

**Isolation of *C. elegans* gene knockouts by PCR screening of chemically mutagenized libraries.**

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## **ABSTRACT**

**Here I describe how to generate *C. elegans* deletion mutants by chemical mutagenesis and how to detect them by PCR screening. Approximately 600,000 worms are grown synchronously, mutagenized with ethyl methane sulfonate, divided in groups of 500 and allowed to self-fertilize for 2 generations. DNA is prepared from a fraction of each worm population, pooled into a 96-well plate, and screened by PCR with primers positioned 2.5-3.5 kb apart. Cultures containing deletion mutants are subdivided in small worm populations and tested again by PCR to identify positives. Single animals are then cloned from positive cultures, allowed to self-fertilize and identified by PCR genotyping. This method, which takes about a month, gives approximately a 50% chance of finding a deletion of interest bigger than 500-600 bp. If a deletion cannot be found, the library can be pooled at lower complexity and screened for smaller deletions using an alternative PCR-based method.**

## INTRODUCTION

The availability of a complete genome sequence for many genetic organisms has greatly increased the possibility of studying biological processes utilizing a reverse genetic approach (i.e. from a gene of interest to the phenotype caused by the elimination of that gene) rather than a more traditional, forward genetic approach (i.e., from a phenotype of interest to the gene that causes it). Clarifying genetic interactions in reverse genetic studies often requires stably reducing or eliminating the activity of a gene. This is achieved by physically removing a portion or the entire coding sequence of that gene from the genome.

In the *C. elegans* field we are fortunate that many deletion mutants are already available and others can be ordered from laboratories dedicated to isolating *C. elegans* knockouts.

Information about the deletions already available and an explanation on how to request them or how to suggest new gene targets can be found at <http://shigen.lab.nig.ac.jp/c.elegans> and at <http://celeganskoconsortium.omrf.org/>.

There are two methods for generating gene knockouts in *C. elegans*: transposon insertion followed by imprecise excision<sup>1,2</sup> and chemical mutagenesis. The protocol presented here uses chemical mutagenesis. Treatment with ethyl methane sulfonate (EMS) and trimethyl psoralen (TMP) followed by UV irradiation are the two chemical mutagen-based methods normally used to generate small, detectable deletions in *C. elegans*<sup>3</sup>. EMS has a high mutation frequency per gene ( $5 \times 10^{-4}$ ) and approximately 13% of the mutations produced are deletions<sup>4</sup>. TMP has a lower mutation frequency, but is thought to generate a proportionally higher number of small deletions than EMS<sup>5,6</sup>. However TMP seems to cause more generally deleterious effects on worms, thus making it more difficult to recover mutants<sup>7</sup>. Indeed, both chemicals have been successfully used to isolate gene knockouts in *C. elegans* (see for example<sup>3,7-12</sup>). It was estimated that the average deletion size for both chemicals is ~1,300 bp. This estimate did not take into account deletions of ~500 bp or smaller because they are difficult to detect. However some of these deletions were fortuitously found, so it is likely that the average deletion size is actually smaller<sup>7</sup>.

The protocol described below (**Fig. 1**) is derived from one originally developed by Gary Moulder and Robert Barstead at the Oklahoma Medical Research Foundation<sup>11</sup>. It involves a random chemical mutagenesis of approximately 600,000 worms, which are then subdivided in 1,152 cultures of 500 worms and allowed to self-fertilize for 2 generations. Genomic DNA is then obtained from ~20% of each worm population while the remaining ~80% is kept alive at 15 °C. DNA is pooled into a single 96-well plate, the pool of rows (PR) plate (**Fig. 1** and **2**), so that each well contains ~12,000 haploid genomes. Deletions in this pool are detected by PCR with primers positioned 2.5-3.5 kb apart. A gene with a deletion between the primers will generate a PCR band smaller than the wild-type gene (**Fig. 3**). Once a deletion is detected in one of the DNA pools, the single worm culture containing that deletion is identified (**Fig. 2**). Worms from this population are subdivided into small cultures and tested by PCR. Single worms are then cloned from positive cultures and PCR genotyped. In this way one identifies clones containing 1, 2 or no copies of the deleted gene (**Fig. 4**).

Because deletions are rare, it is highly unlikely that a well in the PR plate contains more than one deleted gene of interest, which is therefore mixed with ~12,000 other wild-type genes. To detect such a rare template it is essential that PCR conditions favour amplification of the deleted gene over the wild-type gene. This is achieved by carrying out two consecutive PCRs with nested primers using very short extension times. PCR detection is the limiting step and deletions smaller than ~500bp cannot be revealed, presumably because such small difference in size does not give a big enough advantage to the deleted gene. However, one can greatly

increase the chances of finding deletions shorter than ~500 bp by employing the poison primer technique, a sensitive PCR-based method, which allows the detection of deletions shorter than 1 kb<sup>13</sup>.

The protocol described here gives approximately a 50% chance to find a deletion of interest<sup>11</sup>. If a deletion is not found in the first instance, the library can be pooled at a complexity of ~4,000 genomes/well and re-screened for small deletions (150-1,000 bp) using the poison primer method<sup>12,13</sup>. Alternatively, a new deletion library can be constructed.

To increase the probability of isolating knockout clones after screening the PR plate, a live, rather than a frozen *C. elegans* library is employed. Recovering live worms from a frozen population is extremely inefficient, especially after chemical mutagenesis<sup>7</sup>. On the other hand a live library has a limited lifetime and must be screened within ~6 weeks. This is usually much longer than needed, even if one screens the library for many deletions. However, protocols are available that utilize frozen libraries<sup>12</sup>.

This protocol is simple but has a few critical steps, most involving *C. elegans* manipulation, which require a high level of accuracy. It is therefore advisable that a researcher with several years of lab experience, preferably with *C. elegans*, supervises the work. This protocol should be performed by two people, since the DNA preparation from the 1,152 worm cultures is particularly demanding, and would require approximately 12 consecutive hours of work for one person.

