Protocol

High-Throughput RNAi Screening for Germline Apoptosis Genes in *Caenorhabditis elegans*

Benjamin Lant[1] and W. Brent Derry[2,3]

[1]*Developmental and Stem Cell Biology Program, The Hospital for Sick Children, Toronto, Ontario M5G 1L7, Canada;* [2]*Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S 1A1, Canada*

Among the greatest tools that *Caenorhabditis elegans* can provide researchers are the capabilities to perform high-throughput, genome-wide screens. Using bacterial RNAi libraries, which cover the majority (>85%) of the worm genome, genes can be rapidly and systematically evaluated for apoptosis phenotypes in the germline. Screens can be designed to directly assess the levels of apoptotic corpses under normal physiological conditions using transgenic strains expressing fluorescent reporters that mark apoptotic bodies. Vital dyes that are selectively retained in apoptotic cells, such as acridine orange (AO), can also be used to screen for genes that regulate germline apoptosis. Using these reagents, screens can be performed in wild-type worms or mutant backgrounds that suppress or enhance apoptosis phenotypes. This protocol describes methods for designing and carrying out high-throughput or targeted RNAi screens for germline apoptosis regulators.

Cold Spring Harbor Protocols
www.cshprotocols.org
CSH PROTOCOLS

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Acridine orange (AO) (10-mg/mL stock solution) (Molecular Probes A3568) (optional)

Immediately before use, dilute 7.5 µL of AO stock solution into 1 mL of M9 buffer for worms <R>.

C. elegans strain of interest (e.g., fluorescent reporter or mutant strain)

Before screening, synchronize tens of thousands of worms at the L1 stage using the hypochlorite method (see WormBook *[Stiernagle]). Worms should be prepared no more than 3 d before use (Step 8); see Discussion.*

Recommended fluorescent strains are described in Protocol: ***Fluorescent Visualization of Germline Apoptosis in Living*** **Caenorhabditis elegans** *(Lant and Derry 2014a).*

If you are screening a mutant strain, you should simultaneously run controls (typically of the N2 strain) to assess the individual effects of gene ablation on apoptosis. If your purpose is to identify genes that restore apoptosis to a resistant strain, this may not be necessary.

Isopropylthio-β-galactoside (IPTG) (0.1 M)

IPTG induces expression of the T7 RNA polymerase in the RNAi bacteria (Timmons et al. 2001).

Luria Bertani (LB) + amp and tet (liquid) <R>

[3]Correspondence: brent.derry@sickkids.ca

© 2014 Cold Spring Harbor Laboratory Press

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080234

M9 buffer for worms (for use with AO staining) <R>

Nematode growth medium (NGM; liquid) <R>

The number of genes you plan to screen will determine how best to culture the bacteria. The method provided here assumes that a large number of genes (>1000) will be screened; hence, liquid culture in 96-well plates is appropriate. Alternatively, if you are screening <500 genes, solid medium in 24-well plates can be substituted (but can be costly in terms of time and money). To screen a small number of genes (<100), see Protocol: ***Fluorescent Visualization of Germline Apoptosis in Living*** **Caenorhabditis elegans** *(Lant and Derry 2014a).*

Plates containing solid NGM (see Step 13) <R>

RNAi clones from the *C. elegans* RNAi feeding library (Source BioScience LifeSciences)

Tetramisole (20 mM)

Equipment

Automated liquid media dispenser

Centrifuge

Flask (large)

Fluorescence dissecting microscope

Incubator at 37°C

Opaque bag or tin foil (for optional AO staining)

Orbital shaker (lateral only) at 37°C and room temperature

P200 tips (sterile)

Parafilm

Plastic bags (sealable)

Plates (conical- and flat-bottom), 96-well

Replicator, 96-pin

We recommend the disposable 96 LONG-PIN REPAD from Singer Instruments, which can be cleaned with 70% ethanol and reused many times.

Slides

Cold Spring Harbor Protocols
www.cshprotocols.org

METHOD

Careful handling of liquid cultures must be used at all times. Although it seems obvious, the bacteria in plates can very easily be spilled and enter surrounding wells. Take special care in keeping the plates horizontal, especially in transition to and from the orbital shaker.

