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RNAi Screening: Automated High Throughput Liquid RNAi Screening in *Caenorhabditis elegans*

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Abstract

RNA interference (RNAi) is a powerful reverse genetic tool that has revolutionized genetic studies in model organisms. The bacteriovorous nematode *Caenorhabditis elegans* can be genetically manipulated by feeding it an *E. coli* strain that expresses a double-stranded RNA (dsRNA) corresponding to a *C. elegans* gene, which leads to systemic silencing of the gene. This chapter describes a protocol for performing an automated high throughput RNAi screen utilizing a full-genome *C. elegans* RNAi library. The protocol employs liquid handling robotics and 96-well plates.

Keywords

RNAi; feeding RNAi; reverse genetics; automated high throughput screening; *C. elegans*

INTRODUCTION

Genetic analysis in the free-living non-parasitic nematode *Caenorhabditis elegans* has been used extensively to study a variety of complex biological processes. There are several advantages of using *C. elegans* as a model system, including: (1) *C. elegans* animals are small (1.3 mm in length) and can easily be maintained in the lab. (2) *C. elegans* has a short 12–18 day lifespan. (3) *C. elegans* are transparent and individual cells can be readily visualized. (4) About 60–80% of *C. elegans* genes have human counterparts (Consortium, 1998; Kuwabara & O'Neil, 2001; Lai, Chou, Ch'ang, Liu, & Lin, 2000; Sonnhammer & Durbin, 1997). (5) *C. elegans* are self-fertilizing hermaphrodites, enabling easy genetic analysis of large clonal populations. (6) Due to their small size, more than 100 worms can fit in one well of a 96-well plate, making them an ideal whole-animal model system for high throughput screening. (7) Reverse genetics using “feeding” RNA interference (RNAi) is straightforward and facilitated by commercially available whole-genome RNAi feeding libraries (Fire et al., 1998; Fraser et al., 2000; Kamath & Ahringer, 2003; Kamath, Martinez-Campos, Zipperlen, Fraser, & Ahringer, 2001; Rual, Hill, & Vidal, 2004).

In 1998, Fire and Mello discovered that introduction of exogenous double-stranded RNA (dsRNA) into *C.elegans* results in the rapid and sequence-specific degradation of corresponding endogenous mRNA (Fire et al., 1998). The process was termed RNA interference (RNAi). RNAi-mediated gene knockdown typically phenocopies the genetic mutation. Importantly, RNAi knockdown occurs systemically and can be heritable for three or more generations (Fire et al., 1998; Grishok, Tabara, & Mello, 2000). RNAi mediated gene silencing can be achieved by three methods: injection of dsRNA (Fire et al., 1998), soaking worms in high concentrations of dsRNA (Tabara, Grishok, & Mello, 1998), and feeding *E. coli* expressing dsRNA to the worms (feeding RNAi)(Kamath et al., 2001; Timmons, Court, & Fire, 2001). Feeding RNAi is the least laborious and most cost effective method.

Normally, feeding RNAi is carried out on solid agar medium, making full genome RNAi screening methods relatively tedious because they cannot take full advantage of liquid handling robots. In this unit, we provide a detailed protocol for high throughput RNAi screening in liquid using 96 well-formatted plates. The protocol can be adapted to 384 well-formatted plates with some optimization. The protocol focuses on advance planning, setup, data acquisition, and data analysis. The unit also discusses general issues to consider when carrying out RNAi based high-throughput screening. An overview of the protocol is outlined in Fig 1.

STRATEGIC PLANNING

The combination of feeding RNAi and the availability of near full-genome RNAi libraries makes high-throughput genome-wide RNAi screens feasible. To create bacterial clones that express dsRNA, a DNA fragment corresponding to a target gene is cloned between two phage T7 RNA polymerase promoters in the L4440 vector. This vector is then transformed into *E. coli* strain HT115 (DE3)(Timmons et al., 2001). The genotype of HT115 is: F-, *mcrA*, *mcrB*, IN(*rrnD-rrnE*)1, *rnc*14::Tn10(DE3 lysogen: *lavUV5* promoter -T7 polymerase) (IPTG-inducible T7 polymerase) (RNase III minus). The HT115 strain carries an isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible T7 polymerase transgene for inducible dsRNA expression. The bacterial strain also contains a deletion in the RNase III-encoding *rnc* gene to improve dsRNA stability and feeding RNAi efficacy (Timmons et al., 2001; Timmons & Fire, 1998). Currently, there are two commercially available RNAi feeding libraries that were constructed in the Ahringer (Fraser et al., 2000; Kamath et al., 2001) and Vidal (Rual et al., 2004) laboratories. Both libraries are provided as frozen stocks in 96- or 384- well formats (Source Bioscience). The Ahringer library targets around 87% of currently annotated *C. elegans* genes and the Vidal library covers 55% of the 19,920 predicted unique protein-coding genes. Sub-libraries containing specific gene sets are also available, including phosphatases (166 clones), chromatin-related proteins (257 clones), and transcription factors (387 clones) (Source Bioscience).

This protocol takes advantage of the fact that 15–25 L1 larval stage worms can be readily pipetted into the wells of micro-titer plates with relatively good well-to-well accuracy. In our hands, when attempting to deliver 15 or 25 L1 stage worms per well, the standard well-to-well deviation is ± 5 worms. Once delivered into the wells, the L1 stage worms are fed with

E. coli strain HT115 (DE3) expressing a particular dsRNA corresponding to a *C. elegans* gene. The worms are then grown in liquid to at least the L4 larval stage in the presence of the *E. coli* RNAi clone to allow the *C. elegans* RNAi machinery time to knockdown expression of the targeted gene.

Before starting a large-scale screen, it is important to optimize the screening conditions in the desired well format (typically 96 or 384). This will allow maximizing the hit rate while minimizing the rate of false positives and negatives. The protocol described below utilizes half area 96 well plates, but the same general method can be adapted for other well formats. 15–25 adult *C. elegans* animals fit comfortably per well in half area 96 or 384 well plates. Some factors to consider when setting up a pilot screen include (a) incubation temperature, (b) concentration of RNAi bacteria, (c) worm strain, (d) worm developmental stage, (e) number of worms per well, and (f) length of the assay. It is always advisable to assemble a set of known positive and negative controls that read out the efficiency of RNAi-mediated silencing in liquid. We found that RNAi clones corresponding to *vhp-1*, *pap-1*, and *klf-3* are good positive controls that demonstrate the efficacy of the RNAi knockdown. The clones *vhp-1* and *pap-1* cause larval arrest phenotypes whereas *klf-3* causes uterus prolapse through the vulval opening (burst vulva). As a negative control, we typically use *E. coli* HT115 containing the empty vector L4440, and which is commonly referred to as “L4440”.