Because PCR screening plays such an important role in detecting the deletion of interest, before starting the protocol below tests should be carried out to ensure that the primers chosen for each deletion of interest produce a single, clear band from wild-type *C. elegans* genomic DNA.

Finally, due to the EMS mutagenesis, candidate animals are likely to carry mutations in several genes in addition to the deletion in the gene of interest<sup>4</sup>. Thus, they should be cleaned and outcrossed at least 6 times with wild-type worms. The deletion can be followed by PCR genotyping.

## MATERIALS

### REAGENTS

- Ethyl methane sulfonate (Sigma cat. no. M0880)
- 100 mM dNTPs (Promega cat. No. U1240)
- Taq DNA polymerase (Invitrogen cat. no. 10342-046)
- Gel purification kit (QIAGEN cat. no. 28704)
- Streptomycin (Merk Biosciences cat. no. 5711)
- Nystatin (Merk Biosciences cat. no. 475914)
- 0.5 % Thioglycolic acid (Sigma cat. no. T3758) in 1M NaOH (VWR cat. no. 31627.290)
- Sodium hydroxide pellets (VWR cat. no. 102525P)
- Agarose (Invitrogen cat. no. 15510-027)

### REAGENTS SETUP

**Rich NGM plates** 3 g NaCl (VWR cat. no. 102415K); 15 g electrophoresis grade agarose (Invitrogen cat. no. 15510-027); 7.5 g bacto peptone (BD Biosciences cat. no. 211677); 975

ml distilled water. Autoclave. Let cool to 55-60 °C and add 1 ml 5 mg ml<sup>-1</sup> cholesterol (Sigma cat. no. C-8667) in EtOH, 1 ml 1M CaCl<sub>2</sub> (VWR cat. no. 100704Y), 1 ml 1M MgSO<sub>4</sub> (VWR cat. no. 101514Y), 25 ml phosphate buffer, pH 6.

**M9 buffer** 22mM KH<sub>2</sub>PO<sub>4</sub>, 42mM Na<sub>2</sub>HPO<sub>4</sub>, 85mM NaCl, 1mM MgSO<sub>4</sub>. Weigh 3 g KH<sub>2</sub>PO<sub>4</sub> (VWR cat. no. 102034B), 6 g Na<sub>2</sub>HPO<sub>4</sub> (VWR cat. no. 28026292), 5 g NaCl, 1 ml 1 M MgSO<sub>4</sub>; to 1 litre with sterile distilled water.

**Synchronizing solution** 0.25 M KOH (VWR cat. no. 296274N), 1-1.5 % hypochlorite (commercial bleach contains ~12% hypochlorite).

**Lysis buffer** 50 mM KCl (VWR cat. no. 1049360500), 10 mM Tris HCl pH 8.3 (VWR cat. no. 441514A), 2.5 mM MgCl<sub>2</sub> (VWR cat. no. 101494V), 0.45 % NP-40 (Merk Biosciences cat. no. 492016), 0.45 % Tween-20 (Sigma cat. no. P-7949), 0.01 % gelatin (Sigma cat. no. G-7765), 200 µg ml<sup>-1</sup> Proteinase K (Sigma cat. no. P-6556). Sterilize by filtration. Add Proteinase K solution just before use (20 µl ml<sup>-1</sup> of lysis buffer).

**Proteinase K** 10 mg ml<sup>-1</sup> in sterile distilled water.

**50X TAE** 242 g Tris base (VWR cat. no. 103156X); 57.1 ml glacial acetic acid (VWR cat. no. 10001CU); 100 ml 0.5 M EDTA (Sigma cat. no. ED2SS); to 1 litre with distilled water.

**6X dye** 0.25% bromphenol blue (Bio-Rad Laboratories cat. no. 1610404); 0.25% xylene cyanol FF (Sigma cat. no. X4126); 1 mM EDTA; 30% glycerol (VWR cat. no. 101186M) in distilled water.

## EQUIPMENT

- 55 mm Petri dishes (VWR cat. no. 391-0865)
- 140 mm Petri dishes (VWR cat. no. 391-2028)
- 50 ml Falcon tubes
- 15 ml Falcon tubes
- Disposable plastic Pasteur pipettes
- 250 ml wide-mouth plastic bottles (Fisher Scientific cat. no. BTR-635-090B)
- Carousel (e.g., Stuart Rotator SB3)
- Repetitive pipette (Eppendorf Multipipette Plus)
- 5 ml Eppendorf Combitip Plus tips for repetitive pipette (Eppendorf cat. no. 0030 069.250)
- 10 ml Eppendorf Combitip Plus tips for repetitive pipette (Eppendorf cat. no. 0030 069.269)
- Two water baths (for 65 °C incubations)
- 96-well PCR plates (ABgene cat. no. AB-0800)
- 96-deep well plates (ABGene cat. no. AB-0564)
- Plate thermosealer and carrier plate (ABgene cat. nos. AB-0384/240 and AB-0563/1000).
- Easy Peel polypropylene sealing foil (ABgene cat. no. AB-0745)
- Tip-tub reservoir (Eppendorf cat. no. 0030 058.607)
- 10-100 µl, 8-channel pipette (Eppendorf cat. no. 3114 000.131)

- 0.5-10  $\mu$ l, 12-channel (Eppendorf cat. no. 3114 000.123)
- 10-100  $\mu$ l, 12-channel (Eppendorf cat. no. 3114 000.140)
- 30-300  $\mu$ l, 12-channel (Eppendorf cat. no. 3114 000.166)
- 0.1-10  $\mu$ l tips (Eppendorf cat. no. 0030 073.363)
- 2-200  $\mu$ l tips (~7,500) (Eppendorf cat. no. 0030 073.428)
- 20-300  $\mu$ l tips (~1,500) (Eppendorf cat. no. 0030 073.444)
- PCR thermocycler with 96 well block (e.g., BioRad PTC 200 DNA engine)
- Microplate Replicator (e.g., Grant cat. no. MR96)
- 24-well plates (VWR Cat. No 402/0323/14)
- 2 Owl Centipede gel systems (Cat. No D3-14)

## PROCEDURE

### Before starting worm synchronization 1 Day 0 Timing 2 hours

- 1 Four days before starting prepare 10 140 mm rich NGM plates. Let them dry for 24 hours and spread them with OP50 *E. coli*.
- 2 Put 4-5 L4 N2 hermaphrodites in each of 5 55 mm plates. Grow at 20 °C.
- 3 Prepare M9 buffer (see Materials).
- 4 The day you start worm synchronization 1 prepare 25 more 140 mm rich NGM plates.

