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CeMbio Screen 96WP

Forked from Microbiome Assay 96WP

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Behavioural Genomics

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Protocol status: Working
We use this protocol and it's working

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Abstract

Protocol for behavioural screening of *Caenorhabditis elegans* response to bacteria from its natural microbiome (CeMbio)

Preparing worms

1 Using an eyebrow hairpick, pick 10 L4-stage N2 worms onto each of 10 OP50-seeded 90mm petri plates 4 days prior to bleaching (e.g. on Monday if bleaching on Friday).

2 On day of bleaching (e.g. Friday 2pm) follow the protocol for *Bleach synchronisation of C. elegans:*

Protocol

NAME

Bleach synchronisation of C. elegans

CREATED BY

Ida Barlow

PREVIEW

2.1 Wash hermaphrodites off plate with several ml of M9 solution and transfer to 15ml falcon tube (Fisher Scientific-Falcon 352096)

2.2 Fill falcon tube up to 15ml with M9 solution

2.3 Centrifuge for 2 minutes at 1500 rpm (RCF:210, ascending 9; descending 7) – program 1

Program 1 retains the worms as pellets and the bacteria is suspended as the supernatant

The descending is slow as the worm pellet is lose at this stage which we don't want to break

2.4 Remove supernatant using a plastic Pasteur pipette taking care not to disturb pellet
Leave atleast 0.5ml M9 to avoid disturbing the pellet

2.5 Fill the tube with M9 upto 15ml

2.6 Spin program 1

2.7 *Repeat steps 4-6*

2.8 On final wash remove as much supernatant as possible and add M9 upto 4ml

2.9 Add 4ml 2X Bleach solution (From here onwards try to work as quickly as possible to avoid over-exposure of the worms to the bleach)

USE FRESHLY PREPARED BLEACH EVERYTIME

Note

2X Bleach solution:

5% Sodium hypochlorite solution - 4ml
Sterile water - 3.5 ml
1M NaOH solution - 2.5 ml
TOTAL - 10 ml

2.10 Vortex on maximum setting for 4 min (no more as this will damage the eggs)

Makesure the vortex forms

After vortexing, top up the tube with M9 till 15ml

2.11 Centrifuge for 2 mins at 2500rpm (RCF:590, ascending 9; descending 7) – program 2

(Always check the program on the centrifuge before using it)

2.12 Remove supernatant by pouring into waste bottle – pellet should be compact and yellow in colour at bottom of falcon, but be careful not to lose

2.13 Add 15ml M9

2.14 Centrifuge at program 2

2.15 Repeat steps 12-14 four more times

The number of washes is crucial here as we need to get rid of all the bleach

2.16 After final wash add 15ml M9 and store eggs/larvae in the falcon on the rotator that is constantly spinning at 20^{o}C, until feeding

Note

L1 arrested larvae can be starved for up to 5 days before refeeding

2.17 Centrifuge larvae on program 2 to pellet

2.18 Remove supernatant with plastic Pasteur pipette

The pellet is lose here so makesure not to disturb it

2.19 Add 15ml M9, spin to wash

2.20 On final wash leave 0.5ml M9 in falcon

2.21 Resuspend the pellet by gently tapping the tube/flicking it

2.22 Place droplet containing larvae onto seeded plate and allow to grow to desired developmental state (ie. 2 days for L4s, 2.5 days for young adults)

Use glass pipette to place the droplet onto seeded plate, avoid using plastic pipette as larvae will stick to it

Note

Development times at 20^{o}C:
- 2 days for L4s
- 2.5 days for young adults

Note:
- If you feed larvae within 12hrs of bleaching then they develop faster than the longer arrested ones
- It is a good practice to bleach in two tubes in parallel
- If you drop the tube at any point of the process, makesure to transfer the contents into a new tube as the dropped tube may get cracked resulting in loss of worms during centrifugation/vortexing
- Any unused larvae can be topped up with M9 and stored spinning in the rotator to be re-used
- Use clean autoclaved rubber bulbs for the refeeding everytime to avoid contamination
- Put the used bulb in the box labelled 'Used Teets'

Stages	Grown at 20 C from L1	Grown at 25 C from L1
L1 division	11.7hrs	9hrs
Mid L1	16.9hrs	13hrs
First L2 division	22.1hrs	17hrs
Between L2 divisions	23.4hrs	18hrs
Second L2 divisions	24.3hrs	19hrs
Mid L2	29.9hrs	23hrs
L3 division	32.5hrs	25hrs
Mid L3	37.7hrs	29hrs
L4 division	42.9hrs	33hrs
Mid L4	49.4hrs	38hrs
Early adult	55.9hrs	43hrs
Adult	62.4hrs	48hrs

Table of Development times for different temperatures

3 72:00:00 Keep the tube with bleached worms on a rotator in an incubator at 20°C until re-feeding (maximum 5 days post-bleaching, as behaviour is not consistent after this time frame).

