Journal of Visualized Experiments
Measuring RAN peptide toxicity in C. elegans
--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61024R2
Full Title:	Measuring RAN peptide toxicity in C. elegans
Keywords:	Neurodegeneration, ALS, Huntington's disease,C9orf72, proteotoxicity, repeat expansion disorders
Corresponding Author:	S Todd Lamitina University of Pittsrbugh Pittsburgh, PA UNITED STATES
Corresponding Author's Institution:	University of Pittsrbugh
Corresponding Author E-Mail:	stl52@pitt.edu
Order of Authors:	Paige Rudich
	Carley Snoznik
	Noah Puleo
	S Todd Lamitina
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Pittsburgh, PA USA

University of Pittsburgh

Todd Lamitina, Ph.D.
Associate Professor

Children's Hospital of Pittsburgh of UPMC
One Children's Hospital Drive
4401 Penn Avenue
Rangos, Rm 7122
Pittsburgh Pennsylvania 15224
Tel: 412-692-9437
Fax: 412-641-1844
stl52@pitt.edu

School of Medicine
Cell Biology and Physiology

December 30, 2019

Dear Editor,

I am pleased to submit a revised version of our manuscript entitled "**Methods for measuring RAN peptide toxicity in *C. elegans***" for publication in JoVE. This revision addresses all of the requested editorial comments. This submission is in response to an invitation by Dr. Cindy Voisine for inclusion in the upcoming collection on *C. elegans* methods. We hope that the manuscript is now suitable for publication.

Best Regards,

Todd Lamitina, Ph.D.
Associate Professor of Cell Biology
University of Pittsburgh

Transforming the Present - Discovering the Future

1 **TITLE:**
2 **Measuring RAN peptide toxicity in *C. elegans***
3

4 **AUTHORS AND AFFILIATIONS:**
5 Paige Rudich[1], Carley Snoznik[2], Noah Puleo[2], Todd Lamitina[1,6]
6
7 [1]Graduate Program in Cell Biology and Molecular Physiology, University of Pittsburgh, Pittsburgh,
8 Pennsylvania, USA
9 [2]Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania,
10 USA
11
12 **Corresponding author:**
13 Lamitina T.   (stl52@pitt.edu)
14
15 **Email Addresses of Co-authors:**
16 Rudich, P.   (paige.rudich@mail.mcgill.ca)
17 Snoznik, C.   (carley.snoznik@chp.edu)
18 Puleo, N.   (nrp37@pitt.edu)
19

20 **KEYWORDS:**
21 neurodegeneration, ALS, Huntington's disease, C9orf72, proteotoxicity, repeat expansion
22 disorders
23

24 **SUMMARY:**
25 Repeat-associated non-ATG-dependent translational products are emerging pathogenic features
26 of several repeat expansion-based diseases. The goal of the protocol described is to evaluate
27 toxicity caused by these peptides using behavioral and cellular assays in the model system *C.*
28 *elegans*.
29

30 **ABSTRACT:**
31 *C. elegans* is commonly used to model age-related neurodegenerative diseases caused by repeat
32 expansion mutations, such as Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease.
33 Recently, repeat expansion-containing RNA was shown to be the substrate for a novel type of
34 protein translation called repeat-associated non-AUG-dependent (RAN) translation. Unlike
35 canonical translation, RAN translation does not require a start codon and only occurs when
36 repeats exceed a threshold length. Because there is no start codon to determine the reading
37 frame, RAN translation occurs in all reading frames from both sense and antisense RNA templates
38 that contain a repeat expansion sequence. Therefore, RAN translation expands the number of
39 possible disease-associated toxic peptides from one to six. Thus far, RAN translation has been
40 documented in eight different repeat expansion-based neurodegenerative and neuromuscular
41 diseases. In each case, deciphering which RAN products are toxic, as well as their mechanisms of
42 toxicity, is a critical step towards understanding how these peptides contribute to disease
43 pathophysiology. In this paper, we present strategies to measure the toxicity of RAN peptides in
44 the model system *C. elegans*. First, we describe procedures for measuring RAN peptide toxicity

45 on the growth and motility of developing *C. elegans*. Second, we detail an assay for measuring
46 postdevelopmental, age-dependent effects of RAN peptides on motility. Finally, we describe a
47 neurotoxicity assay for evaluating the effects of RAN peptides on neuron morphology. These
48 assays provide a broad assessment of RAN peptide toxicity and may be useful for performing
49 large-scale genetic or small molecule screens to identify disease mechanisms or therapies.
50
51 **INTRODUCTION:**
52 The inappropriate expansion of DNA repeat sequences is the genetic basis for several
53 neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), frontotemporal
54 dementia (FTD), and Huntington's disease (HD)[1]. While there are established cellular and animal
55 models for these diseases, mechanisms underlying these conditions are not well defined. For
56 example, HD is caused by the expansions of a CAG repeat sequence in the coding sequence for
57 the Huntingtin protein Htt[2]. Because CAG encodes the amino acid glutamine, the CAG repeat
58 expansion results in the insertion of a polyGlutamine, or polyQ, sequence within Htt. Expanded
59 polyQ proteins form length- and age-dependent protein aggregates that are associated with
60 toxicity[3,4]. Surprisingly, two recent studies suggest that the length of the polyQ sequence is not
61 the main driver of HD disease onset, suggesting that polyQ-independent factors may also
62 contribute to the disease[5,6].
63
64 One possible polyQ-independent mechanism involves a newly discovered type of protein
65 translation termed Repeat Associated Non-AUG-dependent (RAN) translation[7]. As its name
66 implies, RAN translation only occurs when an expanded repeat sequence is present and does not
67 require a canonical start codon. Therefore, RAN translation occurs in all three reading frames of
68 the repeat to produce three distinct polypeptides. In addition, because many genes also produce
69 an antisense transcript that contains the reverse complement of the expanded repeat sequence,
70 RAN translation also occurs in all three reading frames of the antisense transcript. Together, RAN
71 translation expands the number of proteins produced from an expanded repeat-containing DNA
72 sequence from one peptide to six peptides. To date, RAN translation has been observed in at
73 least eight different repeat expansion disorders[8]. RAN peptides are observed in postmortem
74 patient samples and only in cases where the patient carries an expanded repeat[9,10]. While these
75 peptides are clearly present in patient cells, their contribution to disease pathophysiology is
76 unclear.
77
78 To better define the potential toxicity associated with RAN peptides, several groups have
79 expressed each peptide in various model systems, such as yeast, flies, mice, and tissue culture
80 cells[11-16]. Rather than utilizing the repeat sequence for expression, these models employ a codon-
81 variation approach in which the repeat sequence is eliminated but the amino acid sequence is
82 preserved. Translation initiation occurs through a canonical ATG and the peptide is typically fused
83 to a fluorescent protein at either the N- or C-terminus, neither of which appears to interfere with
84 RAN peptide toxicity. Therefore, each construct overexpresses a single RAN peptide. Modeling
85 the different RAN products in a multicellular organism with simple assays to measure RAN
86 peptide toxicity is vitally important to understand how the different RAN products from each
87 disease-causing repeat expansion contribute to cellular dysfunction and neurodegeneration.
88

