in the reservoir. We add 3 mL of 0.6 embryo/µL per channel. Then using a 12-channel micropette, 50 µl is dispensed into the corresponding row of the assay plate.
28. Seal all 96-well plates with a gas permeable sealing film (Fisher Cat #: 14-222-043). Try to avoid wrinkles in the film.
29. Shake overnight at 170 rpm at 20ºC (Andersen lab: on the Excella E24R shaker (**SPROUT**) or the INFORS HT Multitron shaker (**POMONA** or **NEVILLE**)) in a freshly made humidity chamber.
    a. To make a humidity chamber, place damp paper towels in a clean (bleached and rinsed) plastic box (IRIS USA CNL clear Latching Box, 6 Qt, 18 Count; or similar box)
    b. After filling the humidity chamber with plates, close the chamber using the lid, and seal the chamber using parafilm.
    c. These chambers hold 18 plates, so scale up the number of boxes according to your experimental setup.

Section 3: Feeding strains

Notes on feeding:

- *We feed plates one day after bleaching, as long as the embryos have hatched to L1 larvae. If larvae do not hatch, you have likely overbleached and cannot continue the experiment.*
- *Plates are imaged using the Molecular Devices ImageXpress Nano at 48 hours after feeding, so time your feedings accordingly.*

30. The morning after bleaching, check one or two plates that were aliquoted the previous day. Visually inspect the plates for successful hatching of L1 larvae using the ImageXpress or under a stereoscope. If there was successful hatching, judged by nicely swimming L1s, continue with protocol. If not, contact Erik.
    a. Also check for leftover chunks/contamination from the bleach and crystals.
31. Make food from frozen HB101 previously prepped (see protocol). If you are running many plates, start making food early in the morning.
    a. Dilute freshly thawed OD100 HB101 to a concentration of OD30 with K medium.

i. Add kanamycin (US Biological, Cat #: K0010) to the OD30 food mix. The final kanamycin concentration in the well should be 50 µM (final concentration in the food dilution, prior to plating, will be 150 µM). That is 18.75 µL of 80 mM stock per 10 mL of solution.

1. Andersen GWA:
OD100: 138 mL (four conicals from each of the three batches of food = 12 conicals; For the large scale HTA, three separate batches of food are prepared. Using equal amounts from each batch reduces the potential variation associated with differences in batch)
K medium: 322 mL
Kanamycin: 862.42 µL

Add ingredients in the order above. This dilution yields 460 mL of OD30 HB101. This dilution is typically made up in an autoclaved Pyrex media storage bottle.

ii. Prepare drug dilutions and add to OD30 HB101 food with kanamycin. It might be helpful to use this shiny app to calculate drug dilutions and amount of food needed.

1. Get fresh milli-Q water and/or DMSO for standardizing solvent conditions. Use a fresh aliquot of milli-Q water. Decant into a 2 mL tube. Pipet from the DMSO stock bottle into a new 2 mL tube. Label the tubes for the solvent.

32. Start with one or two plates. Make sure to resuspend the bacteria before you add it to the plates by swirling (if a large volume of OD30 bacteria is made in an autoclaved Pyrex media storage bottle) or vortexing (if using smaller batches of OD30 bacteria made up in a conical tube or otherwise vortexable container). Feed one plate on average every 3-4 minutes. Use a timer to make sure you do not forget. 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.
    a. Mix enough food and drug (at the appropriate concentration) for three replicate plates. Pour this mixture into a single channel trough (USA Scientific: ChannelMate 100 mL Reservoir, Cat #: 1306-1010). Using a micropipette, add 25 µL of food + drug to every well that has worms in it, for each of the three replicate plates.
33. Seal with a gas permeable sealing film (Axygen BF-400 gas permeable sealing film: Fisher Cat #: 14-222-043), label the plate top with the plate number and any condition information, and record the 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 chamber shut. Return the humidity chamber to the incubator and make sure the shaking restarts.
34. Shake for 48 hours at 180 rpm at 20ºC (Andersen lab: the Excella E24R shaker (**SPROUT**) or the INFORS HT Multitron shaker (**POMONA** or **NEVILLE**)).

Section 4: Scoring

35. Follow the ImageXpress operation protocol here.
    a. Add 167 µL of 50 mM sodium azide in M9 buffer to each well twice 10 minutes before scoring. Use a single-channel trough to hold the sodium azide solution and dispense the azide using either an 8- or 12-channel pipette. For each step, dispense at opposing angles to each well. Opposing angles helps alleviate heterogeneous swirling of well contents and uneven illumination of images. Also, incubating in sodium azide for too long (> 15-20 min) might cause uneven illumination as the bacteria can clump.

RECIPES

Bleach Solution (Per 200 mL):

40 mL NaOCl (in the cold room, from Fisher, cat #SS290-1), add ~ 100 mL ddH_2O
10 mL of freshly made 10 M NaOH (add 4g NaOH pellets to 10 mL of ddH_2O), add some ddH_2O
Add ddH_2O up to 200 mL

Mix well, store at 4ºC (Andersen lab: under the sink in the cold room) until needed. This bleach should be made four days before use. Throw out old bottles of bleach in the fridge as necessary.

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 HTA 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 dH_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.

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 (Andersen HTA: four conicals of 12 mL per food batch, 12 conicals in total). For the large-scale HTA, three separate

batches of food are prepared. Using equal amounts from each batch reduces potential variation associated with differences in batch.

3. Dilute OD100 HB101 with K medium to the appropriate concentration
    a. Dilute 1:3.333 to OD 30
4. Add kanamycin
    a. Add 18.75 µL of 80 mM kanamycin per 10 mL of solution.
5. Vortex to resuspend before pipetting into wells.

