RNAi screens in *Caenorhabditis elegans* in a 96-well liquid format and their application to the systematic identification of genetic interactions

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We describe a protocol for performing RNA interference (RNAi) screens in *Caenorhabditis elegans* in liquid culture in 96-well plates. The procedure allows a single researcher to set-up and score RNAi experiments at ~2,000 genes per day. By comparing RNAi phenotypes between wild-type worms and worms carrying a defined genetic mutation, we have used this protocol to identify synthetic lethal interactions between genes systematically. We also describe how the protocol can be adapted to target two genes simultaneously by combinatorial RNAi.

INTRODUCTION

Caenorhabditis elegans is a very powerful model organism for reverse genetic analysis, because RNA interference (RNAi) can be delivered systemically by feeding worms on bacteria that express double-stranded RNA targeting any gene of interest[1]. The availability of a genome-wide RNAi feeding library[2] has facilitated the completion of multiple genome-wide loss-of-function phenotypic screens (reviewed in ref. 3). RNAi feeding experiments can be performed using bacterial feeding on agar plates ("plate feeding"), but this approach has a throughput that restricts the number of screens that can be performed to one or two genome-wide screens per study.

To improve the throughput of RNAi screens in *C. elegans*, we[4] and others[5–7] have developed protocols for screening in liquid culture in 96-well plates. RNAi feeding bacteria are grown overnight in 96-well plates and resuspended in nematode growth media (NGM). Approximately ten synchronized first larval stage (L1) worms obtained by filtration (or bleaching adults and hatching overnight) are dispensed into each well of a flat-bottomed 96-well plate, to which 40 µl of bacterial suspension is added. This small volume ensures adequate aeration of each well. The plates are incubated for 4 days with shaking at 20 °C, allowing sufficient time for the L1 worms to grow to adults, lay eggs and for these eggs to hatch and develop into larvae. By this stage worms will have consumed most of their food, resulting in clearing of the bacterial suspension and allowing easy scoring of phenotypes under a dissecting microscope. An overview of the protocol can be seen in **Figure 1**. Variations of the protocol are also described at http://www.niob.knaw.nl/researchpages/plasterk/96well%20forma.htm and http://www.wormbook.org/chapters/www_introreversegenetics/introreversegenetics.html.

There are two main advantages to this protocol. First, all steps of the protocol use 96-well plates, allowing both an easy setup using multichannel pipettes and several thousand experiments to be performed in parallel by a single researcher. An experienced screener should be able to complete the equivalent of a genome-wide screen in about 2–3 weeks (although additional time will be required to verify positives). Second, the assay uses a population of worms in each well, thus avoiding the animal-to-animal variation of RNAi phenotypes that is observed with single worm plate feeding protocols[2].

We primarily developed this assay to facilitate the use of RNAi screens to systematically identify genetic interactions between genes. In this approach, the RNAi phenotype of a gene in a worm strain carrying a defined genetic mutation is directly compared to that in wild-type worms and also to the phenotype of the mutant worm strain alone—a synthetic phenotype is defined here as a phenotype that is stronger after the double perturbation than the expected additive phenotype of the two single perturbations. Additionally, liquid feeding can be used for screens that use the expression of stable fluorescent constructs as reporters[6] or for chemical genetic screens.

As an alternative approach to identifying genetic interactions, two genes can be targeted simultaneously by RNAi ("combinatorial RNAi")[8]. The protocol we describe here works well for combinatorial RNAi, although it is much more effective when using an RNAi hypersensitive worm strain such as *rrf-3* (ref. 9), *eri-1* (ref. 10) or *lin-35* (refs. 11, 12). Combinatorial RNAi is useful as it allows genetic interactions to be identified for genes for which a viable loss-of-function genetic allele is not available.