89   Like other model systems, *C. elegans* provides a flexible and efficient experimental platform that
90   enables studies of new disease mechanisms, such as RAN peptide toxicity. Worms offer several
91   unique experimental attributes that are not currently available in other models of RAN peptide
92   toxicity. First, *C. elegans* are optically transparent from birth until death. This allows for simple
93   visualization of RAN peptide expression and localization, as well as in vivo analysis of
94   neurodegeneration in live animals. Second, transgenic methods for generating RAN peptide
95   expression models are inexpensive and fast. Given the short three-day life cycle of *C. elegans*,
96   stable transgenic lines expressing any given RAN peptide in a cell-type specific manner can be
97   produced in under a week. Third, simple phenotypic outputs can be combined with genetic
98   screening methods, such as chemical mutagenesis or RNAi screening, to rapidly identify genes
99   essential for RAN peptide toxicity. Finally, the short lifespan of *C. elegans* (~20 days) allows
100   investigators to determine how aging, which is the greatest risk factor for most repeat expansion
101   diseases, influences RAN peptide toxicity. Together, this combination of experimental attributes
102   is unmatched in any other model system and offers a powerful platform for the study of RAN
103   peptide toxicity.
104
105   Here we describe several assays that leverage the experimental advantages of *C. elegans* to
106   measure the toxicity of RAN peptides and to identify genetic modifiers of this toxicity. The codon-
107   varied ATG-initiated RAN peptides are tagged with GFP and expressed individually in either
108   muscle cells under the *myo-3* promoter or in GABAergic motor neurons under the *unc-47*
109   promoter. For expression in muscle cells, it is important that toxic RAN peptides are tagged with
110   green fluorescent protein (GFP), or other fluorescent protein (FP) tag that can be targeted with
111   an RNAi feeding vector. This is because toxic RAN peptide expression usually blocks growth,
112   rendering such strains nonviable. The use of *gfp(RNAi)* conditionally inactivates RAN peptide
113   expression and allows strain maintenance, genetic crosses, etc. For assays, these animals are
114   removed from *gfp(RNAi)*, which allows expression of the RAN peptide and the resulting
115   phenotypes. In addition to the molecular strategy for designing codon-varied RAN peptide
116   expression constructs, we describe assays for measuring developmental toxicity (larval motility
117   and growth assay), post-developmental age-associated toxicity (paralysis assay), and neuron
118   morphological defects (commissure assay).
119
120   **PROTOCOL:**
121
122   **1. Generating codon-varied RAN peptide expression constructs**
123
124   1.1. Design the individual RAN peptide coding sequence utilizing synonymous codons to eliminate
125   the fundamental repetitive DNA/RNA structure but preserve the overlying amino acid sequence.
126
127   1.2. Order the custom codon sequences commercially at the repeat lengths needed for the
128   studies (typically 5–100 repeats). Include a HindIII restriction site at the 5' end and a BamHI
129   restriction site at the 3' end to facilitate cloning into *C. elegans* expression vectors, such as
130   pPD95.79.
131
132   NOTE: If synthesis of larger constructs proves difficult, smaller building block sequences can be

133   synthesized and then built into larger repeats using a directional ligation strategy[17].
134
135   1.3. Subclone the peptide sequence into a *C. elegans* expression vector using standard T4 ligation
136   methods.
137
138   1.4. Subclone a cell type-specific promoter sequence generated by PCR in front of the RAN
139   peptide sequence to drive tissue-specific RAN peptide-GFP expression.
140
141   1.5. Microinject the RAN peptide construct into the gonad of wild type *C. elegans* to generate
142   transgenic strains containing extrachromosomal arrays using standard methods[18]. For a co-
143   injection marker, the muscle-specific RFP reporter (*myo-3p::RFP* (*pCFJ104*)) was utilized,
144   although other markers can be utilized as well.
145
146   1.6. Integrate a stable transgene into the genome using standard *C. elegans* integration screening
147   methods[19].
148
149   1.7. If the RAN peptide is toxic and transgenic animals do not survive, feed animals *gfp(RNAi)*
150   expressing bacteria pre- and post-injection to silence the expression of the toxic protein.
151
152   NOTE: Removal of animals from *gfp(RNAi)* enables RAN peptide expression for phenotypic
153   analyses using the assays described below.
154
155   **2. Measuring the developmental toxicity of RAN peptides following RNAi-based gene**
156   **knockdown: Video speed analysis protocol**
157
158   2.1. Pour standard nematode growth medium (NGM) RNAi 24 well plates with 1 mM isopropyl β-
159   D-1-thiogalactopyranoside (IPTG) and 25 µg/mL carbenicillin.
160
161   2.2. Streak out RNAi-feeding clones in HT115 bacteria to be tested onto Luria broth (LB) and
162   carbenicillin (25 µg/mL) agar plates and grow bacteria at 37 °C for 24 h. Include *empty*
163   *vector(RNAi)* as the positive control for toxicity and *gfp(RNAi)* as the negative control.
164
165   2.3. For each clone, pick a single colony into 1 mL of LB + carbenicillin liquid media and grow
166   bacteria overnight for 18 h at 37 °C while shaking at 250 rotations per minute.
167
168   2.4. Spot four individual wells of the NGM RNAI 24 wells with 20 µL of the following overnight
169   bacterial cultures: column 1 wells = *gfp(RNAi)*; column 2 wells = *(EV)RNAi*; columns 3–6 wells =
170   RNAi against genes to be tested. Allow RNAi bacteria to induce dsRNA production overnight at
171   room temperature (RT).
172
173   2.5. Isolate eggs from day one adult *C. elegans* expressing the integrated RAN peptide transgene
174   using the standard hypochlorite method and seed ~30 *C. elegans* eggs into each well of the NGM
175   RNAi 24 well plate.
176