### Worm synchronization 1 Day 4 Timing 7 days

- 5 Put ~30 L4 N2 (wild-type) worms in each of 10 140 mm rich NGM plates spread with a lawn of OP50 *E. coli*.
- 6 Grow for 4 days at 20 °C. Check under a dissecting microscope that most of the worms are gravid adult hermaphrodites bloated with eggs.
- 7 Collect worms in M9 buffer and transfer them to 2 50 ml Falcon tubes. CRITICAL STEP. Make sure to collect worms when the plates contain mostly adult hermaphrodites full of eggs. You need as many eggs as possible at this stage since only eggs will survive treatment with synchronizing solution.
- 8 Spin at 500g for one minute. Carefully remove and discard supernatant. Combine the content of the 2 tubes into a single Falcon tube.
- 9 Spin at 500g for one minute. Carefully remove and discard supernatant
- 10 Add 10 volumes of freshly prepared synchronizing solution. CAUTION! Synchronizing solution contains KOH and bleach; both cause severe burns.
- 11 Leave for 5-7 minutes inverting the tube once or twice a minute until ~30-40% of the worms open up and start losing eggs. CRITICAL STEP. This step kills everything except eggs. However, eggs left in contact with synchronizing solution for more than a few minutes are killed too. Check progression under dissecting microscope and spin as soon as you see 30-40% of fragmented worms.
- 12 Spin at 500g for one minute.
- 13 Carefully remove supernatant. Wash by adding ~10 volumes of M9 buffer.
- 14 Spin at 500g for one minute.
- 15 Wash 3 additional times by repeating steps 13 and 14.

- 16 Resuspend pellet in ~3 ml of M9 buffer. Put tube on a carousel and leave it rotating overnight at room temperature. Eggs will hatch and worms will arrest at the L1 stage.
- 17 Take 5  $\mu$ l of worm suspension and count worms under dissecting microscope.
- 18 Distribute a volume containing ~30,000 L1s in each of 25 140 mm rich NGM plates spread with a lawn of OP50 *E. coli*.
- 19 Grow for ~48 hours at 20 °C, until most of the worms are at the late L4 stage. CRITICAL STEP: Worms at this stage have a white crescent at the developing vulva with a black dot in the middle. Worms should be mutagenized at this stage because they are likely to produce the highest number of independently mutagenized genomes<sup>4</sup>.
- 20 Collect worms in M9 buffer and transfer them to 2 50 ml Falcon tubes.
- 21 Centrifuge at 500g for one minute and carefully remove supernatant.
- 22 Combine the content of the 2 tubes into a single 50 ml Falcon tube.
- 23 Centrifuge at 500g for one minute and carefully remove supernatant.

### **Before starting EMS mutagenesis Day 8 Timing 1 hour**

- 24 Three days before starting EMS mutagenesis prepare 20 140 mm rich NGM plates. Let them dry for 24 hours. Seed them with OP50 *E. coli*.
- 25 You also need the equipment listed in box 1.  
BOX 1. Equipment needed for EMS mutagenesis
  - 100  $\mu$ l Eppendorf pipette
  - Gloves (1 box).
  - Plastic Pasteur pipettes in a plastic beaker.
  - NaOH pellets.
  - Two 250 ml wide-mouth plastic bottles that can contain, capped, a 50 ml Falcon tube. These bottles will be used, with NaOH pellets inside (to inactivate EMS), to centrifuge the Falcon tube with worms in EMS solution.
  - Parafilm.
  - 1X M9 buffer to wash worms (4 X 50 ml).
  - Disposable holders for 15 ml and 50 ml tubes (polystyrene tube holders, for example).
  - A horizontal shaker. This is not essential (see step 31)
- 26 Just before mutagenesis mix 5 ml thioglycolic acid with 1 litre of 1M NaOH. CAUTION: Make solution under the fume hood and use gloves. When working with EMS do not bring to the fume hood anything that you are not prepared to leave there. Wear a lab coat and two pairs of gloves; remove external gloves each time you leave the fume hood. Use disposable plastic Pasteur pipettes. Put everything that has been in contact with EMS in a 2 litre plastic beaker half full of the NaOH/thioglycolic acid solution. Leave in solution for 24 hours.

### **EMS mutagenesis (mutagenesis can be carried out with TMP/UV<sup>6,11,12</sup> instead). Day 11 Timing 1 day**

- 27 Resuspend worms in 8 ml of M9. In a separate 15 ml tube put 8 ml of M9.
- 28 Prepare 8 ml of 100 mM EMS in M9 by adding 85  $\mu$ l of EMS (9.42 M) to the tube without worms. Close tube and seal it with parafilm. Mix the tube by inverting a few

times. Make sure that the oily-looking sphere at the bottom is dispersed. Wash the pipette tip in NaOH/thioglycolic acid. Put tip in solid waste. CAUTION! EMS is a carcinogen and everything that has been in contact with it must be inactivated for 24 hours in a solution of NaOH/thioglycolic acid. NaOH causes severe burns.

Thioglycolic acid is caustic.