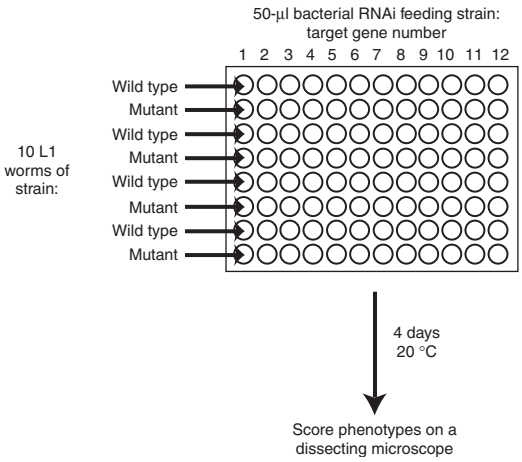

Figure 1 | Overview of 96-well liquid culture RNAi feeding protocol.

Finally, we note that the protocol as described here is not suitable for scoring phenotypes that can be detected only in adult progeny (e.g., behavioral phenotypes), because in a 50-µl volume progeny worms starve before reaching adulthood. However, it is possible to grow progeny to adults by spotting each well onto seeded agar plates or by increasing the volume of each assay to 150 µl. However this introduces more well-to-well variation, and it is still probably best to use plate feeding experiments for scoring adult phenotypes. For researchers who are new to using *C. elegans*, there are several excellent collections of basic protocols available online (e.g., http://www.wormbook.org/toc_wormmethods.html and http://info.med.yale.edu/mbb/koelle/protocol_list_page.html).

MATERIALS

REAGENTS

- Worm strains can be obtained from the *C. elegans* Genetics Center (http://www.cbs.umn.edu/CGC/) and the National Bioresource Project (http://shigen.lab.nig.ac.jp/c.elegans/index.jsp); for combinatorial RNAi, we use the strain NL2099 *rrf-3(pk1426)*, although the strains *eri-1*, *lin-35* or *eri-1; lin-15B* or *eri-1;lin-35* are also suitable
- The Ahringer RNAi feeding library consisting of 16,757 bacterial clones is available from Geneservice Ltd (http://www.geneservice.co.uk/products/rnai/); as an alternative, the Vidal ORFeome feeding library consisting of 11,804 bacterial clones is available from Open Biosystems (http://www.openbiosystems.com/rnai/)
- LB (Luria–Bertani) media plus 100 µg ml^{-1} ampicillin rectangular agar plates for replicating the RNAi library
- 2× TY (tryptone/yeast extract) plus 100 µg ml^{-1} ampicillin (or LB plus 100 µg ml^{-1} ampicillin) media for growing bacterial feeding strains overnight
- NGM agar plates seeded with *Escherichia coli* OP50 (available from the *C. elegans* Genetics Center) for growing worms
- IPTG to induce bacteria
- Ampicillin

EQUIPMENT

- Deep-well (2 ml) 96-well plates for growing bacteria (e.g., Corning Costar 3961)
- Flat-bottomed 96-well tissue culture plates for liquid culture RNAi incubations (e.g., Falcon 353072). ▲ CRITICAL Do not use round-bottomed plates
- Plastic dishes for dispensing media and worms using a multichannel pipette (e.g., Nunc 176597)
- 10-µm mesh filters for purifying L1 worms (e.g., Millipore S5EJ008M04)
- Multichannel pipette (50–300 µl) for dispensing media
- Multichannel pipette (5–50 µl) for dispensing worms and bacterial suspensions
- 96-pin replicating tool for replicating the RNAi library and inoculating cultures (a multichannel pipette can also be used for this purpose)
- Plastic boxes for holding stacked 96-well plates to prevent evaporation of worm/bacterial suspensions (e.g., BDH BUH4900716)
- A 37 °C shaking incubator to grow bacteria
- A 16–25 °C shaking incubator for liquid culture RNAi incubations
- A dissecting microscope to score phenotypes
- A bench-top centrifuge for pelleting bacteria and filtering worms (e.g., Eppendorf 5810R)

REAGENT SETUP

- NGM (per liter: 3 g NaCl, 2.5 g peptone, 975 ml H_2O, 1 ml cholesterol (5 mg ml^{-1} in ethanol), 800 µl fungizone, 1 ml $CaCl_2$ 1 M, 1 ml $MgSO_4$ 1 M, 25 ml KH_2PO_4 pH 6; the last five ingredients should be added after autoclaving).
- M9 buffer (per liter: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml $MgSO_4$ 1 M, 1 liter H_2O) for washing and aliquoting worms.
- Bleach solution (per 1,000 µl: 250 µl NaOH, 150 µl sodium hypochlorite, 600 µl water).