A pilot screen can be carried out to identify the most appropriate positive and negative controls for a particular assay as well as for assay optimization. For example, in our lab, we used RNAi to identify genes that are involved in the host response to a bacterial (*Enterococcus faecalis*) infection. To identify a positive control for the genome-wide screen, we first carried out a small-scale pilot screen in duplicate using a customized sub-library consisting of 3,236 clones. The readout for the *E. faecalis* infection assay is worm longevity. Under these assay conditions, 80–90% of the worms feeding on *E. faecalis* die by day 4 of infection whereas essentially none of the worms feeding on *E. coli* are dead at that time. Hit wells had 40% of worms surviving on day 3 of infection. We cherry-picked 161 primary hits and made a “hit library” in 96 well plate format and repeated the assay for verification. We re-picked the verified hits and repeated the assay. We chose a hit (in our case *cap-2*) that had a highly reproducible phenotype as the positive control for a genome-wide screen.

To evaluate the robustness of the assay in the high-throughput screening setup, half of the wells in the plate contained the negative control (L4440, only vector control) and half the wells contained *cap-2* RNAi. It is convenient to store a glycerol stock of this control plate at –80°C so that it can be replicated and included as a control plate during the large-scale genome-wide screen as the RNAi library is unlikely to contain appropriate controls in every plate. The robustness of a high throughput RNAi assay can be quantified using the Z'-factor metric, a statistical method often used in chemical screening during assay optimization to evaluate the quality of an assay.

The Z'-factor uses the means and standard deviations of positive controls and negative controls to calculate how well the two signals are separated from each other. The formula used to derive the Z'-factor is as follows,

$$Z' - factor = 1 - (3\sigma_p + 3\sigma_n) / |\mu_p - \mu_n|$$

Where μ indicates mean and σ indicates the standard deviation of both positive (p) and negative (n) controls. The Z' -factor coefficient considers the dynamic range of the assay (differences of means) and the variation associated with both positive and negative reference controls. In the case of a biochemical assay, a Z' -factor above 0.5 is considered to be very good, a score between 0–0.4 is considered marginal, and a value less than 0 is unacceptable (J. H. Zhang, Chung, & Oldenburg, 1999). For phenotypic assay using cells (or whole animals such as *C. elegans*), a Z' -factor of 0.5 is considered very good and 0–0.4 acceptable (Birmingham et al., 2009). One concern with the Z' -factor metric for assay assessment is that it is possible to obtain a high Z' -factor using a strong positive control that might not accurately represent the reality of the screen. This can lead one to miss weak hits that might be biologically meaningful. Thus, it is recommended to use controls similar to the predicted screen hits (Birmingham et al., 2009; J. H. Zhang et al., 1999) such as *cap-2* in the screen described in this Unit.

Statistical methods in addition to the Z' -factor metric have been recently introduced to evaluate the robustness of assays, including “strictly standardized mean difference” (SSMD) and “receiver operator characteristics” (ROC) curves (Birmingham et al., 2009; J. H. Zhang et al., 1999). SSMD is the ratio between the difference of the means and the standard deviation of the difference between two populations, in this case, the positive and negative controls.

$$SSMD = (\mu_1 - \mu_2) / \sqrt{(\sigma_1^2 + \sigma_2^2)}$$

The strength of the positive controls determines the acceptable values of SSMD. It has been shown that SSMD is a more accurate quality indicator than the Z' -factor (X. D. Zhang, 2007). ROC curves, which are widely used as a quality metric for microarray transcriptomics (Forster, Roy, & Ghazal, 2003), plots sensitivity versus 1- specificity. The area under the ROC curve can be used as a quality metric, where an area of 1 represents a perfect predictor, whereas an area of 0.5 represents random chance. Even though the SSMD and ROC methods address some limitations of the Z' -factor metric, they are not widely used because they are relatively user-unfriendly. See Table 1 for definitions and advantages and disadvantages of these three statistical methods (Birmingham et al., 2009).

Another important consideration when designing a genome-wide RNAi screen is whether to carry out the screen in duplicate (or even triplicate) or not. The advantage of having replicates for each RNAi clone tested is that it provides an empirical estimation of the intrinsic variance of the assay and thus helps to predict the probability of detecting true hits. On the other hand, although replicates eliminate false positives, it is considerable more effort and more expensive. It may be less time consuming to not have replicates and to simply cherry pick and retest all of the potential positive hits in duplicate or triplicate, but this will depend on the rate of obtaining false positive hits in a particular assay. The reproducibility of

an assay can be calculated using correlation coefficient methods (r), where r ranges from -1 , a perfect negative correlation, to $+1$ a perfect positive correlation. For genome RNAi screening, r -values above 0.7 indicate a highly reproducible assay with relatively low variance. If the genome-wide screen is not done in replicates, it is advisable to carry out a pilot screen with a sub-library as described above to obtain a rough estimate of the variance of the assay.

Once the assay is optimized using pilot screens with positive and negative controls, a test run of a large-scale whole-genome screen should be carried with a limited number of the RNAi plates (for example, the first 5 or 10 96 well plates of the whole-genome RNAi library corresponding to genes on chromosome I). If the screen is not done in duplicate, the positive hits obtained from this test run can then be rescreened to determine the variance/reproducibility of the assay.

Important criteria for the success of a high-throughput screen are automated assay readouts and analyses. Manual visual inspection of a phenotype is typically a major bottleneck in high-throughput screening. Automated image processing and data analysis algorithms have vastly improved in recent years, and taking advantage of these developments helps improve the quality and throughput of *C. elegans* RNAi screens. An automated quantitative assay such as the level of fluorescence of a reporter gene allows the implementation of statistical metrics such as Z scores to identify hits and calculate their statistical significance, thereby reducing bias. In the protocol described here, we briefly describe the automated data analysis sources available to quantitate assays based on growth or fluorescence readouts such as GFP, Nile Red, or MitoTracker.

BASIC PROTOCOL 1

Feeding RNAi using L1 stage *C. elegans* in liquid culture in 96 well plates

Basic protocol 1 describes a method for carrying out a large-scale high throughput RNAi screen in liquid culture in 96-well format to take advantage of automated microscopes and imaging processing software.