3d

4 Re-feed arrested L1 larvae approximately 72 hours prior to tracking (e.g. on Monday if tracking on Thursday), following the steps outlined in the above protocol for *Bleach synchronisation of C. elegans.*

5 Store the refed L1 plates in 20°C incubator.

Making LB NGM agar imaging plates (96WP)

2h 30m

6 02:30:00 2 days prior to tracking (e.g. on Tuesday if tracking on Thursday), make 500ml LB NGM agar following the protocol *Making LB NGM Plates:* 2h 30m

Protocol

NAME

Making LB NGM Plates

CREATED BY

Priota Islam

PREVIEW

1.5 g Sodium Chloride (Sigma-Aldrich-71376-1KG)

8.5 g Bio Agar (Biogene-400-050)

1.25 g LB Broth Powder (Fisher. BioReagents-BP-1426-2)

0.5 mL Cholesterol, 5mg/ml in EtOH (Sigma-C1145-250MG)

487.5 mL Sterile Water

Post-autoclave salts:

0.5 mL Calcium Chloride, 1M (CaCl2; Sigma-C3881-1KG)

0.5 mL Magnesium Sulphate, 1M (MgSO4; Fisher-M/1050/53)

12.5 mL Potassium Sulphate, 1M, (KPO4, pH 6.0; Sigma-Aldrich-P0662-500G-M)

6.1 Book the autoclave (notebook on top of the machine).

6.2 Take clean flasks from the glass kitchen (Only the ones with autoclave tape on are sterile)

6.3 Measure all the pre-autoclave reagents and add to the flask (Use a new weighing boat and spatula for each reagent. Also, the cholesterol is kept in the fridge.)

6.4 Once water is added mix thoroughly and label with autoclave tape ('LB NGM Rm 5020'). Makesure the bottle is not screwed completely when placing it inside the autoclave machine.

6.5 Turn ON the autoclave

6.6 Make sure that the autoclave's probe bottle is the same size as the largest bottle you use and fill it with water.

6.7 Place the temperature probe in it.

6.8 Fill up the autoclave with water until it reaches the grill.

6.9 Place the bottles in the autoclave and make sure that the cap is not screwed completely.

6.10 Check the waste flask is not too full

6.11 Use 'media' program.

6.12 Press START.

6.13 It will take about 2.5 hours for 1L or larger bottles.

6.14 When autoclave is complete, remove the probe flask

6.15 Make sure to wear gloves as the flask will be hot

6.16 Let the agar to cool to around 55°C, ie the bottle is cool enough to hold for a second with a gloved hand.

6.17 Add the post autoclave reagents.

6.18 Mix it well and start pouring onto desired sized plates (See Protocol for plate pouring)

6.19 Try not to shake the bottle too much while mixing to avoid air bubbles.

6.20 The agar needs to be warm to be poured without blocking the tubings, so try to pour as quickly as possible and if not poured immediately put the bottle on a waterbath set to 60C until being used.

7 Using the Integra ViaFill, dispense 200ul of LB NGM agar into 96-well imaging plates, following the protocol *Dispensing agar into multiwell plates:*

Protocol

NAME

Dispensing agar into multiwell plates

CREATED BY

Saul Moore

PREVIEW

7.1 Prepare a 250ml bottle of hot milliQ water in the microwave and keep in the waterbath along with the agar. The water is important to have on hand in case of tubing blockages.

7.2 Insert large cassette into the machine

7.3 Configure X, Y, and Z settings for the multiwell plate by clicking on tool symbol → stage alignment.

Note

For UNIPLATE96SQWLF 650U:

X = 95.6
Y = 4.2
Z = -22.5

For 48WP:

7.3.1 Put the plate into the stage and then press 'Move' so that the plate moves so that it is under the dispensing cassette.

7.3.2 Use the up and down arrows to move the pipette tip so that they hover just over the plate and make note of the height (this will be entered into the dispensing program at a later step). Press 'Fast/Slow' button to switch between fast and slow movements.