- 29 Carefully transfer the EMS solution into the tube with the worms. Close tube, seal with parafilm and mix well by inverting a few times.
- 30 Wash the empty tube with NaOH/thioglycolic acid and put it in solid waste.
- 31 Leave worms in EMS solution for 4 hours. Attach tube horizontally on a horizontal shaker with tape. Make sure the tube is sealed well. Shake very mildly (1 Hz). If a shaker is not available, the Falcon tube with worms in EMS solution can be left horizontal.
- 32 Take away outer glove and wash with NaOH/thioglycolic acid. Put in solid waste.
- 33 Close fume hood, put inner glove in normal waste, and wash hands thoroughly with soap.
- 34 Back after 4 hours with 2 x 250 ml wide-mouth plastic bottles in which you have previously put a layer of NaOH pellets. Tubes with worms/EMS solution will be put into these bottles during centrifugation to avoid EMS spillage.
- 35 Put tube with worms into 1 250 ml wide-mouth plastic bottle. Put an identical tube with 16 ml of water in the other bottle (centrifuge balance). Spin at 500g for a minute.
- 36 Carefully remove supernatant leaving ~0.5 cm of liquid over the worms. Wash mutagenised worms 4 times with 10 volumes of M9.
- 37 Plate the mutagenized worms in 20 140 mm rich NGM plates seeded with OP50 *E. coli*.
- 38 To assess whether the mutagenesis has been effective check: F1 viability and F1 sterility. Check F1 viability by picking ~50 worms to fresh plates, allow them to lay eggs overnight and then remove the adults. Let plates sit 24 hours at room temperature then count the number of hatched larvae and dead eggs among the F1s (see Anticipated Results). Check F1 sterility by cloning, 2 days later, 100 F1s and checking how many are sterile (see Anticipated Results).
- 39 Grow for 24 hours. At this point the plates should ideally be recently starved with mostly bloated adults (P0 generation) and some L1 progeny (F1 generation). Proceed immediately to step 43.

#### **Before starting worm synchronization 2/plating Day 9 Timing 1.5 days**

- 40 Three days before starting worm synchronization 2, pour ~1,200 55 mm rich NGM plates (~15 litres of media). You will need 1,152 55 mm plates but it is advisable to pour more in case some plates get contaminated with mould.
- 41 Prepare 15 96 deep-well plates and number them.
- 42 Anytime in the following 4 days number 1,152 55 mm plates. A fraction of worms grown in each of these 55 mm plates will later be transferred to a well in a 96-deep well plate (**Fig. 2**). You will use 12 96-deep well plates for a total of  $12 \times 96 = 1,152$  wells. Number 55 mm plates according to the position of the well in the 96-well plate where the worms will be placed. 1A1, 1A2....1A12, 1B1, 1B2....1B12 and so on until 12H12. The first number indicates the 96-deep well plate number, the letter indicates the row and the second number the column number (**Fig. 2**).

### **Worm synchronization 2/plating Day 12 Timing 6 days**

- 43 Collect worms from the 140 mm plates with ~5 ml of M9 buffer per plate. Put them in 2 50 ml Falcon tubes.
- 44 Spin at 500g for one minute and carefully remove and discard supernatant.
- 45 Combine the content of the 2 tubes into a single Falcon tube.
- 46 Spin at 500g for one minute and carefully remove and discard supernatant.
- 47 Synchronize worms as in steps 10 to 15. CAUTION! Synchronizing solution contains KOH and bleach; both cause severe burns.
- 48 Resuspend pellet in ~30 ml M9. Put tube on a carousel and leave it rotating overnight at room temperature. Eggs will hatch and worms will arrest at the L1 stage.
- 49 Take ~5  $\mu$ l of L1 suspension and count worms under dissecting microscope. Dilute with M9 so that there are ~500 L1s per 50  $\mu$ l.
- 50 Use repetitive pipette and dispense 50  $\mu$ l of worm suspension in the middle of each of the 1,152 plates. Make sure to maintain good worm suspension by inverting the tube several times before loading repetitive pipette.
- 51 Let worms grow for 5 days at 20 °C.

### **Before starting DNA preparation Day 13 Timing 4-5 days**

- 52 Design nested PCR primers for the gene of interest 2.5-3.5 kb apart (two external primers for primary PCR, two internal primers for secondary PCR). Primers should be ~20 bp long with ~50% G-C content. Two consecutive PCRs with these primers are run to reveal the deletion of interest (**Fig. 3** and supplemental file 1). Test primers and PCR conditions with wild-type *C. elegans* genomic DNA and make sure that you obtain a single, clear band.
- 53 Prepare 4 x 50 ml tubes of lysis buffer (each 50 ml tube should include 1 ml of 10 mg ml<sup>-1</sup> Proteinase K solution). CAUTION! Proteinase K is harmful and irritating to eyes, respiratory system and skin. Wear suitable protective clothing. Do not breathe dust.
- 54 Prepare 240 tip-tub reservoirs for sample pooling, 96 for PR plate and 144 for PC plates (**Fig. 2**).

### **DNA preparation Day 18 Timing 1 day**

- 55 Dispose numbered 55 mm plates from step 42 in stacks of 6.
- 56 Using a repetitive pipette add 0.8 ml of sterile distilled water containing 100  $\mu$ g ml<sup>-1</sup> streptomycin and 12.5  $\mu$ g ml<sup>-1</sup> nystatin to each plate. Work with one stack of 6 plates at the time.
- 57 Rock plates to detach worms and transfer 150  $\mu$ l of the worm suspension from each of the six plates to the corresponding wells of a 96-deep-well microtitre plate. To keep track of the filled wells, eject the micropipette tip into the well you have just transferred the worms into. CRITICAL STEP: Make sure to be extremely accurate. Transferring worms from a plate to a well different from the corresponding one will result in being unable to recover candidate knockout worms later.
- 58 Once you fill a 96 deep-well plate with the worm suspension, add 150 $\mu$ l of lysis buffer to each well and mix. Use 12-channel pipette and tip-tub reservoirs.