Materials

NOTE: All solutions and equipment must be sterile and proper sterilization methods should be used accordingly.

Nematode Growth Media or NGM agar plates (see recipe)

M9 buffer (see recipe)

Luria-Bertani agar

Luria Broth

Ampicillin or Carbenicillin

Nystatin

RNAi library in 96-well format frozen at -80°C

Isopropyl β -D-1-thiogalactopyranoside (IPTG)

Buffered bleach (see recipe)

S-basal complete buffer (see recipe)

96 Solid Pin Multi-Blot Replicators (V&P Scientific, catalog number: VP 408)

96-deep-well assay blocks (1 ml or 2ml) (Costar; catalog numbers: 3958 or 3960)

96-half-well area plates with transparent lids for manual phenotypic analysis (Corning, catalog number: 3697)

96-half-well area plates with lids, black-walled wells, and clear bottoms for imaging and analysis (Corning, catalog number: 3882)

96-well-flat-tissue culture treated sterile plate for RNAi library glycerol stock preparation (Corning, catalog number: 353916)

Breathe-Easy-Gas permeable sealing membranes for 96 well plates

50 ml sterile disposable reagent reservoirs for media and worms (Corning, catalog number: 4870)

Aluminum seal for frozen stocks (Costar, catalog number: 6570)

Cell Strainer 40 μ m Nylon (Falcon, catalog number: 08-771-1)

Dry ice

Tube roller

Multi-channel pipette (5–50 μ l and 50–200 μ l)

Incubators (15°C, 20°C and 25°C)

Plastic assay boxes (commercial food storage boxes)

Preparation of a control plate in 96-well format—Typically, in high throughput chemical screening protocols, positive and negative controls are included in every plate. However, for genome-wide RNAi screening, the RNAi library will most likely not contain appropriate controls in every plate. In this case, it is necessary to set up an independent plate with positive and negative controls. For creating a customized control plate, consider using *E. coli* strain HT115 containing the feeding RNAi vector L4440 (Timmons et al., 2001) referred colloquially as L4440, as the negative control. For positive controls, we recommend *vhp-1* RNAi (which causes larval arrest, lethality) and *klf-3* RNAi (which causes uterus prolapse through the vulval opening when the RNAi is administered at the L1 stage), which work well in liquid medium. Alternatively, positive and negative controls can be chosen based on the data obtained from a pilot screen or from the literature. Setting up a full plate with controls will also help in assay optimization and to determine the robustness of the assay using the Z'-factor metric as described above. In addition, having controls in the same format as other RNAi library stock plates will allow the control clones and RNAi library clones to be treated similarly to reduce artifacts caused by a difference in handling between the RNAi controls and experimental RNAi clones. Normally, in our lab, we use at least one

negative control and two positive controls with different degrees of activity. Following is the protocol for preparing a glycerol stock control plate.

1. Remove the appropriate frozen RNAi library stock plates from -80°C containing the RNAi clones of interest and put them on dry ice.
2. Remove the aluminum foil cover from the frozen plate carefully to avoid aerosols that might cross contaminate the wells.
3. Pick the clone(s) from the appropriate wells in the frozen glycerol stock library plate using a 1 ml pipette tip with filter and streak on LB agar plates containing 200 $\mu\text{g/ml}$ of carbenicillin and 15 $\mu\text{g/ml}$ tetracycline. Incubate at 37°C overnight.

Note: As there is typically up to a ~20% error rate in RNAi genome libraries, sequence the clones to make sure you picked the right ones.

4. Seal the library stock plate with a new aluminum foil cover and return the plate immediately to -80°C .
5. The next day, prepare a 96 deep well assay plate (2 ml) containing 1 ml of LB broth containing 200 $\mu\text{g/ml}$ of carbenicillin.
6. Design a plate map for your control plate. For example, if you have only two controls, one positive and one negative, divide the plate into two (e.g., wells A1 to D12 for the negative control and wells E1 to H12 for the positive control). If you have four controls, divide the plates into four parts, etc.
7. Pick a single colony from the LB agar plate corresponding to each control and dilute in 5 ml of LB broth. Inoculate 50 μl per well to the appropriate wells based on your plate map and incubate at 37°C with shaking, overnight.
8. The next day, centrifuge the bacterial culture at 4000 rpm for 10 min and discard the supernatant carefully without causing cross contamination.
9. Add 500 μl of LB containing 20% glycerol to each well and resuspend the pellet using a multichannel pipette.
10. Transfer 200 μl bacterial culture with glycerol from the 96-deep well plate to a 96-well flat bottom sterile plate (in duplicate).

Note: Care should be taken to avoid cross-contaminating the wells.

11. Seal the control plates with a new aluminum foil cover and carefully place them in -80°C rack. The plates should not be disturbed for at least 12 hours.
12. Use one frozen glycerol stock control plate as a working stock and store another as a master plate stock. If RNAi efficiency goes down in the control plate, likely due to damage caused by frequent freezing and thawing, use the master plate and make a new glycerol working stock plate.

Worm propagation and egg production

13. Prepare 10 cm NGM agar plates with 1 ml of 50X *E. coli* OP50 (see reagents and solutions) to propagate worms. Drop 1000–2000 L1 larval worms per plate according to the required number of worms for the experiment.

Note: Normally 1000 wild-type worms will yield at least 10,000 embryos, which will produce ~10,000 L1 larval worms. For each 96-well plate, you will need approximately 2,500 L1 worms.

14. Incubate the worms at an appropriate temperature and for an appropriate time (generally 2–5 days) according to the worm strain used for the screen. The worms are ready for egg preparation when you observe embryos inside the gravid animals.

NOTE: It is recommended to do the egg preparation soon after the worms become gravid. If you prepare eggs after seeing many embryos, you will end up with a lot of dead eggs in your egg preparation, which might interfere with the future phenotypic analysis.

15. Treat the worms using hypochlorite bleach (see Support Protocol 1)

Bacterial Preparation and IPTG Induction for screening plates

16. Prepare a sufficient number of rectangular LB agar plates containing 200 µg/ml carbenicillin and 15 µg/ml tetracycline.

- a. Dry plates with the lids off on a clean bench for 10 min and make sure there are no condensed water droplets on the plates or lids before using them.
- b. Label plates before spotting them and always label the position of the A1 well, which will help keep the plates orientated correctly and minimize labeling errors in subsequent steps.