PROCEDURE

Preparation of bacteria

1| Replicate RNAi library bacterial glycerol stocks onto LB plus ampicillin plates using a 96-pin replicating tool and grow overnight at 37 °C.

2| The day before setting up a screen, inoculate bacteria into 400–800 µl (depending on the final volume required; 40 µl of bacteria is required for each individual feeding experiment) of 2× TY plus 100 µg ml^{-1} ampicillin in a 2-ml 96-well plate and grow overnight in a shaking incubator at 220 rpm at 37 °C.

▲ CRITICAL STEP Use freshly grown bacteria.

Induction of bacteria

3| The following morning, induce transcription of double-stranded RNA by adding IPTG to each well of the bacterial cultures to a final concentration of 4 mM and incubate at 37 °C for 1 h.

4| Pellet bacteria by spinning at 2,500*g* for 5 min. Discard the supernatant by rapid inversion to avoid cross-contamination and resuspend in a volume of NGM equal to the volume of 2× TY that the bacteria were grown in overnight (i.e., 400–800 µl) plus 100 µg ml^{-1} ampicllin plus 4 mM IPTG.

Preparation of worms

5| For worm strains with a brood size similar to wild-type worms, approximately ten L1 worms should be added to each well of a 96-well plate.

Prepare worms by filtering (option A) or bleaching (option B). When using filtered worms, it is recommended that one use plates approaching starvation to avoid contamination of RNAi feeding experiments with OP50.

▲ CRITICAL STEP For worm strains with a reduced brood size, more worms should be used per well such that the total brood per well is similar to that of wild-type worms—this allows easy comparison between wells and the robust identification of genetic interactions.

? TROUBLESHOOTING

(A) L1 worms can be easily prepared by filtering mixed-stage or starved populations of worms through a 10 µm mesh

(i) Wash worms off plates in M9 buffer.

(ii) Isolate L1 worms by centrifugation through a 10 µm mesh for 10 s at 200*g*.
(iii) Resuspend L1 worms to a final concentration of ten L1 worms per 10 µl of M9 buffer.

(B) Alternatively, L1s can be prepared by bleaching gravid adults on the previous day and hatching worms overnight in M9 buffer

(i) Wash off gravid adult worms in M9, pellet by centrifuging for 1 min at 200*g* and add 2 ml bleach solution to 0.25 ml worm pellet. Incubate for 5–10 min with occasional vortexing until only embryos remain.
(ii) Pellet embryos by centrifuging for 1 min at 200*g* and resuspend in M9 buffer three times to remove bleach.
(iii) Hatch worms overnight in M9 buffer with gentle shaking to allow aeration.
(iv) Pellet L1 worms by centrifuging for 1 min at 200*g* and resuspend at a concentration of approximately ten L1 worms per 10 µl of M9 buffer.

RNAi feeding

6| Pipette approximately ten L1 worms in a volume of 10 µl of M9 buffer into each well of a 96-well plate from a plastic tray using a multichannel pipette. To avoid settling of worms, the plastic tray should be agitated while pipetting. For genetic interaction screens, it is best to compare directly the RNAi phenotypes observed in a mutant strain with that seen in wild-type (N2) worms. This can be achieved by alternating rows of wild-type and mutant worms in a 96-well plate, each row fed with the same bacterial clones (see **Fig. 1**).

7| For combinatorial RNAi, add an equal volume of each bacterial feeding clone to each well (20 µl of each strain, grown overnight and induced independently). For effective combinatorial RNAi, an RNAi-hypersensitive strain, such as *rrf-3(pk1426)*, should be used[8,9].

Incubation

8| Grow worms in a shaking incubator at 150 rpm for 4 days at 20 °C. To avoid evaporation of liquid from wells, 96-well plates should be stacked in plastic boxes (see 'Equipment') within the incubator.