- 59 Seal each 96-deep well plate with thermosealer/sealing foil. Press plate/sealing foil against hot plate for 7 seconds and immediately after press the sealing foil with carrier plate for a few seconds to ensure good sealing. Seal edges with autoclave tape.
- 60 Place 96-deep well plates at -80 °C for at least 30 minutes to freeze worms. PAUSE POINT; plates can be left indefinitely at -80 °C.
- 61 Incubate 96-deep-well plates for a minimum of 4 hours to overnight at 65 °C in a water bath.
- 62 Centrifuge 96-deep well plates at 500g for 10 seconds to pellet worm carcasses. The centrifugation concentrates worm carcasses at the bottom of the well and removes DNA solution on the sealing foil at the top of well. CRITICAL STEP If you can't centrifuge, hit plate vertically on bench and carefully remove the sealing foil to avoid cross contamination. In addition when transferring DNA solution, pay attention that each tip contains approximately the expected volume. Worm carcasses in suspension could block the tip resulting in less DNA solution being collected into the tip.

### Sample pooling Day 19 Timing 1 day

- 63 Dilute samples with 300 µl of sterile distilled water. Use multichannel pipette.
- 64 Pool samples from the 12 deep-well plates by transferring, with a multichannel pipette, 50 µl of each row and each column to a clean tip-tub reservoir. Then use a 1 ml Gilson pipette to transfer to the appropriate wells of 2 96-deep-well plates: the PR plate (pool of rows) and the PC plates (pool of columns; **Fig. 2**).
- 65 Use multichannel pipette to transfer 150 µl of each pooled sample to a new 96-well PCR plate and seal with thermosealer/sealing foil.
- 66 Heat to 95 °C for 15 minutes in PCR machine to inactivate Proteinase K.
- 67 Store the 96-deep-well plates at -80 °C. Store the 96-well PCR plates with inactivated Proteinase K at 4 °C.
- 68 Use PR plate for PCR screening.

### Screening DNA pools by PCR Day 20 Timing 2 days

- 69 Primary PCR: transfer 5 µl from the PR plate (**Fig. 2**) to a new 96-well PCR plate. Add 20 µl of master mix with external primers (**Fig. 3** and supplemental file 1).
- 70 Seal 96-well PCR plate with thermosealer/sealing foil and run PCR (**Fig. 3**).
- 71 Secondary PCR: prepare master mix with internal primers. Add 25 µl of master mix per well (**Fig. 3**) to a new 96-well PCR plate. Use a 96-pin replicator to transfer a small volume of each well from the primary PCR to the corresponding well of the secondary PCR. Wash 96-pin replicator in sterile water, dip it in ethanol and sterilize it by flaming.
- 72 Seal 96-well PCR plate with thermosealer/sealing foil and run PCR (**Fig. 3** and supplemental file 1).
- 73 When PCR has ended, add 5 µl of 6X dye per well with a multichannel pipette.
- 74 Load 10 µl of each well on a 1% TAE agarose gel.
- 75 Run gel at 125 V. PCR bands shorter than wild-type indicate the presence of putative positive clones (**Fig. 3 c**). TROUBLESHOOTING!
- 76 Re-check each putative positive by re-PCR amplifying DNA from the well in the PR plate where the positive was detected and by PCR amplifying the corresponding 12

wells of pooled columns in plate PC1 or PC2. Positive wells in both situations indicate the address of the original well containing the deleted gene and therefore the corresponding worm population (**Fig. 2**). It is necessary to verify the putative positives in this way because ~50% of the bands shorter than wild-type are false positives and can not be reproduced.

- 77 If deleted PCR bands can be re-amplified in PR and PC plates, pool positive PCR reactions, run on an agarose gel and extract DNA with gel extraction kit. The DNA obtained is usually enough to obtain a good DNA sequence. Sequence in both directions with primers internal to the ones used for secondary PCR.

### **Isolating small populations containing deleted worms Day 22 Timing 6 days**

- 78 Three days before starting to isolate small populations of deleted worms, prepare 16 24-well rich NGM plates (a total of 384 worm cultures). Use 2 ml of medium per well and spread with 5  $\mu$ l of OP50 *E. coli*. You can start doing this after having performed step 62.
- 79 Design primers for PCR genotyping (legend of **Fig. 4 b** for details).
- 80 Collect worm in ~2 ml M9 buffer from the plate corresponding to the well with candidate deletion. Transfer to a 15 ml Falcon tube.
- 81 Observe 5  $\mu$ l of worm suspension under dissecting microscope and count worm number to determine worm concentration
- 82 Transfer 25 worms to each well of 16 24-well rich NGM plates spread with OP50 bacteria.
- 83 Let grow for 5 days.
- 84 Add 100  $\mu$ l of sterile distilled water per well with repetitive pipette.
- 85 Rock the plates to detach worms from bacteria.
- 86 Transfer 25  $\mu$ l of worm suspension to the corresponding wells of 4 96-well PCR plates.
- 87 Add 25  $\mu$ l of lysis buffer per well (remember Proteinase K) with multichannel pipette and mix.
- 88 Seal plates with thermosealer/sealing foil.
- 89 Place 96-well PCR plate at -80 °C for at least 30 minutes to freeze worms. PAUSE POINT; plates can be left indefinitely at -80 °C.
- 90 Place 96-well PCR plate in PCR machine at 65 °C for 4 hours followed by 95 °C for 15 minutes.
- 91 Use 5 $\mu$ l from each well and carry out primary and secondary PCRs as above to identify cultures with deleted worms (**Fig. 3** and Supplemental file 1).

### **Isolating clones of deleted worms Day 28 Timing 7 days**

- 92 Three days before starting to isolate clones of deleted worms, prepare 16 24-well rich NGM plates (total 384 wells). You can start doing this while performing step 83.
- 93 Collect worms in M9 buffer from culture(s) with candidate deletions.
- 94 Transfer ~100 L1-L2 worms per plate to 6 55 mm rich NGM plates. Spin down the rest of the worms and freeze them.
- 95 Let the worms grow for 1-2 days.
- 96 Clone 384 single L4 worms to 16 24-well plates.
- 97 Let grow for 4 days.