17. Remove the frozen RNAi library stock plates and control plate from –80°C and put them on dry ice.

18. Remove the aluminum foil covers from the frozen plates carefully to avoid aerosols that might cross contaminate the wells.

19. Replicate the RNAi library stock plates onto the square agar plates using a sterile 96 well pin replicator. The 96 well pin replicator must be sterilized after each inoculation. Use three sterile empty tip boxes to sterilize the replicator: fill the first reservoir with 10% bleach, the second with sterile water, and the third with 90% ethanol. Dip the pin replicator successively in these liquids and then flame thoroughly. Let the replicator cool down before use.

20. Seal the library stock plates with a new aluminum foil covers and return the plates immediately to –80°C.

- a. After placing the aluminum foil cover on the plate, use a hand roller to thoroughly seal the foil to the plate to prevent cross-contamination between wells, even if a plate is accidentally dropped.

- b.** Incubate the rectangular LB agar plate upside down at 37°C overnight to avoid condensed liquid cross contaminating the RNAi clones.
- c.** The next day, check the spotted plate and make a note of clones that didn't grow. Discard plates that have overlapping clones.
- d.** RNAi clones grown on agar plates can be stored at 4°C, upside down for 3 weeks. After 3 weeks of storage, RNAi efficiency may decrease. RNAi efficiency can also be decreased a replicating an agar plate to a new agar plate as well as replicating RNAi clones grown in liquid to agar plates.

21. Prepare an appropriate number of 96 deep well assay blocks containing LB broth with 200 µg/ml of carbenicillin. For 1 ml and 2 ml assay plates, respectively add 500 µl or 1.2 ml of LB media per well so that 96 well replicator pins can reach the media for inoculation.

Note: Carbenicillin is the preferred antibiotic compared to ampicillin because it is more stable.

22. Inoculate the RNAi clones and controls from the rectangular agar plates into LB liquid medium in the 96 deep well plates using a 96-pin replicator. Sterilize the replicator as in step 4 and then place the replicator perpendicular to the agar plate while applying gentle pressure. Raise the replicator straight up from the rectangular plate (to avoid cross contamination) and inoculate the block plate by swirling the replicator gently.

23. Take the replicator carefully out of the deep well plate to avoid cross-contamination between wells.

24. Seal the deep well blocks with Breathe-Easy sealing films and incubate at 37°C overnight in a shaking incubator for 12–16 hrs at 300 rpm.

25. For 2 ml deep well blocks filled with 1.2 ml culture, use a multichannel pipette to add 19 µl of 250 mM IPTG to each well to induce the RNAi cultures. Incubate 3–4 hrs at 37°C in a shaking incubator at 950 rpm (final IPTG concentration is 4 mM). Care should be taken not to cross contaminate the wells. Use one pipette tip per well.

26. Pellet the bacteria by centrifuging the deep well blocks for 15 min at 3000 rpm and discarding the supernatant immediately by rapidly inverting the block upside down into a container. Alternatively, the supernatant can be removed with a 96 well-formatted automated plate washer. You should sterilize the plate washer for each block using 10% bleach followed by sterile water, 90% ethanol, and sterile water.

Note: It is advisable but not necessary to use an automated plate washer to remove the supernatants, which helps in minimizing the chance of cross-contamination between the wells.

27. Tap the plate upside down on a paper towel to remove as much of the media as possible to avoid further growth of bacteria. Use a multi-channel pipette to add 120 µl or 50 µl (1/10th volume) of S-basal buffer containing IPTG and antibiotics to each

well of a 2 ml or a 1 ml deep well block, respectively. The recipe for S-basal buffer containing IPTG and antibiotics is as follows:

For each deep well plate (2 ml) with 1.2 ml wells, prepare 15 ml of S-basal buffer with IPTG and antibiotics:

IPTG (1M)	180 μ l (12 mM)
Carbenicillin (100 mg/ml)	90 μ l (600 μ g/ml)
Nystatin (62.5 KU)	45 μ l (187.5 U)
S-basal complete	14.73 ml

NOTE: The concentration of IPTG and antibiotics are at 3x in this buffer, as it will be diluted by a factor of 3 in the assay plates by adding worms.

- a. For a small number of plates, a multi-channel pipette can be used to resuspend the pellets. For a large number of plates, using a multi-channel pipette is very laborious. The use of a microplate dispensing robot is recommended to add the S-basal buffer to the blocks and the pellets can be resuspended by shaking the deep well blocks sealed with a Breathe-Easy membrane or aluminum foil at 300 rpm for 5 min.
- b. Nystatin is added to inhibit fungal contamination.
- c. The concentration of bacteria, IPTG and the number of worms given here is just a starting point; concentrations can be optimized according to the assay.

Preparation of *C. elegans* at larval stage L1

28. Collect embryos using the hypochlorite bleach protocol from 10 cm NGM plates (Support protocol 1). Incubate the embryos in M9 media in a 15 ml falcon tube in a tube roller at 15°C or room temperature for 1–2 days respectively. This allows the embryos to hatch and arrest the worms at the L1 stage.

- a. Make sure the falcon tube contains only 5–7 ml of buffer with embryos as more volume results in less aeration.
- b. Make sure the rotor is always on. If the rotor stops for an extended period, the embryos will die without aeration.

29. After hatching, filter the L1 worms using a cell strainer (see Materials). This step will get rid of all the debris from the embryo preparation, including debris from adult worms.

30. Dilute the L1 worms in S-basal complete buffer to approximately 25 worms per 20 μ l.

Adding L1 *C. elegans* and bacteria to screening plates

31. Label an appropriate number of 96 half-well black plates using a silver (so that it can be read on the black plate) Sharpie marker.

- a. Choose the type of plate that is most appropriate for your particular screen. If you are only going to screen manually and do not require automated

imaging, you can use 96 half-well clear plates. These plates are inexpensive compared to black well plates.

- b.** If you are planning to use fluorescence labeling or further phenotypic analysis using an imaging program such as Image J or CellProfiler, it is advisable to use black well plates because the reflection of the worms in clear plates will hinder further analysis.

32. Transfer diluted worms (25 worms per 20 μ l) into a sterile 50 ml tube or flask and add 20 μ l of the diluted worms to each well of the screening plates.

- a.** You can increase or decrease the worm number per well according to the screening assay. It is best not to overcrowd the well as overcrowding can affect the way worms respond to their environment. The maximum number of worms per half well is in the range 25–50 for assays that do not involve the production of progeny.
- b.** Once dispensed, use a light microscope to check several wells to make sure that there is a uniform number of worms per well ± 5 . It is difficult to see L1 worms once you add bacteria to the well.