7.3.3 Use the X, Y arrows to move the plate so that the pipette tips are centered in the middle of column 5.

7.3.4 Save all settings.

7.4 Exit settings by pressing the back button

7.5 Press on the program you wish to use (see later for configuring your own program)

7.6 Make sure that the correct cassette is listed and change if necessary

7.7 Select the volume you wish to dispense

Note

For 96WP:
200 µL

7.8 Select 'set height' and set the appropriate height for tip height (usually all the same)

7.9 Place the end of the tubing from the casettee into the agar that is being kept warm in the water bath

7.10 Press 'Prime' to prime the tubing and allow to finish so that agar flows from the pipette tips.

Note

IMPORTANT:
Once the agar is in the tubing it is important to act quickly to avoid agar solidifying and causing blockages. If you are particularly concerned about agar cooling in the tubing, wrap the tubing in aluminium foil to keep hot.

STEP CASE

Unblocking the tubing 9 steps

If the tubing does block, clear the blockage by 'reverse priming' as much of the agar as possible.

Then place tube ends in the hot water and prime continuously with hot water until the water runs all the way through.

If you are having trouble getting the water through, squeeze and massage parts of the tubing where you can see blockages to force the agar along and allow the water to pass.

Once all cleared, 'reverse prime', and reprime with the agar

7.11 Place a clean plate in the stage

7.12 Press run and then plate should fill with agar

7.13 Repeat steps 11-12 until all the plates have been filled.

Note

Little drops of agar can solidify on the tip ends. It is often good to remove these drops using a pipette tip every few runs so that blockages do not occur.

7.14 'Reverse prime' all the agar

7.15 Place the tubing ends into the hot water.

7.16 Prime so that the water runs through and clears all the agar

7.17 Reverse prime to remove the water

7.18 Release tension from the tubing and remove cassette

7.19 Double wrap the cassette in aluminium foil for autoclaving

8 Leave the plates on the lab bench (with lids on) until the agar is dry.

9 Measure the weight of 3 plates (with lids on) and record average plate weight on day of pouring.

10 Store the plates upside-down at 4°C (plates can be stored for up to 1 week prior to use).

Inoculating bacterial cultures (96WP)

11 3 days prior to tracking (e.g. on Tuesday if tracking Thursday), inoculate overnight cultures of bacterial strains in 96-well from 96-well CeMbio frozen stock plates, following the protocol for *Growing overnight bacterial culture in 96WP:*

Protocol

NAME

Growing overnight bacterial culture in 96WP

CREATED BY

Priota Islam

PREVIEW

Place inoculated cultures in 96WP in a shaking incubator at 25°C, 200rpm.

11.1 Obtain LB Broth from the Media kitchen

LB Broth contents:
4gNaCl
4 g Tryptone
2 g Yeast Extract
Add dH2O to 400 mL

11.2 Wipe the work area with 70% ethanol and create a relatively sterile environment on the laboratory bench by using a simple gas-powered burner. Work under the hood if you have a large number of plates. Book the hood in advance in that case

11.3 Label the inoculation plates and the lids with the corresponding frozen library plate ID

11.4 Add 200ul liquid LB to each well of the 96-well plate using a multichannel pipette and a sterile reservoir.
Details of the plates used:
Name- Nunc MicroWell Plates with Nunclon Delta Surface-C (ThermoFisher Scientific)
Catalogue number- 143761
Well capacity- 300ul

11.5 Take the frozen library plates out of the freezer just before doing the inoculation to avoid the wells thawing completely (We want to avoid repetitive freeze thawing of the bacterial strains). You can also use dry ice to take all the plates out of the freezer at once. In that case, just before inoculation take the respective plate off the dry ice and leave at bench top for 2mins

11.6 Position the plates so that well A1 of every plate is at the top left. You can also put sticker on the replicator to mark A1 and H12, to avoid confusion and contamination

11.7 To use the replicator, strap some laboratory/masking tape around your index and middle finger together with the adhesive part on the outer side. Use the stickiness of the tape to pick the replicator by gently pressing on the surface

11.8 Insert the sterile replicator into the frozen library plate (make sure to touch the surface or puncture into the frozen wells to obtain sufficient bacterial cells

11.9 Dip the replicator into the inoculation plate with the LB broth (try to swirl the replicator slightly while inside the wells to ensure some bacteria has mixed with the liquid LB)

11.10 Put the replicator inside the autoclavable box to be sterilised for later use (Try to use one replicator for every inoculation plate to avoid contamination)

11.11 Cover the inoculation plates with plastic lids and/or breathable seals (Axygen Rayon breathable film sterile 50um, Catalogue number-AXY2008)

11.12 If the plates are not intended to be shaken then place them in an airtight box and put inside the incubator to avoid uneven evaporation of the LB and the wells drying up

11.13 Incubate the bacterial culture at the required growth temperature overnight (i.e. 12-18 hrs in general) with/without shaking depending on the growth conditions of the bacterial strains (If shaking is required at any temperature other than 37C, make sure to book the shaking incubator in advance)

11.14 Also, if the plates are meant to be shaken, make sure the wells have a maximum of 2/3 volume of their capacity (i.e. For wells with 300ul capacity do not exceed the volume of 200ul) Shaking rpm should be between 150-180rpm

11.15 After incubation, check for growth, which is characterized by a cloudy haze in the media

12 Separately inoculate 100ml *E. coli* OP50 bacteria in an Erlenmeyer flask and place in a shaking incubator at 37°C, 200rpm.