177   2.6. Incubate the 24 wells at 20 °C for 7 days.
178
179   NOTE: This incubation time is for strains expressing toxic C9orf72 RAN dipeptides, such as PR50
180   or GR50. Other strains may require shorter incubation times to prevent exhaustion of the
181   bacterial food source and subsequent starvation.
182
183   2.7. Acquire transmitted light videos using a stereo dissecting microscope fitted with a
184   monochrome camera connected to a computer running video acquisition software.
185
186   2.7.1. Acquire two videos from two separate wells for each RNAi condition using consistent video
187   acquisition settings between wells. For each video, acquire 10 s at 800 x 600 pixel resolution and
188   13.16 frames per second (time voxel dimension = 0.076 s) using 18.6x magnification (1.49 zoom
189   setting in AF software). Set the image exposure time to 4 ms. Label each video immediately with
190   the strain, RNAi condition, and well number.
191
192   2.8. Blind the experimenter to the genotype associated with each video file by randomizing the
193   order of the videos and changing the name of the videos to numbers. Save this group of videos
194   as a new file. Unblind the experimenter after the analysis is complete.
195
196   2.9. Measure the distance traveled and the length of each animal for 20 different animals from
197   each video, examining a total of ~40 worms for each RNAi condition.
198
199   2.9.1. In the software, select the distance measurement tool. Click on a point in the center of the
200   worm being measured in the first frame of the video. Advance the video to the end while visually
201   tracking the movement path of the worm. In last frame, click on a point in the center of the worm
202   being measured.
203
204   2.9.2. "Distance Traveled" is the distance between the two centroid points. This distance is shown
205   adjacent to the measurement line. Record this measurement in a spreadsheet. Repeat this
206   measurement for 19 other worms in the video while preserving each individual measurement in
207   the analysis window.
208
209   2.10. Make measurements from animals exhibiting the most robust movement to avoid the
210   selection of animals exhibiting no movement and biasing the data towards paralysis. Once the
211   measurements are complete, take a snapshot of the final video frame illustrating all of the
212   measurement lines so that the data can be traced back to the animal from which they originated.
213
214   2.11. Analyze the data using a four column spreadsheet. Column one is the "Worm Identifier"
215   (name or number), column two is the "Speed" (distance traveled/time), column three is the
216   "Animal Length", and column four is the "Normalized Speed" (speed/animal length). Analyze the
217   normalized speed data using a One-way ANOVA with post-hoc testing versus *empty vector(RNAi).*
218
219   **3. Measuring developmental toxicity of RAN peptide: Growth assay**
220

221 3.1. Pick ~30 gravid animals into a 50 µL spot of hypochlorite solution (10 mL of bleach, 2.5 mL of
222 10 N NaOH, 37.5 mL of dH$_2$O) on either a *gfp(RNAi), (EV)RNAi,* or *(gene-specific)* 6 cm *RNAi* plate.
223
224 3.2. After 24 h, move six transgenic progeny that have crawled out of the hypochlorite solution
225 spot to a new 6 cm *RNAi* plate identical to the RNAi conditions on the plate on which they were
226 subjected to the hypochlorite solution treatment. Put these progeny (F1) at 20 °C to grow and
227 reproduce.
228
229 3.3. After 24–48 h, pick 10 transgenic L4 animals onto 6 cm RNAi matching the RNAi conditions
230 that the animals have been growing on. Allow the animals to pulse lay eggs for 24 h in a 20 °C
231 incubator.
232
233 3.4. After 24 h, remove the adult animals and discard them. Count the number of eggs and L1
234 larvae laid during the 24 h period using a mechanical handheld counter. This is the total brood
235 size.
236
237 3.5. Over the next 72 h, count the number of animals that reach the L4 or older stage. As each
238 animal is counted, remove it from the plate.
239
240 3.6. Quantify the "Percent Growth" as the percentage of animals that reach the L4 or older stage
241 out of the total brood size.
242
243 3.7. Perform a 2 x 2 Fisher's exact test versus *empty vector(RNAi)* to determine if the percent
244 growth is statistically significant. The categories for comparison are "Growth" (# of animals that
245 reached the L4 or older stage in 72 h) and "No Growth" (total brood size minus the number of
246 animals reaching L4 or older stage in 72 h).
247
248 **4. Post-developmental RAN peptide paralysis assay**
249
250 4.1. Maintain integrated *C. elegans* transgenic strains expressing RAN peptide-GFP in the muscle
251 on *gfp*(RNAi) by placing 4–6 transgenic L4's on a 6 cm *gfp*(RNAi) plate and grow the worms at 20
252 °C.
253
254 4.2. If the experiment is utilizing RNAi to test for genetic effects on RAN peptide toxicity, move
255 10 transgenic gravid adults from an unstarved plate to each of two 6 cm *empty vector*(RNAi)
256 (positive control for paralysis, negative control for genetic effects), *gfp(RNAi)* (negative control
257 for paralysis, positive control for genetic effects), *gene-specific*(RNAi) (i.e., 10 gravid adults per 6
258 cm plate).
259
260 4.3. If mutants are being used to analyze genetic effects on RAN peptide toxicity, place 10 gravid
261 WT or mutant animals expressing the same RAN transgene on each of two 6 cm *empty*
262 *vector*(RNAi) or *gfp(RNAi)* plates. Grow the worms at 20 °C for 48 h.
263