33. Add 10 μ l of the bacterial RNAi cultures in S-basal with IPTG per well and seal the screening plate with a Breathe-Easy film. Pulse spin the plates in a centrifuge to mix the contents of the wells and to ensure that the entire assay mix is at the bottom of the wells.

Note: The amount of the RNAi food and volume of the assay can be adjusted according to the number of worms in the well and/or the duration of the assay. For example, for lifespan assays that may last a couple of weeks or more, 25 L1 worms can be added in 40 μ l with 20 μ l of bacterial culture because the food has to last a relatively long time and because some of the assay mix may be lost to evaporation. Additional RNAi bacterial food can be added during the course of the assay if necessary. In general, it is important to start the assay with enough bacteria to prevent the worms from consuming them all and becoming starved, which might influence the phenotype.

34. To avoid evaporation during the course of the assay, prepare a humid chamber by lining a sealable storage box with wet paper towels. Place the plate lid on the paper towel and then place the assay plate on top of the lid to keep the bottom of the plate dry. All the plates should be stacked at the same level for better aeration. Cover the box tightly and incubate at a temperature appropriate for the particular assay without shaking.

NOTE: It is important to plan the experiment timing, so that your L1 worms are ready to plate on the day of the bacterial IPTG induction, which mostly depends on the worm strain used in the experiment. This protocol does not require a shaking incubator. Make sure the box is tightly covered to prevent any potential dehydration of the sample. By placing the plate on top of the cover, the bottom of the plate remains dry and does not have to be dried off before imaging, which can scratch the plate.

35. Phenotypes can typically be scored 4–5 days after wild-type control worms reach adulthood as described below in Basic Protocol 2.

BASIC PROTOCOL 2

Phenotypic scoring of RNAi assays

There are several ways to score *C. elegans* phenotypic assays. The phenotype can be scored using a COPAS Biosort robot (Conery, Larkins-Ford, Ausubel, & Kirienko, 2014; Moy et al., 2009) because the robot can measure length, optical density and fluorescence of a single worm (Squiban, Belougne, Ewbank, & Zugasti, 2012; Zugasti et al., 2016). Alternatively, an assay can be scored manually on a light microscope or by using an image-based microscopy platform. Using automated image-based microscopy platforms has several advantages: they are relatively fast, images can be stored for reanalysis, the assay can typically be quantitated, and multiple readouts can be obtained from a single imaging run. For example, even in a fluorescence-based assay, phenotypes such as sterility and developmental stage will also be captured. Image-based screening also allows the same readout for different treatments carried out in parallel, such as RNAi and compound libraries. The following protocol focuses on image-acquisition and analysis methods available for high-throughput screening.

Image acquisition—One of the major advantages of the liquid RNAi screening system described here compared to conventional RNAi screens on agar plates, is that liquid assays generate much higher quality image data which greatly facilitates automated data analysis. In the case of RNAi screens on agar, images of worms typically have high background levels as well as focal plane changes from well to well due to uneven media levels.

There are several automated microscopes designed for cell-based screens that can also image worms in 96 or 384 well plates. In our laboratory, we use a Discovery-1 automated microscope or an Image Xpress Micro automated microscope (Molecular Devices) to capture both transmitted light and fluorescence (GFP and/or RFP) using a 2X objective. A benefit of using 96-half well plates, apart from low evaporation rates, is that more than 80% of the well can be captured in a single image with a 2X objective. This way you can obtain one image per well, whereas normal 96 well plates require multiple images per well and stitching to obtain a full well image, thereby making analysis tedious. For 384-well plates, an entire well can be captured in a single image.

Image analysis—It is advisable to initiate image analysis while the screen is still running to make sure all the positive and negative controls are working as expected. Determining the distribution and number of hits per plate is one of the most effective methods to detect technical problems. For example, some problems such as extreme edge effects, library plates with unusually high hit rates, and variations in controls wells between experiments are usually readily apparent. For *C. elegans* image analysis, a “worm toolbox” has been developed for image-based high-throughput screening in *C. elegans* using the open source cell image analysis software CellProfiler (Wahlby et al., 2012). The worm toolbox can be used to measure phenotypes that are based on shape, biomarker intensity, and organismal staining patterns. A detailed tutorial on creating and customizing an optimized pipeline for your HTS assay can be found at www.Cellprofiler.org.

Hit Identification—Hits can be chosen qualitatively by arbitrarily selecting the top hits manually or by using a statistical method with a defined quantitative cutoff. Some commonly used statistical methods are the Z-score, robust Z-score, strictly standardized mean difference (SSMD), robust strictly standardized mean difference, and *t* statistics (Birmingham et al., 2009). A Z-score, which is distinct from the Z' factor statistic described above, is assigned to each sample well in an assay and is defined as the number of standard deviations that an individual assay in a particular well differs from the mean value of each 96 or 384 well assay plate. Z scores are the most widely used metric for identifying hits in an RNAi screen, but it is worth noting that the Z-score is sensitive to outliers (which are likely to be hits) because outliers will disproportionately affect the mean value of the assay plate. To compensate for the effects of outliers, the “robust Z-score” metric can be used instead, which utilizes outlier insensitive median and median absolute deviation (MAD) instead of the mean values. The strictly standardized mean difference (SSMD) metric, which was developed for use with RNAi screening, is defined as the mean divided by the standard deviation of the difference between two populations. This method can be used both to evaluate the robustness of a screening assay and to identify hits. The advantage of the SSMD metric is that it allows control of both the false positive and false negative rates and doesn't depend on sample size. The disadvantage of this method is that the software necessary to calculate SSMD values is not readily available and is not intuitive. For some screening assays such as those comparing two conditions such as plus or minus compound, the significance of hits can be determined using a standard t-test. A t-test can be used only if the assay is done in triplicate and if the data are normally distributed. The advantages and disadvantages of different statistical methods are summarized in Tables 1 and 2 (Birmingham et al., 2009; Bray & Carpenter, 2004; X. D. Zhang, 2011).