Seeding bacterial lawns with ViaFlo (96WP)

13 The next day (e.g. Wednesday, 1 day prior to tracking), remove overnight cultures from the shaking incubators and replace the LB in each of the wells of the inoculated 96WPs (that are reserved for the control bacterial strain) with 200ul OP50 bacterial culture that was inoculated separately.

14 Store the bacterial cultures at 4°C until seeding bacterial lawns later that day.

15 Remove imaging plates from 4°C storage and record the average weight of 3 of the plates.

16 Place the imaging plates in a drying cabinet for approximately 2-3 hours (until the plates have lost approximately 3-5% of their original weight).

17 Once imaging plates are dry, remove bacterial cultures from 4°C and seed 10ul bacterial culture into 96-well imaging plates, following the protocol *Seeding bacterial lawns using Integra ViaFlo (96WP)*

18 Place seeded plates to dry under a hood for about 30 minutes.

19 04:00:00 Leave seeded plates on the lab bench (with lids on) for 4 hours to allow lawns to grow. 4h

20 Store seeded plates at 4°C overnight.

Dispensing Day1 worms onto seeded plates using COPAS (96WP) 30m

21 On the day of tracking (e.g. Thursday, if re-feeding was performed on Monday), remove seeded plates from 4°C, and dry for 30 mins under a hood. 30m

22 Remove worms on 90mm Petri plates from 20°C incubator, and follow the protocol *Preparing worms for the COPAS (wormsorter):*

Protocol

NAME

Preparing worms for the COPAS (wormsorter)

CREATED BY

Priota Islam

PREVIEW

22.1 Wash the worms off the plates using few milliliters of M9 and collect in a 15ml falcon tube and fill up till 15ml with M9

22.2 Centrifuge the tube at 1500rpm for 2mins followed by removal of the supernatant using a pasteur pipette and addition of another 15ml of M9

22.3 Repeat the steps 2 more times

22.4 After the final wash remove the supernatant as much as leaving behind 10ml in the falcon tube

22.5 Consolidate all the worm liquid in a 50ml tube and dilute if necessary, for the COPAS

22.6 Ideally make 2×50ml Falcon tubes

22.7 Dispense the worms using the COPAS following the protocol *COPAS wormsorter*

23 Dispense Day1 worms onto seeded plates for imaging, following the protocol *Using the worm sorter (COPAS):*

Protocol

NAME

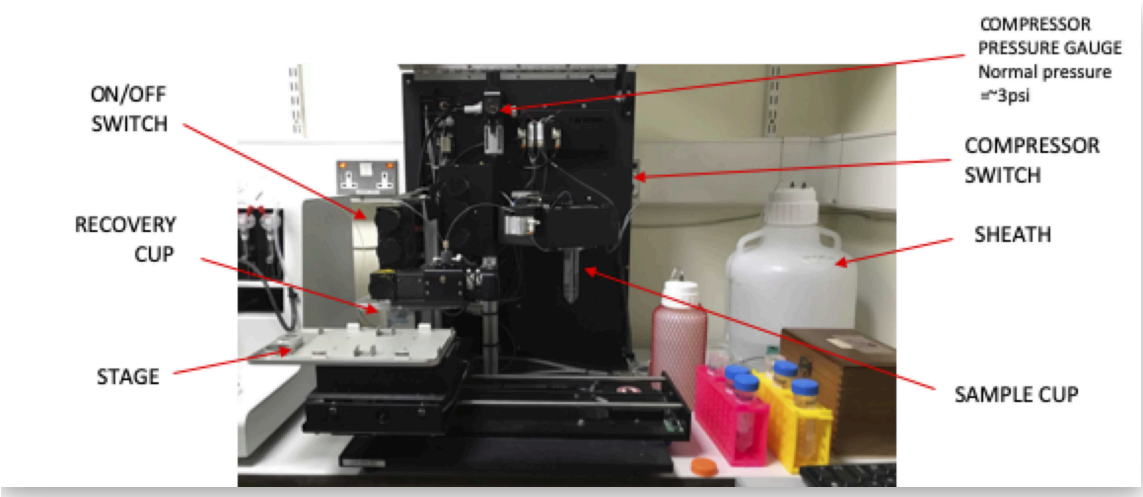
COPAS wormsorter

CREATED BY

Ida Barlow

PREVIEW

23.1



COPAS wormsorter indicating key components

Turn on the compressor at the wall – it should show a pressure of 40psi after switched on