264 4.4. Pick 10 sets of 10 transgenic L4's from the 6 cm RNAi plates and place each set on a 3 cm
265 RNAi plate (i.e., n = 100 animals for each genotype). Ensure that the L4's selected for the assay
266 all have superficially normal motility. Place the plates within a zipper storage bag to retain
267 moisture.
268
269 4.5. Place the bagged plates with L4's in the 25 °C incubator.
270
271 4.6. Remove the animals 24 h later from the 25 °C incubator one strain at a time to minimize the
272 amount of time they are out at RT.
273
274 4.7. Tap the plates on the dissecting microscope to check for movement. If the animals move
275 more than a body length, count the animal as mobile and transfer it to a new 3 cm RNAi plate.
276 Use a platinum pick to tap the remaining worms on the head or tail. If the animal moves more
277 than a body length, count the animal as mobile and transfer it to a new 3 cm RNAi plate.
278
279 NOTE: Do not exceed 10 worms per plate when transferring. Be careful to avoid moving progeny
280 to the new RNAi plate because the assay depends on following aged animals only.
281
282 4.8. Group all of the nonmoving worms together so that movement of more than a body length
283 can easily be detected. Give the animal at least one minute to move more than one body length.
284 If it still does not move, it is paralyzed, bagged (i.e., larvae hatched inside mother), or dead.
285
286 4.9. Censor animals that exhibit bagging, desiccate on the sides of the plate, exhibit extruded
287 intestines, burrow, become lost, or die from the assay at the time of detection. Paralyzed worms
288 are counted, left on the old RNAi plate, and discarded.
289
290 4.10. Score the animals for paralysis every day for 5–7 days as described in steps 4.6–4.9.
291
292 4.11. Analyze paralysis data with a log-rank test identical to the one used to analyze lifespan. In
293 this statistical analysis, moving worms are scored as "Alive", paralyzed worms are scored as
294 "Dead", and dead, bagged, gut extruded, desiccated, burrowed, or otherwise lost worms are
295 scored as "Censored".
296
297 NOTE: In this analysis, the "Percent Alive" number indicates the "Percent Moving" animals. The
298 inverse represents the "Percent Paralyzed". Use the online analysis tool OASIS
299 (https://sbi.postech.ac.kr/oasis2/) to perform the log-rank statistical analyses.
300
301 **5. Measuring neuron pathology: Commissure assay**
302
303 5.1. Generate transgenic *C. elegans* expressing the RAN peptide of interest in the GABAergic
304 neurons using the *unc-47* promoter. Also express *unc-47p::GFP* or *unc-47p::RFP* to reveal the
305 cellular morphology of the GABA neurons.
306
307 5.2. Pick 50 transgenic L4 animals and place them on a 6 cm OP50 plate at 25 °C for 24 h.

308

309   5.3. Transfer the 50 transgenic animals onto a new 6 cm OP50 plate at 25 °C for 24 h.

310

311   5.4. Make agarose pads for imaging the animals under widefield microscopy.

312

313   5.4.1. Place two pieces of tape on top of two microscope slides. Place a cleaned slide without
314   tape in the middle of the two taped slides.

315

316   5.4.2. Dispense ~100 µL (1 drop from a disposable plastic Pasteur pipette) of 3% molten agarose
317   onto the middle slide with a disposable sterile transfer pipette.

318

319   5.4.3. Immediately place a second clean slide across the drop of molten agarose so that it rests
320   on the taped slides and creates a thin and even layer of agarose between the slides.

321

322   5.4.4. After the agarose has cooled and solidified, carefully remove the two slides with the layer
323   of agarose between them, without separating them, and place them in a plastic bag with a damp
324   piece of paper under them. Make one slide per 10 worms to be examined along with three extra
325   slides.

326

327   5.5. Remove the transgenic *C. elegans* from the 25 °C incubator and pick 10 transgenic *C. elegans*
328   into a 100 µL drop of 10 mM levamisole in a glass depression slide. Incubate for 10 min or until
329   animals are paralyzed.

330

331   5.6. Remove a slide pair from the plastic bag and carefully separate them. Label the slide with
332   the agarose with the genotype and add 2 µL of 10 mM levamisole to the middle of the agarose.
333   Move the 10 animals into the levamisole on the agarose pad. Cover the animals with a #1
334   thickness coverslip.

335

336   5.7. Image animals in which the vulva is oriented on the right side of the animal. In this
337   orientation, the commissures of the motor neurons are nearest to the coverslip and are clearly
338   visible on an inverted widefield fluorescence microscope. If the vulva is on the left side of the
339   head, the commissures of the motor neurons will be underneath the animals and not as clearly
340   visible, making accurate quantification difficult. Omit quantification of these animals.

341

342   5.7.1. Visualize the neuron commissures expressing GFP with an inverted widefield fluorescence
343   microscope with a 63x 1.4x NA oil immersion lens and a GFP filter set (Ex480/40 nm; Em527/30
344   nm). If the neuron commissures express RFP instead of GFP, utilize a red fluorescent protein (RFP)
345   filter set (Ex560/40 nm; Em 645/75 nm).

346

347   5.8. Image the worms within 45 min of placing the coverslip on the worms to minimize toxicity
348   from immobilization.

349

350   5.9. For each worm, count the number of visible commissures. There are 16 visible commissures
351   in wild type animals. Also count the number of commissures that have large beads (i.e., blebs) in

the commissures as well as the number of commissures that are broken. To confirm the commissure is broken, follow the commissure from the dorsal cord to the ventral cord by adjusting the focal plane.

NOTE: Commissures in which the *unc-47p::GFP* fluorescence exhibits a gap are considered broken. A broken commissure typically has a bleb on either side of the break, helping show that the commissures is broken. Count the amount of blebbing and breaks for 20 worms.

5.10. Calculate the fraction of commissures with blebs or breaks over the total number of observed commissures for each animal. The absolute number of commissures counted per animal (including wild type, blebbed, and broken events) can also be measured.

5.11. For each category, calculate the mean ± SD and statistically analyze with either a Student's t-test for comparison between two populations, or a one-way ANOVA for comparisons between 3 or more populations.

REPRESENTATIVE RESULTS:
We used the assays described here to evaluate the effect of different gene inhibitions on the toxicity of RAN dipeptides that are found in ALS patients with a G_4C_2 repeat expansion. Using the growth assay to measure developmental toxicity, we analyzed the effects of several genetic knockout mutants identified in a genome-wide RNAi screen suppressors of muscle-expressed PR50-GFP toxicity. While expression of PR50-GFP alone resulted in a completely penetrant growth arrest, loss of function mutations in several genes suppressed PR developmental toxicity from 12–94% (**Figure 1A**).

We also measured the effect of specific gene knockdowns on the developmental motility of PR50-expressing animals using the video speed analysis method. As expected, *gfp(RNAi)* resulted in a large increase in motility compared to *empty vector(RNAi)* due to inhibition of PR50-GFP expression. We also discovered that RNAi against the proteasome subunit *rpn-7* resulted in a significant increase in PR50 motility (**Figure 1B**).

