Preparations of animals for assay:

1. Cultivate worms on 100 mm high growth media (HGM) plates seeded with 1 mL OP50 *E. coli*.

   Avoid starvation as this will affect the worm's behavior

   Do not dry your plates in the hood as this might dry out your plates and affect the worms' behavior
2. Hypochlorite-treat two to four densely populated HGM plates containing gravid adults. Divide the obtained eggs onto three to four seeded HGM plates and incubate at 20°C for at least 72 hours.

   Use large orifice tips when handling the worms

   Work quickly

Day prior to the assay

1. Place the chemotaxis plates at room temperature. You will need twenty five nematode growth medium (NGM) plates per genotype.
2. Seed five NGM plates (your conditioning (2) and hold plates (3)) with 1 mL OP50 *E. coli*.

   The seeded bacteria on the plates might need more than one day to dry, in this case, plates should be seeded earlier.

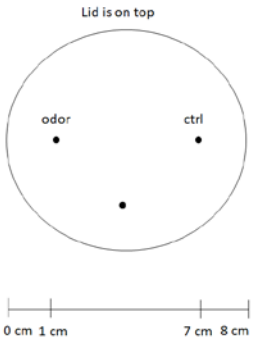
Morning before the assay

1. Prepare 500 µL of a 10% butanone solution in ethanol and vortex

   Use filter tips
2. Put 500 µl of NaN3 (1 M) in 1.5 ml vial
3. Put 500 µl of Ethanol in 1.5 ml vial

   Use filter tips
4. Label your plates, chemotaxis plates, tubes and vials

   Chemotaxis plates should be labeled as depicted here:



5. Check your plates and make sure you have **a)** enough worms (4 well-populated plates per genotype), **b)** your worms are not starved and **c)** that the worms are at the young adult stage

Pre- conditioning starve

1. Put one of the four plates plate aside (for assaying naïve chemotaxis) and wash worms off of your plates with M9 buffer into a 15 mL conical tube.

   Gently apply the M9 buffer to your plates (by pouring or using a squirtbottle)

   Gently swirl your plate to free the worms from the bacteria

   Tilt your plate and pour the M9 buffer/worm mixture into a 15 mL conical tube
2. Let the worms settle by gravity, remove the supernatant by vacuum and wash 3 more times with 4 mL M9 buffer.

   Do not centrifuge

   Make sure settled worms become mixed when adding M9 buffer. IF not, gently invert the tube
3. Add 4 mL M9 buffer to the 15 mL conical tube and let the worms starve in M9 buffer for 1 hour at room temperature.

NOTE: it is recommended to test not more than three genotype at the same time. It is however possible to work in parallel, but six genotypes (A, B & C and D, E & F) should be the limit. In this case, the following scheme can be used:

Assay steps	genotype
pre-conditioning starve	A, B & C
naïve chemotaxis	D, E & F
pre-conditioning starve	A, B & C
naïve chemotaxis	D, E & F
STAM training part 1	A, B & C
STAM training part 1	D, E & F
STAM training part 2	A, B & C
STAM training part 2	D, E & F
STAM training part 3 & 4	A, B, C, D, E & F

Naïve chemotaxis assay

1. Remove the condensation from the chemotaxis plates and spot 1 µL of 1 M NaN_3 at the odorant and control spot.
2. Wash worms off of the conditioning plates with 1 mL M9 buffer into a 1.5 mL microcentrifuge tube using a P1000 pipetman and let worms settle by gravity.

   Use the same gently washing methods as described earlier

   Use large orifice tips

   Tilt your plate to pull of the M9 buffer/worm mixture
3. Wash the pellet with M9 buffer and let worms settle by gravity
4. While the worms are settling, spot 1 µL of 95% ethanol at the control sport.
5. Wash the pellet with M9 buffer and let worms settle by gravity
6. While the worms are settling, spot 1 µL of 10% butanone at the odorant sport

7. Remove the supernatant by vacuum

   Remove as much M9 as possible

8. Using a P20 pipetman, apply 5 µL of the worm pellet onto the origin spot of the chemotaxis plates.

   Use large orifice tips

   Go to the middle of the pellet when pulling up the M9 buffer/worm mixture

   Make sure you pulled up enough worms; if not, discard these worms and use the remaining worms

9. Twist a piece of KimWipe to a small point and use it to remove excess fluid on the origin spot. Incubate for 1 hour at room temperature and proceed to part 4.

   Gently tap the drop of worms to remove the excess fluid

   Do not try to remove all the excess fluid as this might affects the worms' chemotaxis

   Keep the chemotaxis plates in a closed container

Short-term associative memory training

Part 1

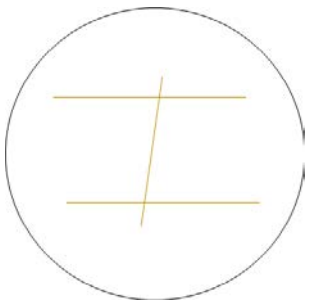
1. Remove the condensation on the lids of the conditioning plates (NGM plates seeded with 1 mL *E. coli* OP50) at the end of the starvation period.
2. Make three streaks on the inside of the lids of the conditioning plates with 2 µL of 10% butanone.

   Per genotype, at least two conditioning plates are required to support the entire population during the conditioning phase

   Close the lids as quick as possible after applying the butanone

   This should be performed right before applying the worms onto the conditioning plates

   The depicted pattern allows the butanone to be evenly distributed throughout the plate



3. Remove as much M9 buffer as possible from the 15 mL conical tube of starved worms by vacuum. Using a P20 pipetman, apply 100 – 150 µL of the worm pellet onto two (or more) condition plates.