Selecting the correct statistic for hit identification is critical, as misuse of a statistical method will result in too many false positives or false negatives. For example, the Z-score and SSMD methods can be used for RNAi screens that do not include replicates whereas *t* statistics can only be used for screens with replicates. Another consideration in screens without replicates is whether to calculate Z-scores or SSMD metrics on a plate-to-plate basis or for all the plates together. In general, considering that feeding RNAi is an inherently variable process that depends on environmental factors and typically exhibits plate-to-plate variation, it is preferable to calculate Z-scores and SSMD statistics for each plate. Because most RNAi clones should behave similarly to the negative control, it is typically acceptable to calculate Z-scores using the mean value of all wells in a plate as the negative control. On the other hand, if a subset library is enriched for specific categories of genes such as kinases that might not follow a normal distribution, it may be preferable to include a relatively large number of negative and positive controls (usually 4–8 wells for each control) on each assay plate and use control-based statistics.

In conclusion, although there are a variety of statistical methods to analyze RNAi screen data, a Z-score metric will often identify statistically significant hits. It is worth noting, however, that most statistical methods described here were developed for screening chemical libraries with the goal of minimizing false positives. In an RNAi screen, these statistical methods may not identify weak hits or hits that exhibit a significant amount of variance even

though they are biologically meaningful. It is important, therefore, to have a clear understanding of the assumptions and limitations underlying a statistical method and apply threshold levels that are suitable for a particular screen. Finally, it is important to emphasize that a successful screening outcome depends on a robust experimental design and appropriate assay development before the primary screen is carried out as well as rigorous secondary screening to validate the primary screening hits.

SUPPORT PROTOCOL 1

Worm egg preparation by Hypochlorite bleach treatment

Hypochlorite bleach kills *C. elegans* adults and larvae, whereas the embryos are resistant to bleach treatment. Thus, the embryos can be collected after treating gravid worms with bleach and hatched in M9 with gentle rocking for 1–2 days to obtain synchronized L1 worms.

Materials

10 cm plates with gravid animals

M9 buffer

Buffered bleach (freshly made; recipe below)

15 ml falcon tube

1. Add 15 ml M9 to a 10 cm plate of gravid worms and wash the worms from the plate into a 15 ml falcon tube.
2. Allow the worms to settle in the 15 ml falcon tube for at least 5 min.
3. Prepare a buffered bleach solution (see recipe).
4. Aspirate the supernatant from the tube that contains the settled worms, leaving ~1 ml, so that you do not disturb the worms at the bottom of the tube.
5. Add an additional 5 ml of M9 to the tube with the worms.
6. Add 6 ml of buffered bleach solution to make the total volume ~12 ml.
7. Gently rock the tube for 4 min until the worms break open.
8. Spin the tube for 30 s at 3000 rpm to pellet the eggs and discard the supernatant.
9. Wash the eggs by suspending them in fresh M9 (10 ml) and spin them for 30 s at 3000 rpm.
10. Wash the pellets 3 times with M9.
11. After the last wash, add 5 ml of M9 to the pellet, resuspend and rotate the tube at RT or 15°C according to the strain for 1–2 days to hatch the embryos to L1s.

REAGENTS AND SOLUTIONS

Buffered Bleach

15 ml M9 buffer
1.3 ml NaOH, 50% (w/w)
8.7 ml Bleach (5–6% sodium hypochlorite)
(Always prepare fresh)-25 ml

E. coli OP50 food

Using sterile technique, inoculate 1L of LB broth with a single *E. coli* OP50 colony from an LB plate. Incubate the culture at 37°C, overnight in a shaking incubator. The next day, pellet the culture and discard the supernatant. Resuspend the pellet in 20 ml of M9 buffer to make a 50X concentrated culture. Aliquot 1 ml of 50X culture per 10 cm NGM plate and dry the plate in a sterile hood keeping the plate open until the surface is dry. The plates can be stored in a sealed container at 4°C for several weeks.

M9 buffer

3.0 g monobasic potassium phosphate (KH_2PO_4)
6.0 g dibasic sodium phosphate (Na_2HPO_4)
5.0 g sodium chloride (NaCl)
1 ml 1 M magnesium sulfate (MgSO_4)
Adjust volume to 1 liter with H_2O
Sterilize by autoclaving
Store up to 2 months at 20°C

NGM plates

3.0 g sodium chloride
17 g agar
2.5 g Bacto-peptone
975 ml H_2O

Add a magnetic stir bar and sterilize by autoclaving. Cool to ~55°C, place on a magnetic stirrer, and add 25 ml of 1 M potassium phosphate buffer, pH 6.0 (see recipe), 1 ml of 1 M calcium chloride (CaCl_2), 1 ml of 5 mg/ml cholesterol in 100% ethanol, and 1 ml of 1 M MgSO_4 . Dispense into Petri dishes (~20 ml per 10-cm plate). Store the plates in a sealed container for up to 1 month at 4°C.

Potassium phosphate buffer, 1 M, pH 6.0

136.1 g monobasic potassium phosphate (KH_2PO_4) per liter of H_2O . Start with 800 ml H_2O and adjust to pH 6.0 with solid KOH (approx. 15 g). Add H_2O to a final volume of 1. Store up to 2 months at 20°C .

S-basal Complete buffer

5.85 g NaCl

1 g K_2HPO_4

6 g KH_2PO_4

Adjust volume to 1 liter with H_2O

Sterilize by autoclaving

Store up to 2 months at 20°C

Before using the buffer add

1 ml cholesterol (5 mg/ml in ethanol)

1 ml of 1M CaCl_2

1 ml of 1 M MgSO_4

COMMENTARY**Background Information**

RNA interference is a biological phenomenon in which double-stranded RNA (dsRNA) facilitates the effective knockdown of a target gene through degradation of the corresponding endogenous messenger RNA. In the 1990s, apparent gene-silencing phenomena were observed independently by several laboratories. Working with petunias, Napoli and Jorgensen were the first to report a gene silencing mechanism in 1990 (Napoli, Lemieux, & Jorgensen, 1990). In 1992, Romano and Macino reported a similar phenomenon in *Neurospora crassa* and termed the process “quelling” (Romano & Macino, 1992). Guo and Kemphues were the first to document gene silencing in *C. elegans* (Guo & Kemphues, 1995). In 1998, Fire and Mello discovered that double-stranded RNA was the source of sequence-specific inhibition of protein expression and they termed it “RNA interference” (Fire et al., 1998). Following this, RNAi was used as a powerful reverse genetic tool in *C. elegans* due to its rapid and efficient inactivation of genes. While the studies in *C. elegans* were encouraging, the introduction of long dsRNA in mammalian cells resulted in nonspecific inhibition suggesting that the RNAi-mediated gene silencing might be limited to lower organisms. However, further studies in plants and invertebrates showed that the actual molecule that induces RNAi is a small dsRNA, which is internally processed by an enzyme called Dicer. Dicer cleaved products are called siRNAs. The discovery that siRNAs could directly trigger RNAi in mammalian cells revolutionized the way gene function is studied in mammalian systems and is the basis for promising RNAi based therapeutics for treating human diseases (Sen & Blau, 2006; Wilson & Doudna, 2013).