ALS, like many neurodegenerative diseases, occurs in adults. Therefore, we analyzed adult phenotypes using the age-dependent paralysis assay. PR50-GFP exhibited up to 80% paralysis by 5 days of age. However, RNAi directed at the gene *cul-6* significantly delayed paralysis, suggesting that *cul-6* is required for PR50-GFP toxicity (**Figure 1C**). This effect was specific to *cul-6(RNAi)* because *cul-1(RNAi)* did not alter PR50-GFP toxicity.

Neurodegenerative proteins, such as toxic RAN peptides, are commonly modeled in the body wall of *C. elegans*[4,20-22]. However, it is also important to determine if RAN proteins cause neuropathology when expressed in *C. elegans* neurons, because neuron-specific toxicity is a common feature of many neurodegenerative diseases. We examined neuron-specific toxicity using the commissure assay. In day 2 adults, PR50-GFP expression in the motor neurons led to a significant increase in motor neuron blebbing. This neuropathology was significantly suppressed by a mutation in the insulin/IGF receptor gene homolog *daf-2* that delays the toxic properties of

396   several neurodegenerative proteins[23] (**Figure 2B**).

397

398   **FIGURE AND TABLE LEGENDS:**
399   **Figure 1: Assays to evaluate the toxicity of muscle-expressed RAN peptides.** (**A**) RAN peptide
400   growth assay. The indicated mutants were crossed into the *drIs34* (*myo-3p::PR50-GFP*) genetic
401   background under *gfp(RNAi)* conditions. Numbers above each genotype indicate the number of
402   progeny scored for growth. All genotypes have p < 0.05 using the Fisher's exact test as compared
403   to wild type. (**B**) Video speed analysis to measure RAN peptide toxicity during development.
404   *drIs34 (myo-3p::PR50-GFP)* animals were grown under *gfp(RNAi)*, *empty vector(RNAi)*, or *rpn-*
405   *7(RNAi)* conditions and then scored for motility as described. Speed was normalized to *gfp(RNAi)*
406   treated animals. Data shown are mean ± SD, n = 40 for each genotype. **-p < 0.01, ***- p < 0.001,
407   one-way ANOVA with Tukey post-test. (**C**) Paralysis assay to measure age onset toxicity. *drIs34*
408   *(myo-3p::PR50-GFP)* animals were grown on *empty vector(RNAi)* ('WT'), *cul-1(RNAi)*, or *cul-*
409   *6(RNAi)* and paralysis was scored as described every 24 h. ***-p < 0.001, log-rank test with
410   Bonferroni post-test correction vs. WT. n = 100 animals per genotype.

411

412   **Figure 2: Assay for measuring neuropathology of motor neuron expressed RAN peptides.** (**A**)
413   Images of adult *C. elegans* expressing the *unc-47p::GFP* motor neuron reporter in wild type or
414   *unc-47p::PR50-GFP* expressing animals. The images for wild type illustrate normal commissure
415   morphology. Images represent flattened Z-series stacks obtained by widefield fluorescence
416   microscopy. PR50 animals demonstrate examples of commissure breakage and blebbing. (**B**)
417   Effect of *daf-2(e1370)* on PR50 commissure blebbing. Points represent the percent blebbing
418   commissures from a single animal, with the mean and SD shown. n = 20 animals per genotype
419   **-p < 0.01, one-way ANOVA with Dunn's post-hoc test.

420

421   **DISCUSSION:**
422   Here we report methods that can be used to assay RAN peptide toxicity modeled in the muscle
423   or in the neurons of *C. elegans*. While neurodegenerative proteins have an age onset phenotype
424   in human patients, they can also exhibit developmentally toxicity when overexpressed in model
425   systems. Overexpression has significant interpretive limitations, but it also provides a powerful
426   starting point for genetic or pharmacological screens aimed at identifying genes or drugs that can
427   reverse toxic phenotypes. This is especially important given that most precise animal models of
428   disease have either no phenotype or weak phenotypes not suitable for unbiased screening
429   approaches[24-26]. Our *C. elegans* RAN peptide models and assays represent a powerful,
430   complementary approach to other RAN peptide model systems, such as yeast and *Drosophila*,
431   for understanding the cellular pathways important for the toxicity of these newly described
432   proteins.

433

434   **Conditional toxicity of muscle-expressed RAN peptides**
435   Measuring RAN peptide toxicity requires a chase period for elimination of the effects of
436   *gfp(RNAi)*. However, the time between removal from *gfp(RNAi)* and the emergence of
437   phenotypes can be inconsistent if care is not taken to precisely time the initiation of experiments,
438   selection of staged animals, temperature shifts, etc. As we have become more experienced with
439   these assays, the timing and penetrance of RAN peptide phenotypes has become relatively