Scoring of phenotypes

9| Score plates under a dissecting microscope 4 days after setup (see **Fig. 2** for example phenotypes). We score embryonic lethality (Emb phenotype) and sterility (Ste) semiquantitatively on a scale from 0 (wild type) to 3 (100% Emb or Ste). To identify genetic interactions, an RNAi phenotype observed in a mutant worm strain is directly compared with both RNAi phenotype observed in wild-type worms and the phenotype of the mutant strain fed on a control bacterial strain. Suitable control strains expres a double-stranded RNA that does not target any expressed sequence in *C. elegans* (e.g., clone Y95B8A_84.g in the Ahringer library).

? TROUBLESHOOTING

Replication

10| Repeat the experiment.

Therefore, it is essential to replicate any putative phenotypes and genetic interactions observed.

▲ CRITICAL STEP RNAi has an intrinsic variability.

? TROUBLESHOOTING

● TIMING

Day –4 (approx.): chunk worms to ensure you have enough L1 worms on day 0 (ten large plates of worms will more than suffice for 25 96-well plates).
Day –2: replicate bacterial glycerol stocks onto plates and grow overnight (allow about 1 h to replicate 25 96-well plates; bacterial colonies can be kept for a week on agar plates at 4 °C).
Day –1: inoculate bacteria into 2×_TY plus ampicillin and grow overnight (allow about 1 h to inoculate 25 96-well plates) (optional: bleach worm strains and hatch overnight in M9).
Day 0: induce bacteria, filter worms, add L1 worms and bacteria to 96-well plates and incubate in a shaker (allow about 2 h for 25 96-well plates).

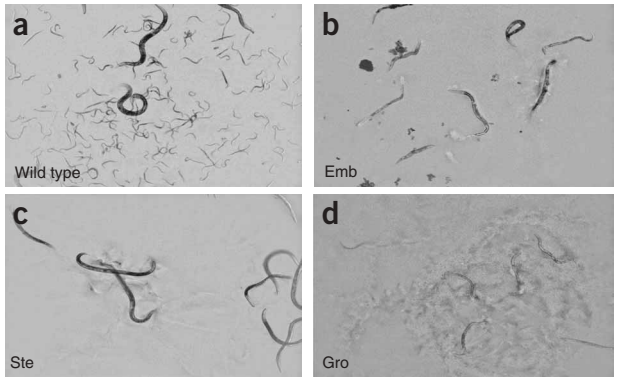

Figure 2 | Example phenotypes. (**a**) Wild-type worms, (**b**) embryonic lethal (Emb), (**c**) sterile (Ste) and (**d**) first-generation growth-defective (Gro).

Day 4: score plates (allow half a day for scoring 25 96-well plates and less time if the plates contain replicates).
Day 5: re-score plates if required.

? TROUBLESHOOTING

Reduced brood size

For worm strains with a reduced brood size, a preliminary experiment should be performed to determine the number of L1 worms to add per well—too few worms per well can result in poor survival rates. To facilitate the identification of genetic interactions, the number of L1 worms added should be adjusted so that the total brood size of each strain fed on control clones is very similar to that of wild type.

Contaminated wells

Contaminated wells are easily recognized in the screen because they will not have cleared after 4 days of incubation at 20 °C and should not be scored.

Temperature sensitive strains

For temperature-sensitive strains, it may be necessary to use a lower or higher incubation temperature and a longer incubation time. Again, the correct conditions should be established in a pilot experiment using control feeding clones.

ANTICIPATED RESULTS

Using wild-type (N2) worms in this assay, we detected phenotypes for 157 of 182 tested genes (86%) with previously reported nonviable RNAi phenotypes from plate feeding assays[4]. Reproducibility between repeats is >90% for the detection of phenotypes. In a screen for synthetic genetic interactions between genes, we identified genetic interactions for 28 of 37 strains screened, and for a total of 0.6% of gene pairs tested[4]. Using combinatorial RNAi, we were able to detect interactions for 7/7 previously known synthetic lethal interactions and for 13/15 synthetic post-embryonic phenotypes and to identify redundant functions for 16 pairs of duplicated genes[8].

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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