Because RNAi in *C. elegans* is systemic, the site of RNAi injection is not critical as the RNAi effect can cross cell boundaries and spread throughout the worm (Fire et al., 1998). In feeding RNAi, dsRNA is absorbed through the gut and distributes to somatic tissues and the germline (Timmons & Fire, 1998). RNAi feeding has several advantages over two other methods, injection of and soaking in dsRNA. First, feeding RNAi is inexpensive and less laborious. Second, it can be adapted for high-throughput genetic screens. Finally, once a bacterial strain expressing a specific dsRNA is generated, it can be replicated and stored and used repeatedly for RNAi experiments. Genome-wide RNAi screening has been greatly facilitated by the Ahringer and Vidal laboratories, which have generated genome-wide RNAi feeding libraries. Kamath and Ahringer generated a library of 16,757 RNAi bacterial feeding clones (Kamath & Ahringer, 2003), which was subsequently expanded to 19,763 clones. The Vidal lab independently generated 11,560 ORFeome RNAi library clones (Ceron et al., 2007), targeting 10,499 individual genes. Together the libraries target ~86% of the 20,466 predicted protein-coding genes in *C. elegans*.

The high throughput RNAi protocol described in this Unit takes advantage of the facts that L1 larval stage *C. elegans* can be readily pipetted into the wells of assay plates and that the *C. elegans* animals mature to the L4 or adult stage feeding on a particular RNAi clone, giving plenty of time for gene silencing to take place. However, because feeding particular RNAi clones to L1 worms results in an embryonic or developmentally delayed or lethal phenotype, some RNAi assays may require worms to be fed on RNAi clones only after they reach L4 or adulthood. This is relevant for genes that not only play key roles in development but also have distinct roles in adult animals. Unfortunately, L4 or young adult worms cannot be transferred to screening plates by pipetting due to their relatively large size, which causes significant variation in the number of worms delivered to each well. To distribute L4 or adult *C. elegans* to assay plates, it is recommended to use a COPAS biosort robot (Union Biometrica) (Conery et al., 2014; Moy et al., 2009; O'Rourke, Conery, & Moy, 2009). The COPAS Biosort instrument is a modified flow cytometer designed to sort small multicellular animals such as *C. elegans* or zebrafish embryos by measuring their size, optical density, or fluorescence intensity. The user can set these parameters for sorting and dispensing worms of interest into microtitre plates at a rate of 100 worms per second, resulting in an equal distribution of worms per well. Using this robot considerably reduces the time required for an experiment, increases accuracy, and allows experiments that are only possible with robotic sorting (Hernando-Rodriguez et al., 2018; Pulak, 2006). Even though it might not be as accurate as the Biosort robot, a microplate dispenser or multi-channel pipette can be used to dispense L1 worms into microtitre plates provided that the worm population is tightly synchronized.

One important factor that need to be kept in mind in large-scale RNAi screening is that genes can have different susceptibilities to RNAi silencing. Genes encoding proteins with long half-lives, for example, are difficult to knock-down completely (Montgomery, Xu, & Fire, 1998). Although RNAi can result in an incomplete knockdown and a hypomorphic phenotype, this can be an advantage when studying genes that cause lethality or major developmental defects when completely eliminated. Another issue is that different tissues have varying sensitivities to RNAi. Neurons, the pharynx, the vulva, and sperm are often resistant to RNAi (Tavernarakis, Wang, Dorovkov, Ryazanov, & Driscoll, 2000; Timmons et

al., 2001). To induce silencing in normally RNAi-resistant tissues, *C. elegans* strains can be used that exhibit an enhanced RNAi-susceptibility phenotype in these cells due to mutations in the *rrf-3* (Simmer et al., 2002), *eri-1* (Kennedy, Wang, & Ruvkun, 2004) or *lin-15* (Lehner et al., 2006) genes.

A problem with using RNAi in any organism is potential off-target effects. As RNA interference is based on sequence recognition, there is always the possibility that a particular RNAi construct might also silence another gene with a similar sequence. Although in most cases an RNAi-mediated phenotype in *C. elegans* is primarily caused by knocking down the expression of the target gene, because of potential off-target effects, it is important to confirm that the observed phenotype is strictly correlated to the target gene. This can be accomplished in many cases by testing a variety of genetic mutations in a targeted gene, although this approach can be difficult for essential genes. A large collection of RNAi phenotypes is available on Wormbase (www.wormbase.org). These datasets can also be used to verify the phenotype obtained in the screen. Importantly, a negative RNAi result is not very informative, primarily because of the issues of RNAi susceptibility described above. In spite of all its limitations, RNAi is a powerful tool for reverse genetic analysis, not only in *C. elegans*, but also in higher organisms due to the rapidity with which genes involved in a given process can be identified.

Critical Parameters

1. For efficient RNAi, always use bacterial clones grown directly from plates streaked out from glycerol stocks and avoid sub-culturing bacteria from plate to plate. Use the bacterial plate containing replicated RNAi clones for screening within 10 days.
2. Make two frozen RNAi library copies, a working copy and one as a main stock. Only use the working copy for repeated freezing and thawing. If you observe a reduction in RNAi efficiency, make another working copy from the frozen stock.
3. To ensure the data resulting from the screen are of high quality, it is important to optimize the assay using the Z'-factor metric or another suitable statistical method before you start the screen. Once the screen is completed, use the Z-score metric to identify statistically significant hits.
4. Because knock down of gene expression by feeding RNAi is inherently variable, it is imperative to develop rigorous secondary screens to validate the primary hits.
5. It is very important to maintain a humid chamber for plate incubation. Failing so might cause uneven evaporation in the plate lead to edge effects and unreliable results.
6. An Important observation in our lab was that growing worms at 15°C rather than at higher temperatures soon after adding the RNAi clones for the first ~18–24 hours yielded healthier worms with better RNAi efficiency.

Troubleshooting

Table 3 describes some causes of and solutions for some of the common problems encountered in RNAi screening.