440 consistent. One of the most critical steps in these assays is the proper shift of animals to 25 °C.
441 Without this shift, RAN peptides exhibit significantly weaker toxicity. However, animals cannot
442 be continually grown at 25 °C because they do not grow and reproduce, presumably due to higher
443 baseline expression of the RAN peptide. This is not unlike the situation in yeast or *Drosophila*
444 where strains expressing toxic RAN peptides are kept under permissive conditions (i.e., low
445 temperature) but then acutely shifted to restrictive conditions (i.e., higher temperature) prior to
446 the assay[13,15] to enhance peptide expression. In such conditional expression systems, genetic
447 conditions that enhance or suppress toxicity could result from changes in peptide expression
448 levels. To determine if genetic modifiers of RAN peptide toxicity act via alterations in transgene
449 expression levels, we take two approaches. First, many of our transgenic strains express a second
450 RFP reporter under the control of the same promoter used to drive expression of the RAN
451 peptide. Changes in promoter activity that cause reduced or increased RAN peptide expression
452 cause similar reductions or increases in RFP levels. We consider mutants or RNAi conditions that
453 significantly alter RFP levels to act nonspecifically. Second, we perform quantitative PCR and
454 Western blotting to determine if the levels of RAN peptide mRNA or protein are altered between
455 conditions. However, Western-based detection of RAN peptides can sometimes prove difficult or
456 impossible, as we and others have observed with the C9orf72-derived RAN peptide PR50-GFP. In
457 such cases, we quantify in vivo GFP levels to determine if protein expression is altered between
458 the conditions being compared.
459
460 **Developmental toxicity: Advantages and limitations**
461 Forced overexpression of toxic RAN peptides in muscle often leads to developmental arrest in *C.*
462 *elegans*. While this clearly does not mimic human disease pathology, it provides a powerful
463 starting point for genetic suppressor screens, because RAN toxicity suppressors are easily
464 identified as animals that grow and reproduce in the absence of *gfp(RNAi)*. Some suppressors
465 will facilitate growth due to reduction of transgene expression. To differentiate between these
466 possibilities, we generally include a second RFP reporter driven by the same promoter in our RAN
467 peptide transgenic strain. Suppressors that reduce or silence transgene expression will also
468 exhibit reduced RFP expression levels. On the other hand, suppressors that exhibit normal or
469 elevated RFP levels are unlikely to function via reduction of transgene expression. Much like the
470 use of yeast growth or *Drosophila* eye morphology[13,15,27], these approaches, while not precisely
471 modeling aspects of the disease in humans, provide a powerful screening tool for the initial
472 identification of RAN peptide modifiers.
473
474 **Post-developmental toxicity (paralysis assay): Advantages and limitations**
475 The advantage of the paralysis assay is that a relative large number of animals (50–100) can be
476 measured with tools present in any worm lab. Paralysis is also a severe phenotype that is easily
477 detected. The main limitation of this assay is that is insensitive to movement defects that do not
478 cause complete paralysis. Such defects may require more sensitive and quantitative approaches
479 to measure, such as the thrashing assays or kinematic movement assays[28]. Paralysis assays should
480 be performed blinded to genotype if possible and care should be taken to ensure that worms are
481 not undergoing any other type of stress (e.g., contamination, starvation), that could significantly
482 impact the assay results. *C. elegans* expressing toxic RAN peptides sometimes fail to exhibit a
483 robust paralysis phenotype. This is likely due to persistent effects of *gfp(RNAi)* continuing to

484   suppress RAN peptide expression. In these cases, if we fail to observe significant paralysis (>10%)
485   in the *empty vector(RNAi)* control animals after day 2, we typically terminate the assay and
486   initiate another replicate. One modification of this assay could involve the use of alternative
487   inducible RAN peptide expression systems. The only other commonly used inducible expression
488   system for *C. elegans* is the heat shock promoter. However, the required heat shock can cause
489   stress responses that might affect the toxicity of the proteins. The future application of other
490   conditional expression systems, such as the auxin-inducible degron (AID) system[29], could
491   significantly enhance our ability to study RAN peptide toxicity.
492
493   **Neurodegeneration/commissure assay: Advantages and limitations**
494   Motor neuron commissures in *C. elegans* represent the axon of one motor neuron passing
495   between the ventral and dorsal nervous cords. Blebbing and breakage can be easily detected in
496   the isolated commissures, allowing neurodegeneration to be quickly quantified in live animals
497   (**Figure 2**). While examining the animals, it is important they do not incubate in levamisole for an
498   extended period of time as this can cause premature animal death, which leads to neuronal
499   blebbing and breakage in the absence of any toxic RAN peptide. In some cases, commissures are
500   completely degenerated and no longer visible. Therefore, they cannot be scored for
501   neuropathological features because our assay measures the percentages of commissures broken
502   or blebbing out of the total number of observed commissures. In these cases, the commissure
503   assay might underestimate the effect of the RAN peptide.
504
505   In conclusion, the assays described in this paper are useful for measuring toxicity caused by RAN
506   peptides in *C. elegans*. Using *gfp(RNAi)* to regulate expression of the proteins allows for post-
507   developmental phenotypes to be observed. Our approaches can be easily adapted to perform
508   large-scale genetic screens for suppressors or enhancers of toxicity. Secondary assays, such as
509   the paralysis assay and commissure assay can confirm that toxicity is suppressed post-
510   developmentally and test if the mechanism of suppression is conserved in neurons.
511