1. Using an automated liquid medium dispenser, aliquot ~150 µL of LB + amp and tet into each well of a conical-bottom 96-well plate.
2. Thaw the RNAi library glycerol stock for 5–10 min at room temperature and inoculate the liquid cultures as follows.
   - If you are targeting a specific class of genes (i.e., the "kinome"), use individual sterile P200 tips to take small samples of bacteria from the target wells. Transfer each tip containing bacteria directly into one well of the 96-well plate containing medium. Incubate at room temperature for 5 min and discard the tip.
   - If you are conducting a full genome screen, use a 96-pin replicator to inoculate the liquid cultures.

     The 96-pin replicator will create a pattern of even and odd numbers and letters from the 384-well library plate. A → C → E …; 1 → 3 → 5 …etc. Hence, rigorous labeling and documentation will be needed, lest you end up in a puddle of misplaced genes!
3. Place the lid on the plate and seal the wells with Parafilm to prevent evaporation. Wrap the plate in a wet paper towel, place in a sealed plastic bag and incubate on an orbital shaker at 37°C.
4. After 24 h of incubation, confirm bacterial growth by observing cloudiness of the medium. Add 6 µL of IPTG (0.1 M) per well. Rewrap the plate and place it back in the orbital shaker for 1 h at 37°C.

 Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080234

5. Following incubation, centrifuge the plates at 3500 rpm to pellet the bacteria. Shake the plates sharply into a large flask to discard the supernatant.

   The pellet should remain in the wells.

6. Add 150 µL of liquid NGM to each well and resuspend the bacteria.

   To delay the screen at this step, plates can be stored at 4°C for up to 7 d.

   Depending on your experimental design, a resuspension volume of 150 µL should allow for creation of both an experimental and a control plate, and provide extra liquid culture for subsequent confirmations on solid plates.

7. Transfer 50 µL of each liquid RNAi culture into individual wells of a flat-bottom 96-well plate.

8. Determine the number of L1 worms per microliter in your sample by placing a small volume (2, 5, or 10 µL) of worms on a slide in tetramisole. Count the worms using a dissecting microscope. Repeat at least five times to obtain an accurate estimate. Dilute the worms to a concentration of 1 worm/µL.

9. Pipette 10 µL of worms into each well of the 96-well plate from Step 7. Seal the plate as above and place on an orbital shaker at room temperature. Grow worms to the young adult stage (~2–3 d).

   The ideal stage for observation of the worms is the young adult stage; exact timing will depend on the growth rate of the strain. Thus, we highly recommend determining the rate of growth of the strain of interest before screening. If you are unsure on the timing of larval stages for your particular worm strains, it may be prudent to quantify growth earlier in the incubation period. It is important to note that as worms age, their intestines will increasingly obstruct the germline, so avoid trying to assess apoptosis >48 h after the young adult stage. Should you wish to determine the effects of stresses such as DNA damage on apoptosis it is important to treat worms with stress 24 h after the L4 stage, then assess apoptosis 24 h later (i.e., 48 h after L4).

   See Troubleshooting.

10. (Optional) Stain the worms with AO.

    i. Add 20 µL of freshly diluted AO solution (75 µg/mL) to each well of the 96-well plate containing worms. Wrap the plate in an opaque bag or tin foil to prevent light from breaking down the dye. Place the plate on an orbital shaker at room temperature for 1 h.

    ii. After 1 h, unpackage the plates and place them at 4°C for 10 min.

       The worms will stop moving at the colder temperature and descend to the bottoms of the wells.

    iii. Carefully remove the liquid from the wells, leaving the worms intact at the bottom of each well. Add 50 µL of M9 buffer to the worms. Place the plates at 4°C for 10 min, remove the liquid, and repeat the M9 wash.

    iv. Repackage the plates (including light resistant covering) and place them on the orbital shaker at room temperature for 3 h to destain. Observe the worms under the fluorescence channel of a dissecting microscope to be sure background has been reduced appropriately.

       The critical factor in apoptosis detection by vital dyes is the stain:destain ratio; see Protocol: ***Fluorescent Visualization of Germline Apoptosis in Living*** **Caenorhabditis elegans** *(Lant and Derry 2014a). A 1:3 ratio is suggested here, but may not be exact for a given experiment or strain. It is important to check staining levels at this step to ascertain whether more destaining time is necessary before proceeding.*

       See Troubleshooting.