- 98 Add 100  $\mu$ l of sterile distilled water per well with repetitive pipette.
- 99 Rock the plates to detach worms from bacteria.
- 100 Transfer 25  $\mu$ l of worm suspension the corresponding wells of 4 96-well PCR plates.
- 101 Add 25  $\mu$ l of lysis buffer per well (remember Proteinase K).
- 102 Seal plates with thermosealer/sealing foil.
- 103 Place 96-well PCR plate at -80 °C for at least 30 minutes to freeze worms. PAUSE POINT; plates can be left indefinitely at -80 °C.
- 104 Place 96-well PCR plate in PCR machine at 65 °C for 4 hours followed by 95 °C for 15 minutes.
- 105 Use 5  $\mu$ l from each well and carry out PCR genotyping (**Fig. 4 b and c** and supplemental file 2). Always include positive (wild-type and deleted DNA) and negative (no DNA) controls.
- 106 Run on a 2% agarose gel. This gel will reveal which clones are homozygous or heterozygous for the deletion of interest or whether they are wild-type.  
TROUBLESHOOTING!
- 107 Outcross the strains carrying the deletion of interest at least 6 times.

## TROUBLESHOOTING

See table 1 for troubleshooting advice.

**Table1. Troubleshooting**

<b>PROBLEM</b>	<b>POSSIBLE REASON</b>	<b>SOLUTION</b>
Too few eggs hatch the day after treatment with synchronizing solution (steps 16 and 48).	The worms were exposed to synchronizing solution for too long and eggs were damaged.	Treat with synchronizing solution for a shorter time.
No PCR bands in secondary PCR (step 75)	Proteinase K was not denatured for 15 min at 95 °C. Active Proteinase K digests Taq DNA polymerase and prevents PCR amplification.	Denature Proteinase K for 15 min at 95 °C.
No PCR bands in genotyping PCR (step 106)	Proteinase K was not denatured for 15 min at 95 °C. Active Proteinase K digests Taq DNA polymerase and prevents PCR amplification.	Denature Proteinase K for 15 min at 95 °C

## TIMING

Before step 1, Testing PCR primers for each deletion: 3 days.  
Steps 1-4, Preparing cultures for worm synchronization: 4 days.  
Steps 5-23, Worm synchronization 1: 7 days.  
Steps 24-39, EMS mutagenesis: 1 day.  
Steps 40-51, Worm synchronization 2/plating: 6 days.  
Steps 52-68, DNA preparation and sample pooling: 2 days.  
Steps 69-77, Screening DNA pools: 2 days.  
Steps 78-91, Isolating small populations containing deleted worms: 6 days.  
Steps 92-106, Isolating single deleted worms: 7 days.  
Step 107, Outcrossing strains carrying the deletion: ~ 6 weeks.

## ANTICIPATED RESULTS

Step 16: Worms arrest at the L1 stage because there is no food. They survive in M9 for several days, so they could be used again if not enough worms are recovered from the second synchronization and you need to start again.

Step 23: You should have approximately 2 ml of packed L4 worms at this stage, which should immediately be used for EMS mutagenesis.

Step 38: Always check the efficiency of the EMS mutagenesis. If mutagenesis has worked efficiently expect ~1-2% F1 lethality and ~5-10% F1 sterility. If you find lower values than these, it is advisable that you re-do the EMS mutagenesis.

Step 51: On the 5<sup>th</sup> day the 1,152 plates should be starved and contain many L1-L2 animals.

Step 70: You don't need to run a gel with samples from the primary PCR because usually no deleted bands are visible.

Step 75: Secondary PCR should produce visible 2.5-3.5 kb bands even with short extension times (40 – 45 sec). A typical gel from a Secondary PCR is shown on **Fig. 3**. Approximately 50% of the smaller bands visible in these gels are false positives. For this reason DNA from putative positive wells in the PR plate, as well as from the corresponding rows in the PC plates, must be re-amplified for verification. If PCR amplification is successful, DNA from putative positive wells containing un-pooled DNA should be tested as well.

Step 106: A typical gel from PCR genotyping is shown on **Fig. 4 c**. Note that in heterozygous animals the band with higher mobility, corresponding to the wild-type gene, suppresses the lower mobility band, which corresponds to the deleted gene. This effect is not always so strong.

## SUPPLEMENTAL MATERIAL

Supplemental file 1: Customizable PCR program for Primary and Secondary PCRs (Excel file).

Supplemental file 2: Customizable PCR program for Genotyping PCR (Excel file).

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## COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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#### BOX 1. Equipment needed for EMS mutagenesis

- 100 µl Eppendorf pipette
- Gloves (1 box).
- Plastic Pasteur pipettes in a plastic beaker.
- NaOH pellets.
- Two 250 ml wide-mouth plastic bottles that can contain, capped, a 50 ml Falcon tube. These bottles will be used, with NaOH pellets inside (to inactivate EMS), to centrifuge the Falcon tube with worms in EMS solution.
- Parafilm.
- 1X M9 buffer to wash worms (4 X 50 ml).
- Disposable holders for 15 ml and 50 ml tubes (polystyrene tube holders, for example).
- A horizontal shaker. This is not essential (see step 31)

## FIGURES LEGENDS

### Figure 1.

Flow diagram of the protocol. Each section (see Procedure) is colored differently and the approximate time required for its completion is shown.

### Figure 2.

Making the deletion library. Grow 1,152 worm cultures containing ~1,000 genomes each in 55 mm plates numbered according to the well where a portion of their worms will be collected for DNA isolation (for example worms from culture 3A1 will be collected in well A1 of the 96-well plate No. 3). DNA isolated from the 1,152 worm cultures is stored in 12 96-deep-well plates. Each row and each column of these 12 96-well plates are then pooled in single wells, thus generating the PR (pool of rows) and PC (pool of columns) plates. The pool of rows results in 96 wells containing ~12,000 genomes each. The pool of columns results in 144 wells containing ~8,000 genomes each. The PR plate is used for the initial PCR screen. If a positive is found (for example 9B, which corresponds to plate 9), then the corresponding row in plate PC1 should be tested (row I in this case). The well in PC positive for the target deletion will indicate the original well, and therefore the worm plate, containing the deletion. For example, if well I8 in PC1 is positive, the worms containing the target deletion are in the worm plate corresponding to well 9B8. DNA from this single well should be tested by PCR before collecting the population from the corresponding worm culture and starting the deconvolution process to isolate single deleted worms.