50 mM sodium azide in 1x M9

6.5 g sodium azide in 2 L 1x M9

PLATE SETUP EXAMPLES:

Dose responses

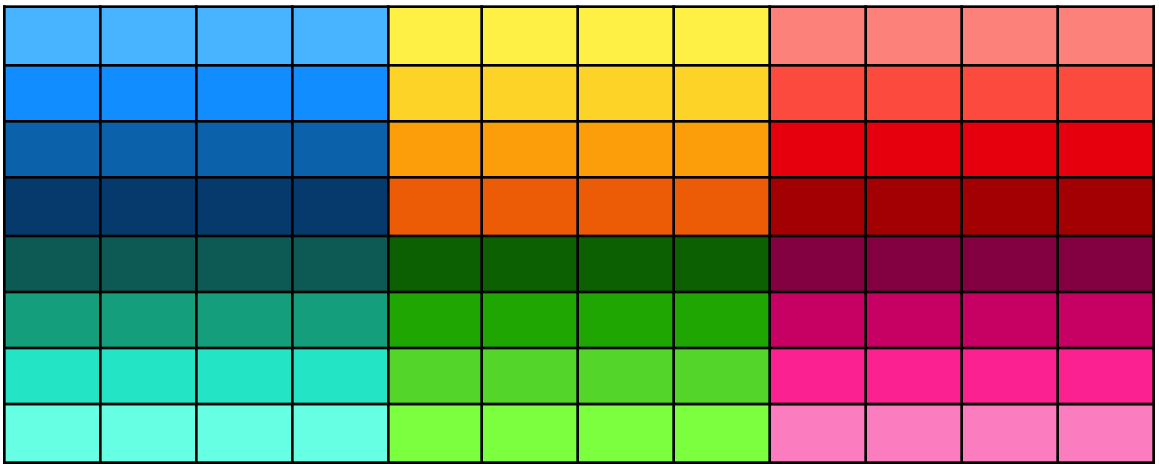
Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6	Dose 7	Dose 8	Dose 9	Dose 10	Dose 11	Dose 12

Columns: increasing doses
Rows: strains
Plates: technical and biological replicates
(i.e. 3 bleaches x 4 biological replicates/bleach)
Single condition per plate

Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6

Columns: increasing doses
Rows: biological replicates
Plates: technical replicates (bleaches)
Colors: conditions
Single strain per plate

Mapping



Colors: strains (note: in this example there are 4 biological replicates of each strain on the same plate)

Single condition per plate

Imager File Naming Conventions:

There are four components to naming captured images on the imager for the current data analysis pipeline. The general outline looks like: **YYYYMMDD_assayname_pXXX_2X**

- **YYYYMMDD** corresponds to the date plates were imaged.
- **assayname** corresponds to the name of the particular experiment conducted in the 96-well assay plate. This area will be titled whatever this assay is called.
- **pXXX** corresponds to the plate number. Plate 1 should be p001. Plate 21 should be p021.
- **2X** corresponds to an example magnification. The imager can take pictures at 2X and 10X magnification

DETAILED HTA SCHEDULE FOR START ON WEDNESDAY

WEEK	Day	Time	Task
1	***Wednesday***	1PM-2PM	Chunk
	Friday	2PM-5PM	Spot Bleach
	Saturday	2PM-5PM	Pick L1s
2	***Monday***	9AM-11:30/noon	Pick L4s
	Wednesday	Anytime	Label Thursdays plates
		After/during labeling	Setup boxes with random strain combinations
	Thursday	9AM-~1PM	Big L4 pick
		Anytime	Make bleach and K medium
	Friday	Anytime	Label next weeks vials
	Sunday	AM	Check development
3	***Monday***	~9AM,10AM,11AM	Take out bleach (30 min pre-bleach)
		9AM-12noon	Wash and bleach, one bleach set at a time
		12noon - 12.30PM	LUNCH -- 30 min
		1230p-130p	Titer
		130p-200p	BREAK -- 20 min (timing flexible)
		Anytime	Unpack plates and make humidity chambers
		2.15pm-5.30pm	Diluting the embryos
		2.30pm-8pm	Loading plates
		8pm-9pm	Finishing up loose ends/sealing boxes/ensure DNA embryos are rotating
	Tuesday	5AM-6AM	Concentrate and plate out embryos DNA/RNA
		8AM-12AM	Prepare food/drugs
		~10AM-6PM	Feed plates
	Thursday	ALL DAY	Check development for DNA/RNA and freeze
		~10AM-6PM	Imaging

Consumables:

- 15 mL bleaching conicals: CELLSTAR®, BLUE SCREW CAP, NATURAL, GRADUATED, WRITING AREA, STERILE, 100 PCS./BAG. Catalog #: 188-271 from Greiner Bio-One
- 15 mL conicals for dilutions: FisherBrand Disposable Centrifuge Tube 15mL Cat #: 07-200-886
- 50 mL conicals for pouring M9 when washing: FisherBrand Disposable Centrifuge Tube 50mL Cat #: 06-443-21
- 12-well reservoirs: USA Scientific 12-well V-Bottom Reservoir Cat #: 3824*3412
- Single reservoir troughs:USA Scientific ChannelMate 100 mL Reservoir Cat #: 1306-1010
- Flat bottomed 96-well plates: USA Scientific 96 well Cellstar Clear TC PLT*FLAT Cat #: 5665-5180, RPI 96 Well Assay Plate, Sterile, Flat Bottom with Lid, Individually Wrapped, 100 per Case, Cat #: 141380
- Axygen BF-400 gas permeable sealing film: Fisher Cat #: 14-222-043
- 500mL Rapid Flow Filter Unit 0.2 µm aPES membrane, 75mm diameter (for filtering K medium): ThermoFisher Cat #: 566-0020