11. Before analysis on the dissecting microscope, add 5 µL of tetramisole to each well.

    Addition of tetramisole will straighten and immobilize the worms, thereby increasing visibility of the germline.

12. Under the dissecting microscope, locate and focus on the worms in the bright field then switch to the green fluorescent protein (GFP) channel to observe the fluorescent corpses. Use the 10× magnification initially to localize the fluorescence and then move to a higher magnification (20×–50×) to look specifically at the corpses in the germline. Look at germlines of multiple worms to avoid recording anomalies.

    The average number of apoptotic corpses is generally equivalent in the anterior and posterior germline regions of the same animals, but we find the posterior gonad easier to visualize because it is less obstructed by the intestine.

    See Troubleshooting.

Cold Spring Harbor Protocols
www.cshprotocols.org
CSH PROTOCOLS

13. Following the screen, or concurrent to running it, confirm any positive "hits" (i.e., candidate apoptosis genes) by reculturing the candidate RNAi strain (or using leftover liquid stock from Step 6, if this is within a week of initial growth) as outlined in Protocol: **Induction of Germline Apoptosis in *Caenorhabditis elegans*** (Lant and Derry 2014b).

    If you have a large number of candidates to test, it may be more convenient to rescreen on 24-well plates containing solid NGM rather than on individual plates. It may also be necessary to cultivate the worms on the RNAi feeding strains for a second generation if the first generation produces inconsistent results.

TROUBLESHOOTING

Problem (Step 9): Contamination is observed after incubation.

Solution: Contamination can be seen as a distinct discoloration in the liquid medium (often brown/yellow, compared to a much lighter cloudy control). In these cases, compare the coloration with surrounding wells to confirm. Often confusion can occur if there are fewer worms in certain wells (because the worms grow slower, or the RNAi induces synthetic lethality), causing bacteria in the well to be consumed more slowly than in surrounding lanes. If indeed contamination is occurring, the well will need to be replicated. There should be excess liquid culture from Step 6, which can be used on solid medium to redo any number of samples. If the stocks are contaminated, it may be necessary to streak out the bacterial strain on selective medium, then pick and culture individual colonies to remove the contamination.

Problem (Step 9): The worms appear to be dead.

Solution: They may very well be! An RNAi screen will produce a number of phenotypes, many of which are lethal. As you will be attempting to view only adult worms, embryonic lethal phenotypes will not be an issue, but synthetic lethality, sterility, and a number of growth defects should be expected. Refer to Wormbase (http://www.wormbase.org/#012-3-6) for detailed information on specific genes and results from previous RNAi screens to confirm an observed phenotype.

Problem (Step 10): There is too much fluorescent background signal in the intestine or too little apoptotic fluorescence after AO staining.

Solution: The stain:destain ratio is critical to successful visualization of corpses. Unfortunately, it requires a level of trial and error (especially if conducted solely in liquid culture), and can vary between samples. Of course, low apoptotic fluorescence may be a symptom of the target RNAi. Hence, it is important to exercise judgment as to whether low germline apoptosis is relative to the background staining or is indeed a real phenotype.

Problem (Step 12): The germline region is too difficult to assess because there are excessive numbers of worms in the well.

Solution: If there are worms settling on top of each other in a well, it may be beneficial to transfer them onto solid plates for visualization. Transferring to solid medium (in 24-well plates) will allow the worms to separate themselves and make detection of apoptotic cells easier, but this requires material and can be labor intensive. Transfer can be done during destaining (in which case the plates must be seeded with OP50 bacteria), or following destaining during visualization (in which case the plates do not need to be seeded with bacteria).