   The total volume of worms should be distributed over at least 2 plates

   Too many worms on one conditioning plate might cause starvation. Therefore, one should make sure to properly distribute the worm population

Remove as much M9 as possible as this will result in less liquid on the plate and therefore allow the worms to reach the OP50 food source quickly

4. Incbuate at room temperature for 1 hour

   Parafilm the conditioning plates

   Conditioning plates can be kept in a closed container to avoid varying environmental conditions to affect the worms' behavior

5. As an additional control, 'part 1' can be performed without the use of butanone. As such, mock behavior can be assessed allowing to test whether the worm population is being stressed during the course of the assay. Next, parts 2, 3 and 4 are performed as described below.

Part 2

1. The condensation is removed from the lids of the hold plates (T30, T60 and T120) and T0 chemotaxis plates at the end of the conditioning period

   For the chemotaxis assay, at least three replicates per genotype must be run but five replicates are recommended (per timepoint)

   T0, T30, T60 and T120 are indicative for the following timepoints:

   T0: 1x associative (massed) learning

   T30: 30 minutes short-term associative memory timepoint

   T60: 60 minutes short-term associative memory timepoint

   T120: 120 minutes short-term associative memory timepoint

2. Spot 1 µL of 1 M NaN_3 at the odorant and control spot.
3. Wash worms off of conditioning plates with 3 mL M9 buffer into a 15 mL conical tube and let worms settle by gravity.

   Use the same gently washing methods as described earlier

4. Wash the pellet with M9 buffer and let worms settle by gravity
5. While the worms are settling, spot 1 µL of 95% ethanol at the control sport.
6. Wash the pellet with M9 buffer and let worms settle by gravity
7. While the worms are settling, spot 1 µL of 10% butanone at the odorant sport
8. Remove the supernatant by vacuum

   Remove as much M9 as possible

9. Transfer approximately 50 µL of the worm pellet onto each hold plate (T30, T60 and T120; NGM plates seeded with 1 mL *E. coli* OP50) and put the plates aside

   Hold plates are kept aside for 30 minutes, 1 hour or 2 hours after which the plates are used in part 3

   The hold plates can be kept in a closed container to avoid varying environmental conditions to affect the worms's behavior

   Use large orifice tips

   Go to the middle of the pellet when pulling up the M9 buffer/worm mixture

Divide the 50 µL over the entire plate by placing little drops on the plate

10. Using a P20 pipetman, apply 5 µL of the worm pellet onto the origin spot of the chemotaxis plates.

    Use large orifice tips

    Go to the middle of the pellet when pulling up the M9 buffer/worm mixture

    Make sure you pulled up enough worms; if not, discard these worms and use the remaining worms

11. Twist a piece of KimWipe to a small point and use it to remove excess fluid on the origin spot. Incubate for 1 hour at room temperature and proceed to part 4.

    Gently tap the drop of worms to remove the excess fluid

    Do not try to remove all the excess fluid as this might affects the worms' chemotaxis

    Keep the chemotaxis plates in a closed container

Part 3

10. After a short-term associative memory training of 30 minutes, 1 hour or 2 hours; the T30, T60 or T120 hold plates, respectively, are used for assessing chemotaxis after a certain period of time on hold plates.

    For the chemotaxis assay, at least three replicates per genotype must be run but five replicates are recommended (per timepoint)

    T0, T30, T60 and T120 are indicative for the following timepoints:

    T0: 1x associative (massed) learning

    T30: 30 minutes short-term associative memory timepoint

    T60: 60 minutes short-term associative memory timepoint

    T120: 120 minutes short-term associative memory timepoint

11. Remove the condensation from the chemotaxis plates and spot 1 µL of 1 M NaN_3 at the odorant and control spot.
12. Wash worms off of conditioning plates with 1 mL M9 buffer into a 1.5 mL microcentrifuge tube using a P1000 pipetman and let worms settle by gravity.

    Use the same gently washing methods as described earlier

    Use large orifice tips

    Tilt your plate to pull of the M9 buffer/worm mixture

13. Wash the pellet with M9 buffer and let worms settle by gravity
14. While the worms are settling, spot 1 µL of 95% ethanol at the control sport.
15. Wash the pellet with M9 buffer and let worms settle by gravity
16. While the worms are settling, spot 1 µL of 10% butanone at the odorant sport
17. Remove the supernatant by vacuum

    Remove as much M9 as possible

18. Using a P20 pipetman, apply 5 µL of the worm pellet onto the origin spot of the chemotaxis plates.

Use large orifice tips

Go to the middle of the pellet when pulling up the M9 buffer/worm mixture

Make sure you pulled up enough worms; if not, discard these worms and use the remaining worms

19. Twist a piece of KimWipe to a small point and use it to remove excess fluid on the origin spot.

    Incubate for 1 hour at room temperature and proceed to part 4.

    Gently tap the drop of worms to remove the excess fluid

    Do not try to remove all the excess fluid as this might affects the worms' chemotaxis

    Keep the chemotaxis plates in a closed container

Part 4

1. Count the number of worms within 1 cm of the control spot, odorant spot and origin and count the total number of worms on the assay plate.
2. Calculate the chemotaxis index: CI = ([($n_{Butanone}$)-(n_{EtOH})]/[(Total-n_{Origin})]