514

517

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588

Figure 1

Figure 1
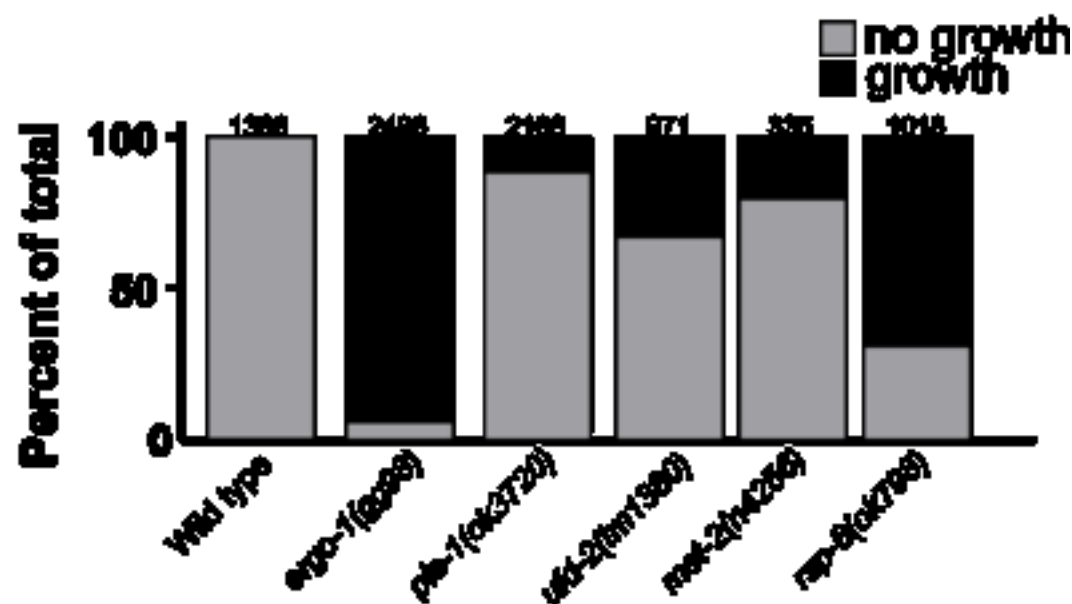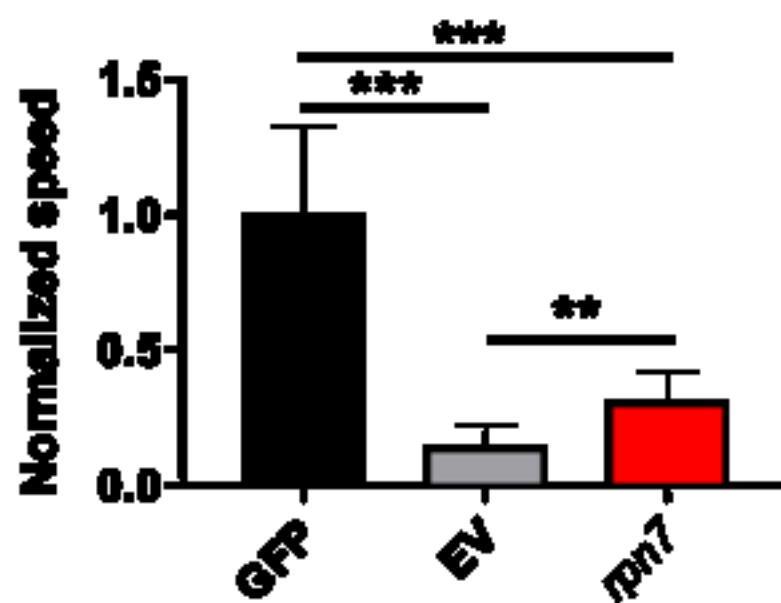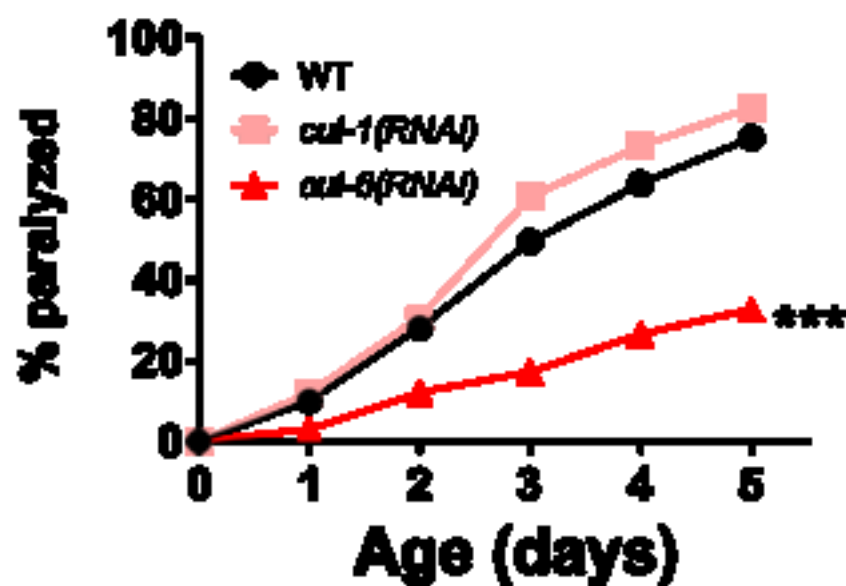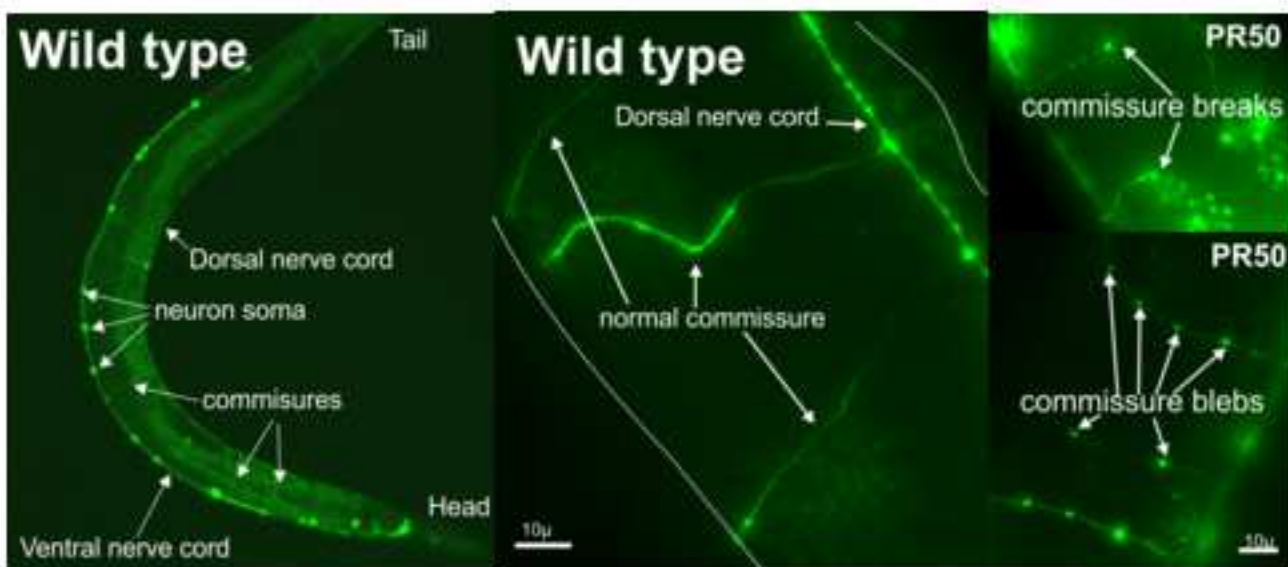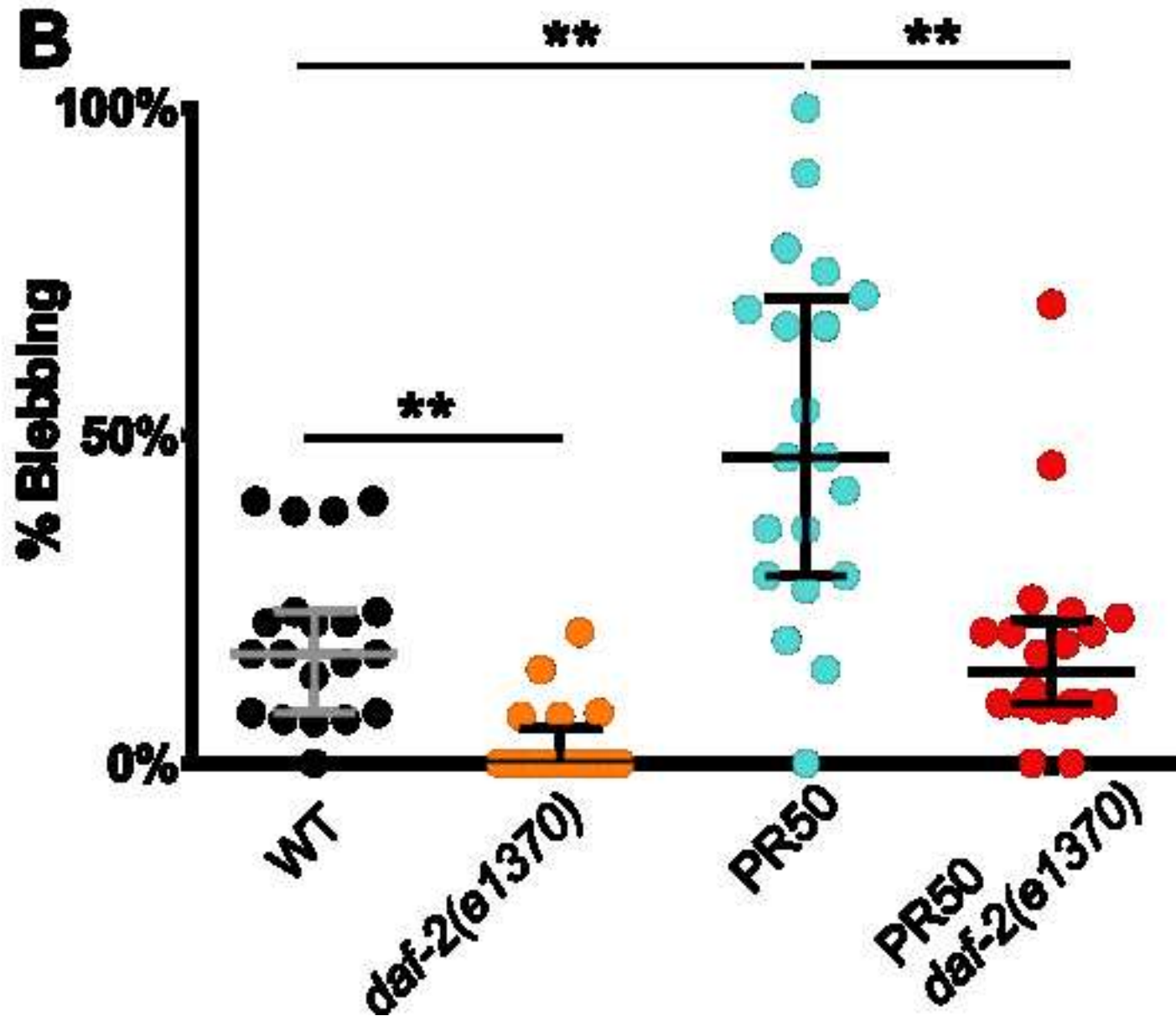
Name of Material/ Equipment	Company	Catalog Number
35mm x 10mm Petri Dish, Sterile	CELLTREAT Scientific Products	50-202-036
AGAR GRANULATED 2KILOGRAM	BD DIAGNOSTIC SYSTEMS	DF0145070
AGAROSE ULTRAPURE	LIFE TECHNOLOGIES	16500500
CARBENICILLIN 5G	THERMO SCI FAIRLAWN CHEMICALS	BP26485
COVER GLASSES NO 1 22MM 1OZ/PK	THERMO SCI ERIE	12542B
FEMOTIPS DISPSBL MICROINJ 20CS	EPPENDORF NORTH AMERICA BIOTOOLS	E5242952008
FF COV GLASS NO1 40X22MM 1OZPK	THERMO SCI ERIE	125485C
Fisherbrand Superfrost Plus Microscope S	THERMO SCI ERIE	12-550-15
Gibco Bacto Peptone	Gibco	DF0118-17-0
HALOCARBON OIL 700	SIGMA-ALDRICH INC	H8898-50ML
IPTG BIOTECH 10G	THERMO SCI FAIRLAWN CHEMICALS	BP162010
Leica Advanced Fluorescence imaging sof	Leica Microsystems	LAS-AF
Leica Immersion type N (Oil)	W NUHSBAUM INC	NC9547002
LEVAMISOLE HYDROCHLORIDE 10GR	THERMO SCI ACROS ORGANICS	AC187870100
MICROLOADER TIPS 2 X 96 PCS	EPPENDORF NORTH AMERICA BIOTOOLS	E5242956003
PETRI DISH, 60X15MM,500/CS	CORNING LIFE SCIENCES PLASTIC	FB0875713A
TISSUE CULT PLATE 24WEL 50/CS	CORNING LIFE SCIENCES DL	87721