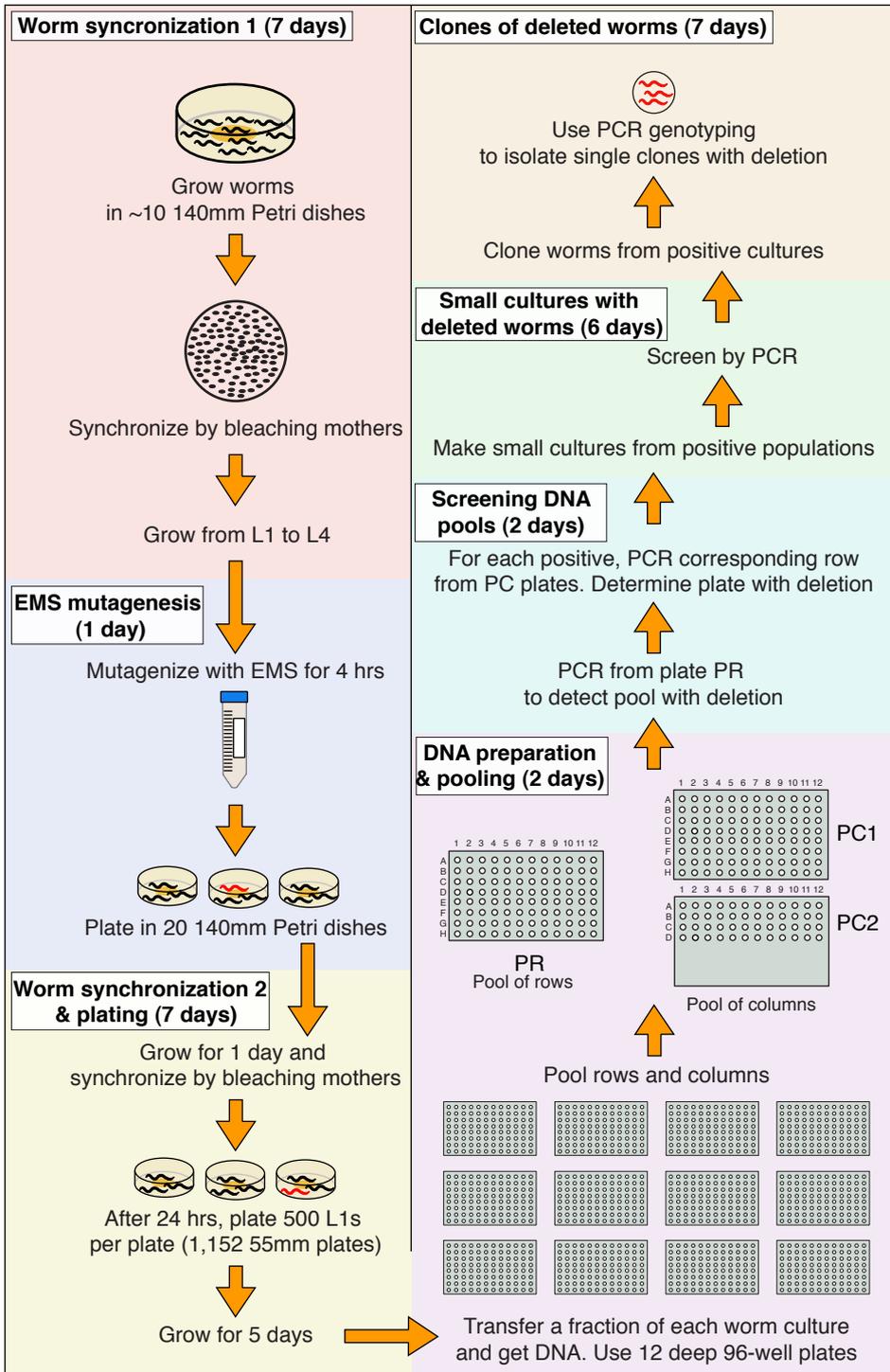
### Figure 3.