DISCUSSION

Using RNAi for High-Throughput Screening

Screening the entire genome for apoptosis phenotypes at high resolution would be a very labor-intensive endeavor, requiring tens of thousands of worms to be mounted on slides to quantify

Cold Spring Harbor Protocols
www.cshprotocols.org
CSH PROTOCOLS

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080234

corpses in each germline. Screening by lower resolution greatly accelerates throughput and reduces handling time significantly, and prevents excess use of vital dyes. The utility of high-throughput screening for apoptosis regulators by fluorescence dissecting microscopy is, of course, subject to the designs of the project, but is generally advantageous because it limits the number of genes to be screened at high resolution. For example, such an approach was used to identify the SCF^{FSN-1} E3 ubiquitin ligase (*fsn-1*) (Gao et al. 2008). Initially, regulators of the Skp-Cullin-F-box (SCF) class of E3 ubiquitin ligases (i.e., *ned-8*) were found to cause lower levels of lethality in *cep-1* mutants compared with wild-type controls in a genome-wide RNAi screen. This suggested that CEP-1 might be under negative regulatory control by a cullin-based E3 ligase, which in turn reduced the number of candidate genes to be screened for apoptosis phenotypes at high resolution. Systematic analysis of ∼300 substrate-specific adaptors of SCF ligases (F-box genes) using DIC microscopy led to the identification of *fsn-1* (Gao et al. 2008). Thus, a first pass screen at low resolution to identify genes that cause selective lethality in wild-type copies but not mutants of proapoptotic genes (such as *cep-1*) narrowed down the search to specific classes of genes (i.e., F-box genes). Targeting high-resolution screens to a limited number of genes has also been successfully used to identify apoptosis regulators from other ubiquitin ligases, such as the HECT domain E3 ligase EEL-1 (Ross et al. 2011).

The high-throughput method described here facilitates the rapid assessment of potentially thousands of RNAi targets, and once the techniques have been learned, the procedure is fairly simple and largely revolves around volume of work rather than specific skills. Data analysis (see below) requires simultaneous microscopy and recognition of apoptotic corpses. In terms of timing, experience and comfort with the techniques are the major determinants of throughput rate. It is a process that requires numerous elements of preparation, so we highly recommend starting slowly then scaling up after the basics have been established. Timing the culturing of the worms and the bacteria must be done carefully, so that neither the worms nor bacteria are compromised by being ready too early. This is particularly pertinent in the case of the worm bleaching procedure, because arrested L1 worms will only survive a limited amount of time in M9 buffer! Not having enough worms to adequately populate hundreds or thousands of wells can seriously delay your screen. Hence, planning both bleaching days and RNAi inoculation days will be critical before starting the project. Subsequent calculations of the number of worm plates, volumes of buffer and tips (and you will go through them at an astounding rate!) that you will need are equally important.

It should be cautioned that the strategy described in this protocol will not always catch all relevant genes because of the inherent variability of RNAi, but exploiting more general phenotypes such as synthetic lethality (or buffering of lethality) or body morphology defects can effectively identify a subset of genes for more tedious high-resolution analysis. In addition, follow-up analysis of reproducible apoptotic phenotypes identified by RNAi should be performed using available mutant strains. Confirmation of results and quantitative analysis should be conducted using high-resolution optics (described in Protocol: **Fluorescent Visualization of Germline Apoptosis in Living *Caenorhabditis elegans*** [Lant and Derry 2014a]). As an alternative to RNAi-induced apoptosis, screening can be performed after exposing worms to various stressors, such as DNA damage via gamma irradiation, hypoxia, or small bioactive compounds (see Protocol: **Induction of Germline Apoptosis in *Caenorhabditis elegans*** [Lant and Derry 2014b]).

Data Analysis

High-throughput screens produce extensive quantities of data, which can be challenging to manage. The details of your screen will determine how much or little you decide to filter when setting thresholds for strong, intermediate, and weak phenotypes. A simple system of qualitative assessment (i.e., increased by twofold or more, decreased by twofold or more, or undetectable differences) of apoptosis is advised. This allows you to pick the so-called low hanging fruit, which enables analysis of a select number of candidate genes for deeper mechanistic studies. For example, AO staining was successfully used to conduct a genome-wide RNAi screen for genes that cause increased levels of

Cold Spring Harbor Protocols
www.cshprotocols.org
CSH PROTOCOLS

apoptosis in the germline, which were then quantified by secondary analysis at higher resolution (Lettre et al. 2004). Alternatively, a screen can use a more global parameter, such as sterility, as a read out for massive apoptosis to reduce the number of hits, with apoptosis being directly assessed on a secondary/confirmatory run. Statistical analysis of apoptosis levels is important if you detect genes that cause intermediate effects on germline apoptosis. Thus, at least three independent replicates must be performed and, as previously mentioned, any gene that is identified as a candidate apoptosis regulator by RNAi should be confirmed in mutant strains, if available.