Time Considerations

A typical RNAi screen will take 3 days to set up each set of assays, which includes bacterial growth, induction, and adding worms and bacteria to the assay plate. There is an additional 3 – 4 days for worm growth and imaging. The amount of time to screen the whole genome will vary depending on the assay and available resources such as incubators. A major advantage of the protocol described here is that it does not require a shaking incubator for worm growth. In our laboratory, roughly 40 96-well RNAi plates can be screened per day (This number is mainly because of the capacity of the bacterial growth incubator). If a sufficient number of incubators are available, it is easy for 3 people to process 100 plates per day, thus allowing a full-genome screening in less than a month.

The time taken for analysis of the screen data depends on the readouts and analysis methods employed. Sometimes data analysis takes longer than the wet-lab screening itself. Therefore, screeners should plan the timeline keeping analysis in mind.

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INTERNET RESOURCES

- <http://www.wormbase.org> WormBase is an international consortium of biologists and computer scientists dedicated to providing the research community with accurate, current, accessible information concerning the genetics, genomics and biology of *C. elegans* and related nematodes.
- <http://www.wormatlas.org> Worm atlas is an online database featuring behavioral and structural anatomy of *C. elegans*.
- <http://nematode.lab.nig.ac.jp/NEXTDB> is an expression pattern database through EST analysis and in situ hybridizations using expressed sequence tags.
- <http://wormfdb.dfc.harvard.edu> Worm ORFeome DataBase (WorfeDB) is a searchable database for *C. elegans* ORF clones.
- <http://www.cbs.umn.edu/research/resources/cgc> The *Caenorhabditis* Genetics Center (CGC), funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440), is the central repository for *C. elegans* strains and distributes strains at a minimal cost to researchers.
- <http://www.lifesciences.sourcebioscience.com/clone-products/non-mammalian/c-elegans/> Source Bioscience sells ORFeome and RNAi reagents, including the genome-wide RNAi feeding library and sub-libraries.
- <http://dharmacon.gelifesciences.com/gene-expression-cdnas-orfs/non-mammalian-cdnas-and-orfs/c-elegans> GE Dharmacon provides ORFeome reagents, including the ORFeome RNAi feeding collection.
- www.cellprofiler.com CellProfiler is open source cell image analysis software developed in Carpenter Lab at Broad Institute of Harvard and MIT.

Figure 1.
Schematic of RNAi screening assay protocol

Table 1:

Summary and comparison of statistical methods for assay quality metrics

Assay Quality Metrics	Definition and Interpretation	Advantages	Disadvantages
Z'-Factor	$Z' = 1 - (3\sigma_p + 3\sigma_n) / \mu_p - \mu_n $ Interpretation: $Z' = 1$ - An ideal assay $1 > Z' > 0.5$ - excellent assay $0.5 > Z' > 0$ - Marginal assay $Z' < 0$ - Unacceptable	Ease of calculation. Accounts for signal dynamic range and data variation. Often found in both commercial and open-source software packages.	Does not scale linearly with signal strength Assumes the positive and negative control follows a Gaussian distribution. Easily leads to an inaccurate measure of control separation distribution in the presence of an outlier.
Strictly standardized mean difference (SSMD, denoted as β)	$\beta = (\mu_n - \mu_p) / (\sigma_n^2 + \sigma_p^2)$ Interpretation: Acceptable screening values for SSMD depend on the strength of the positive controls used.	Ease of calculation. Accounts for signal dynamic range and data variation. Lack of dependence on sample size. Linked to rigorous probability interpretation.	Not available as part of most analysis software packages (Cell Profiler is an exception). Not intuitive. The thresholds are based on a subjective classification of control strength.
Receiver operating characteristic curve (ROC)	plot sensitivity vs. $(1 - \text{specificity})$ where: sensitivity = $[\# \text{ true-(p)}] / [\# \text{ true-(p)} + \# \text{ false-(n)}]$ specificity = $[\# \text{ true-(n)}] / [\# \text{ true-(n)} + \# \text{ false-(p)}]$ Interpretation: ROC = 1 - An ideal assay ROC = 0.5 - Poor assay ROC < 0.5 - worse than random chance	Provides visual quality control allows investigation of the effect of changing hit-threshold can be quantitated as the area under the ROC curve	Ideally, needs many replicates of positive and negative controls. Time-consuming to review and interpret curves. Not available as part of most analysis software packages.

Where μ indicates mean and σ indicates the standard deviation of both positive (p) and negative (n) controls. (Birmingham et al., 2009; Bray & Carpenter, 2004)

Table 2:

Formulas for hit identification metrics in primary screens

Hit Identification Metrics	Formula
Z-score	$X_i - \mu_n / \sigma_n$
Robust Z-score	$X_i - M_n / MAD_n$
SSMD based on method-of-moment (MM) method	$X_i - \mu_n / \sqrt{2} \sigma_n$
Robust SSMD (MM) method	$X_i - M_n / \sqrt{2} MAD_n$
SSMD based on uniformly minimal variance unbiased estimate (UMVUE)	$X_i - \mu_n / \sqrt{2/k(N_n - 1)} \sigma_n$
Robust SSMD (UMVUE)	$X_i - M_n / \sqrt{2/k(N_n - 1)} MAD_n$

Where X_i indicates measured activity value of the i th well of RNAi; N_n indicates the number of negative reference wells in a plate; k is a preset constant often set to be 2 or 3; μ_n , M_n , MAD_n and σ_n indicates mean, median, median of absolute deviation and standard deviation of measured values for the negative control, respectively.

Table 3:

Troubleshooting.

Problem	Possible Causes	Solutions
No phenotype	<ul style="list-style-type: none">• RNAi didn't work• RNAi food contaminated	<ul style="list-style-type: none">• Prepare fresh media and IPTG• Use fresh bacterial culture for induction• Test the assay with positive controls• Make fresh antibiotics and store them at an appropriate temperature• Use carbenicillin instead of ampicillin
Edge Effect	<ul style="list-style-type: none">• The wells in edge dry faster than the wells in the center	<ul style="list-style-type: none">• Incubate the plate in a well-humidified chamber or box• If using 96 well plates, shift to 96 half-area well plates for the screen
The image has lots of debris and background	<ul style="list-style-type: none">• Worm debris, salts in the buffer, and bacteria can increase background	<ul style="list-style-type: none">• Washing with M9 or S- basal will reduce the debris and will give a clean image for analysis
Images with lots of wiggling worms	<ul style="list-style-type: none">• Worms tend to swim more actively under the microscope when illuminated with blue light	<ul style="list-style-type: none">• Add levamisole or keep the worms on ice for few minutes before imaging.