Comments/Description

Nematode growth plates and RNAi

Nematode growth plates and RNAi

Microinjection to generate RAN peptide transgenic strains

Nematode growth plates and RNAi

Imaging for commissure assay

Microinjection to generate RAN peptide transgenic strains

Microinjection to generate RAN peptide transgenic strains

Imaging for commissure assay

Nematode growth plates and RNAi

Microinjection to generate RAN peptide transgenic strains

Nematode growth plates and RNAi

Image acquisition software for video speed analysis and commissure assay

Imaging for commissure assay

Imaging for commissure assay

Microinjection to generate RAN peptide transgenic strains

Nematode growth plates and RNAi

Nematode growth plates and RNAi

Please list affiliations. Please also email addresses for all authors and specify the corresponding author.

- Affiliations and email addresses added. The corresponding author is now noted

Minimum 6 keywords/phrases required.

- Additional keywords added

Define (abbreviations)

- Defined NGM, IPTG, and LB in the text

mention growth conditions.

- Specific growth conditions for bacteria are now mentioned

What kind of videos? Mention imaging equipment used and mention all settings. Is this a standard inverted microscope? If fluorescence imaging is used mention excitation and emission settings.

- Defined the video type. Mentioned all of the equipment utilized and all settings. Method does not use fluorescence imaging so Ex/Em settings not included.

Describe software actions in detail using button click and menu selections in order to enable scripting and filming. List the software used in the table of materials.

- More detail was added describing software actions and the software was added to the table of materials

Please check

- '…plate two each of two…' changed to '…plate to each of two…'

Mention all settings including magnification, lens NA, fluorescence emission and excitation filter settings

- Additional details on magnification, lens NA, and fluorescence filter settings added