While the process may seem daunting, the ability to cover an entire genome in a matter of a few months can provide feedstock for several years of future studies.

RECIPES

Luria Bertani (LB) + Amp and Tet

1. To prepare solid medium, mix 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar, and bring to 1 L with H_2O. (To prepare liquid medium, combine all ingredients except agar.)
2. Autoclave for 30 min.
3. Let cool for 1 h in a 55°C water bath.
4. Add 2 mL of 50 mg/mL ampicillin and 2 mL of 5 mg/mL tetracycline.
5. While the agar-containing medium is still warm, pour a thin layer into each Petri dish and let cool to solidify.
6. Store dishes or liquid medium at 4°C for up to 1 mo.

Cold Spring Harbor Protocols
www.cshprotocols.org
CSH PROTOCOLS

M9 Buffer for Worms

1. Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in 1 L of H_2O.
2. Autoclave for 20 min.
3. Add 1 mL of 1 M $MgSO_4$.
4. Store at room temperature. After 2 wk, check for visible contamination before use.

Nematode Growth Medium (NGM)

1. For solid NGM, mix 3 g of NaCl, 2.5 g of peptone, and 20 g of agar and bring to 1 L with H_2O. (For liquid NGM, prepare without agar.)
2. Autoclave for 1 h.
3. Let cool for 1 h in a 55°C water bath.
4. Add 1 mL of cholesterol (5 mg/mL in ethanol), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M (pH 6.0) KPO_4, mixing after each addition.
5. Using an automated plate pourer (peristaltic pump), pour the medium into sterile plastic plates to set. During pouring, keep the NGM on a hot plate with stirrer to prevent the medium from solidifying.
6. Store NGM plates at 4°C until use. Seed NGM plates with bacteria the day after pouring. Warm the plates to room temperature before adding the worms. (For liquid NGM, store at room temperature for up to 1 mo. Regularly check for excess cloudiness to ensure bacterial contamination is absent.)

 Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot080234

ACKNOWLEDGMENTS

We are grateful to B. Yu and M. Gunda for their insight and help with procedures. This work was funded by grants from the Excellence in Radiation Research for the 21st Century (EIRR21) Radiation Medicine Research Program (B.L.), operating grants from the Canadian Institutes of Health Research (CIHR) (MOP79495) (W.B.D.), and infrastructure grants from the Canada Foundation for Innovation and Ontario Research Fund (Project No. 9782) (W.B.D.).

REFERENCES

Gao MX, Liao EH, Yu B, Wang Y, Zhen M, Derry WB. 2008. The SCFFSN-1 ubiquitin ligase controls germline apoptosis through CEP-1/p53 in *C. elegans*. *Cell Death Differ* **15:** 1054–1062.

Lant B, Derry WB. 2014a. Fluorescent visualization of germline apoptosis in living *Caenorhabditis elegans*. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot080226.

Lant B, Derry WB. 2014b. Induction of germline apoptosis in *Caenorhabditis elegans*. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot080192.

Lettre G, Kritikou EA, Jaeggi M, Calixto A, Fraser AG, Kamath RS, Ahringer J, Hengartner MO. 2004. Genome-wide RNAi identifies p53-dependent and -independent regulators of germ cell apoptosis in *C. elegans*. *Cell Death Differ* **11:** 1198–1203.

Ross AJ, Li M, Yu B, Gao MX, Derry WB. 2011. The EEL-1 ubiquitin ligase promotes DNA damage-induced germ cell apoptosis in *C. elegans*. *Cell Death Differ* **18:** 1140–1149.

Stiernagle T. Maintenance of *C. elegans*. (February 11, 2006), *WormBook*, ed. The *C. elegans* Research Community, wormbook. 1.0101.1, http://www.wormbook.org.

Timmons L, Court DL, Fire A. 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263:** 103–112.

Cold Spring Harbor Protocols
www.cshprotocols.org
CSH PROTOCOLS