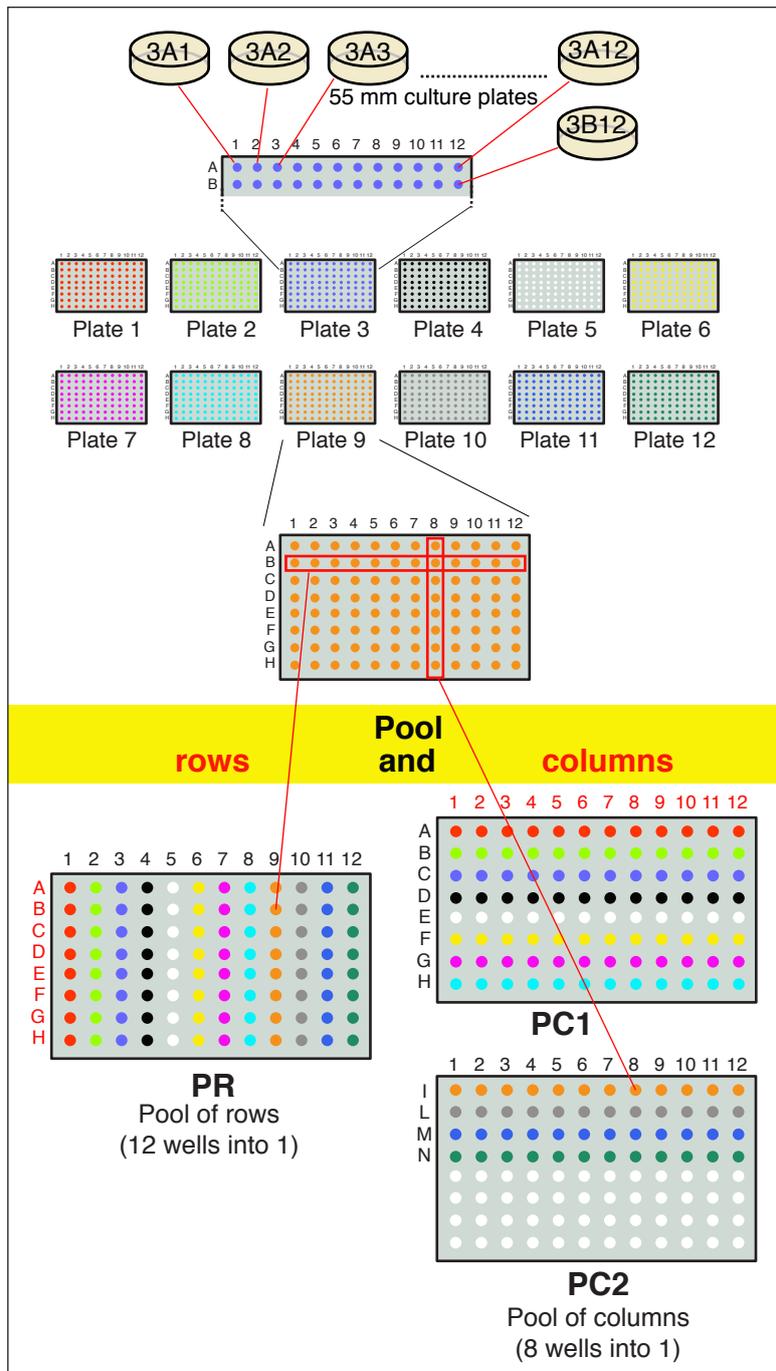
Screening of the deletion library by PCR. (a) Design 2 sets of nested primers, Ext1/Ext2 and Int1/Int2, positioned 2.5-3.5 kb apart. (b) Using the DNA isolated from worm cultures and utilizing the PCR conditions shown, carry out 2 consecutive PCRs. The volume of template used in the secondary PCR is negligible, since it is transferred from the primary PCR with a 96-pin replicator. Notice the low extension time (40 seconds). This allows the shorter DNA from a deleted gene to be PCR amplified more efficiently than the DNA from the wild-type gene. (c) Representative 1% agarose gel with 96 samples from a secondary PCR. Red arrowheads indicate potential positives. Asterisks indicate the 1 kb ladder.

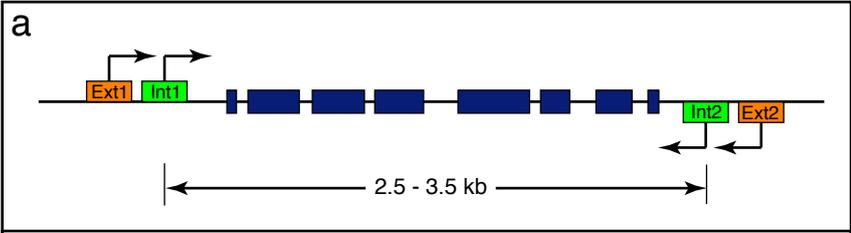
### Figure 4.

Isolation of clones carrying the deletion. (a) The worm culture whose DNA is positive for the deletion of interest is divided into 384 small cultures (in 24-well plates). DNA is extracted from these cultures and PCR screened for the presence of the deletion. 384 single worms from positive wells are then cloned, let grow and PCR genotyped. (b) PCR genotyping. To determine the genotype of a population of worms, 3 PCR primers are designed: Gen1, Gen2 and Gen3 so that the Gen1/Gen3 band (400 – 500 bp in wild-type animals) can only be PCR amplified in the presence of an intact gene and the Gen1/Gen2 band (550-650 bp in deleted animals) can only be PCR amplified in the presence of a deleted gene. Gen1/Gen2 from the wild-type gene is too big to be PCR amplified at detectable levels. Because low molecular weight bands are amplified more efficiently and can sometimes suppress high molecular weight bands, primers are designed so that the band from the deleted gene (Gen1/Gen2) has a higher molecular weight than the band generated from the wild-type gene (Gen1/Gen3). The

red box in i) shows the portion of the gene that is deleted in ii). The PCR program used for PCR genotyping is shown. Note that the PCR is run for a total of 40 cycles. Each of the 3 primers has a final concentration of  $0.4 \text{ pmol } \mu\text{l}^{-1}$  (see Supplemental file 2). (c) Representative 2% agarose gel showing the bands obtained for 19 worm clones by genotyping PCR. Red asterisks indicate worm populations homozygous for the deleted gene.







**b**

Primary PCR	Secondary PCR	PCR program
2.50 $\mu$ l 10X Taq buffer 1.25 $\mu$ l 50mM MgCl <sub>2</sub> 0.20 $\mu$ l 25 mM dNTPs 10 pmol primer <b>Ext1</b> 10 pmol primer <b>Ext2</b> 0.5 U Taq 5.00 $\mu$ l DNA water to 25 $\mu$ l	2.50 $\mu$ l 10X Taq buffer 1.25 $\mu$ l 50mM MgCl <sub>2</sub> 0.20 $\mu$ l 25 mM dNTPs 10 pmol primer <b>Int1</b> 10 pmol primer <b>Int2</b> 0.5 U Taq ~0.05 $\mu$ l DNA (with 96 pin) water to 25 $\mu$ l	1. 94°C for 30 sec 2. 92°C for 30 sec 3. 1°C/sec to 55°C 4. 55°C for 30 sec 5. 1°C/sec to 72°C 6. 72°C for 40 sec 7. To step 2 for 34 times 8. 72°C for 7 min 9. 12°C soak