23.2 Turn on COPAS machine with switch on the left hand side

23.3 Turn on the lasers (488 laser sufficient if using unmarked animals). Add in picture of lasers.

23.4 Turn on the computer

23.5 Discard waste contents that are in the recovery cup (small shallow cup on the left-hand side of the machine)

23.6 Check that there is water in the sheath. If the water is low, fill up with MQH2O (not M9).

23.7 Make sure that the recovery cup and sample cup are securely tightened so that there are no leaks in the system

23.8 Open dbgview – should always be running in the background

23.9 Open FlowPilot software and a prepared experiment with a set gate for eg Adults. :

23.9.1 File → Load Experiment

23.9.2 File → Load sample

23.10 Maintenance → Flush Sample

23.11 Click 'Refill Sample' – the sample cup pressure should decrease. You can see this in the software on the left hand size (include screenshot).

> Note
>
> Sometimes the sample cup pressure doesn't decrease and in fact increases. You can still unscrew the sample cup but if this persists there may be a blockage.

23.12 Unscrew sample cup and replace with falcon filled with cleaning solution (pink in colour)

23.13 Once securely replaced click 'Done refill'

23.14 Check 'Sample on' and 'mixer on' – cleaning solution should now pass through the system; allow a 2-3 ml to pass through (make sure sheath is unchecked)

Note

You will get a warning about contaminating the flow cell, this normal and you can click 'Yes'

23.15 Uncheck ‘Sample on’ or click Abort to stop sample flow.

23.16 Repeat steps 11-15 with water

23.17 Repeat steps 11-13 with sample.

23.18 **Turn mixer ON**. If you do not do this you may lose all your worms that have settled to the bottom of the tube!!!

23.19 Maintenance → Prime Flow Cell; to flush sample through the system and remove air bubbles

23.20 Maintenance → Flush sample

23.21 Check 488nm (and 568nm) laser boxes

23.22 Check ‘Use sort gate’ for stored sort gate – include screenshot of software here

23.23 Click ‘Acquire’ – sample should pass through the system and number of events per second will be shown:

- Aim for 10-20 events per second
- If too few/too many events increase/decrease ‘Sample cup pressure’ so that it is between 1.5-2psi
- To ensure only one event per droplet go to Setup→Coincidence, select ‘Pure, no double’. This increases accuracy in the number of worms dispensed but the time to dispense may increase.

23.24 Click on the plate icon on the top bar

23.25 Select number of objects to sort

23.26 Select the wells you would like to fill (for testing we use a spare 60mm plate and fill wells A1, A2, B1, B2

23.27 Select which gate to use

23.28 Apply

23.29 Place 60mm plate in front left corner of left-hand stage with A1 in the left corner.

23.30 Click ‘Fill plate’

23.31 Keep an eye on the number of events per second

23.32 Ensure the ‘Diverter pressure’ is checked

23.33 Check under microscope that the correct number of objects were dispensed per ‘well’

23.34 If too many objects, decrease sample cup pressure and repeat steps 8-11 or select Pure no double to increase accuracy.

23.35 Click on the plate icon on the top bar

23.36 ‘Clear plate’

23.37 Select number of objects per well and click ‘Apply to All’ or select which wells you would like to fill.

23.38 Apply

23.39 Place 96 well plate in left-hand stage

23.40 Ensure ‘Diverter pressure’ is checked’; if it is not then liquid comes out of the dispenser constantly and you get flooding.

23.41 ‘Fill plate’

23.42 Keep an eye on the number of events per second still and monitor how much sample fluid is coming through the system

23.43 Repeat steps 11-15

23.44 Keep sample cup with water secured so that the system is air-tight and closed

23.45 Turn off all equipment (Computer, lasers, compressor, worm sorter).

24 00:30:00 Leave plates to dry under a hood for 30 minutes. 30m

25 00:30:00 Place plates in the imaging cave under the Hydra rigs to acclimate for 30 minutes prior to imaging (+1hr timepoint). 30m

Tracking using Hydra rigs 15m

26 00:15:00 After acclimation under the Hydra rigs, record worm behaviour on the bacterial food for 15 minutes (25fps, exposure 25000msec, bluelight stimulation). 15m

27 Record 15 minute videos at the following timepoints: 1, 3, 5, and 24 hours on food.

28 After tracking, discard the plates in a biological waste bin.