Isolation of *C. elegans* Deletion Mutants Following ENU Mutagenesis and Thermostable Restriction Enzyme PCR Screening

Chunyi George Huang,[1] Peter Agre,[1] Kevin Strange,[2] and Todd Lamitina[2,]*

Abstract

The ability to generate null mutants is essential for studying gene function. Gene knockouts in *Caenorhabditis elegans* can be generated in a high throughput manner using chemical mutagenesis followed by polymerase chain reaction (PCR) assays to detect deletions in a gene of interest. However, current methods for identifying deletions are time and labor intensive and are unable to efficiently detect small deletions. In this study, we expanded the method pioneered by Wei et al., which used the thermostable restriction enzyme *Psp*GI and tested the usefulness of other thermostable restriction enzymes including *Bst*UI, *Tsp*45I, *Ape*KI, and *Tfi*I. We designed primers to flank one or multiple thermostable restriction enzymes sites in the genes of interest. The use of multiple enzymes and the optimization of PCR primer design enabled us to isolate deletion in 66.7% of the genes screened. The size of the deletions varied from 330 bp to 1 kb. This method should make it possible for small academic laboratories to rapidly isolate deletions in their genes of interest.

Index Entries: Mutation; polymerase chain reaction; ethylnitrosourea.

To generate knockouts in *Caenorhabditis elegans* in a high throughput manner, chemical mutagens are used to induce deletions in a library of worms, followed by polymerase chain reaction (PCR) detection assays designed to screen desired regions of the genome ***(1–5)***. Multiple mutagens, including ethyl methansulfonate (EMS), ethylnitrosourea (ENU), and ultraviolet-activated trimethylpsoralen (UV-TMP) induce detectable deletions at comparable frequencies ***(1–5)***.

Detection of deletion mutants depends on the ability to PCR amplify a smaller-sized template from a background of 1000 or more wild-type (WT) genomes. One approach to detect deletions is to limit the extension time of PCR and allow shorter templates to be preferentially amplified ***(1,2)***. Limitations of this method include low detection sensitivity (1:2000 ratio of deletion to WT), high background from the WT product, and an inability to efficiently detect deletions less than 600 bp ***(2,3)***. Because significant portions of deletions in a given library are less than 600 bp and deletions in small genes do not possess an advantage over WT template, another approach is required. One way to address these limitations involves the use of "poison primers" that handicap the amplification of WT template and allow the detection of small deletions at a sensitivity of 1:5000 ***(3)***.

A limitation of the poison primer method is that significant residual WT and poisoned products may persist ***(3)***. To circumvent these limitations, Wei et al. ***(5)*** made use of the thermostable restric-

*Author to whom all correspondence and reprint requests should be addressed. [1]Department of Biological Chemistry, Johns Hopkins University, Baltimore, MD; [2]Department of Anesthesiology, Vanderbilt University Medical Center, 1161 21st Avenue South, MCN T4202, Nashville, TN 37232-2520. Email: todd.lamitina@vanderbilt.edu

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tion endonuclease *Psp*GI. *Psp*GI has a half-life of 2 h at 95°C and is active during PCR ***(5)***. Digestion of a mutant DNA library with *Psp*GI theoretically cleaves all WT templates so that only deletions removing *Psp*GI sites within the screened regions are amplified successfully. In practice, however, small amounts of undigested WT template frequently persist following digestion. Amplification from these WT templates is further suppressed as a result of the inclusion of *Psp*GI during PCR, cleaving any residual WT templates and suppressing WT amplification. Using this approach, deletion alleles lacking *Psp*GI sites are preferentially amplified with no WT background at a sensitivity of 1 in 40,000 ***(5)***. In a library of 400,000 genomes, Wei et al. ***(5)*** demonstrated that the *Psp*GI method recovered six novel deletions in 15 screening intervals yielding a recovery rate of 30% ***(5)***. This result compares favorably with the poison primer ***(3)*** and limited extension ***(1,2)*** methods, which have recovery rates of approx 10 to 20% ***(5)***. The *Psp*GI method allows for greater pooling during PCR, lower proportion of false-positives, and higher likelihood of isolating a targeted deletion.

One limitation of the *Psp*GI method is the requirement for a *Psp*GI restriction site to be present at a desirable location within the targeted gene ***(5)***. However, not all genes contain such sites. By increasing the diversity of enzymes that can be used with this method, additional genes could be targeted. Even for genes that contain a *Psp*GI site, screening with multiple thermostable enzymes should greatly increase the deletion identification rate in any single gene of interest.

In this study, we examined the compatibility of other commercially available thermostable restriction enzymes for deletion screens. We designed nine sets of primers, corresponding to eight aquaporin (*aqp*) genes and a gene encoding a glycerol-3-phosphate dehydrogenase (*gpdh-2*). The average size of the genes was 1.6 kb making detection of deletions with limited extension methods difficult. In addition, two of the genes lacked *Psp*GI sites. We constructed an ENU-mutagenized library containing approx 800,000 genomes and pooled the DNA into approx 8000 genomes per PCR reaction, using established protocols ***(5,6)***. Prior to PCR, the library DNA was divided into five plates and each digested by one enzyme **(Fig. 1A)**. PCR primers were designed to flank single or closely spaced sites in one or more thermostable restriction enzymes including, *Psp*GI, *Bst*UI, *Ape*KI, *Tfi*I, and *Tsp*45I **(Fig. 1B)**. Increased detection sensitivity was obtained when the distance between PCR primers and the closest restriction enzyme site was ≥200 bp. Importantly, all of the restriction enzymes tested are active during PCR and effectively destroyed WT templates **(Fig. 1C–E)**.

Each set of digested DNA was used as a template for nested PCR reactions. Wells containing only WT copies of a targeted gene product produced no PCR bands while deletions were amplified **(Fig. 1C)**. For example, the *C. elegans aqp-5* gene contains recognition sequences for *Tsp*45I, *Ape*KI and *Tfi*I but not *Psp*GI. Following PCR screening of each set of template DNA, a deletion that eliminated the *Tsp*45I site, but not the *Ape*KI or *Tfi*I sites, was detected readily **(Fig. 1D)**. Similarly, primers to the *aqp-2* gene were designed to encompass one *Psp*GI, one *Bst*UI, and multiple *Tsp*45I sites. The deletion removed the single *Bst*UI site while leaving the *Psp*GI and two *Tsp*45I sites intact **(Fig. 1E)**. Therefore, without the use of multiple thermostable enzymes, these deletions would not have been detected.

Deletions were detected in six out of nine genes, with a total of nine alleles, yielding a recovery rate of 66.7% **(Table 1)**. Deletions were isolated in three out of nine genes using only *Psp*GI sites. Without designing primers to utilize other thermostable restriction enzyme sites, deletions would have been recovered in only 33% of the targeted genes. The size of deletions varied from 330 bp to 1 kb and we were able to isolate more than 70% of our initial positives as single mutant animals through three rounds of sib selection using previously established methods ***(1–5)***.

In conclusion, by expanding the repertoire of thermostable restriction enzymes and optimizing the design of PCR primers, we were able to isolate a diverse array of deletion mutants in greater than 60% of genes screened and double our yield

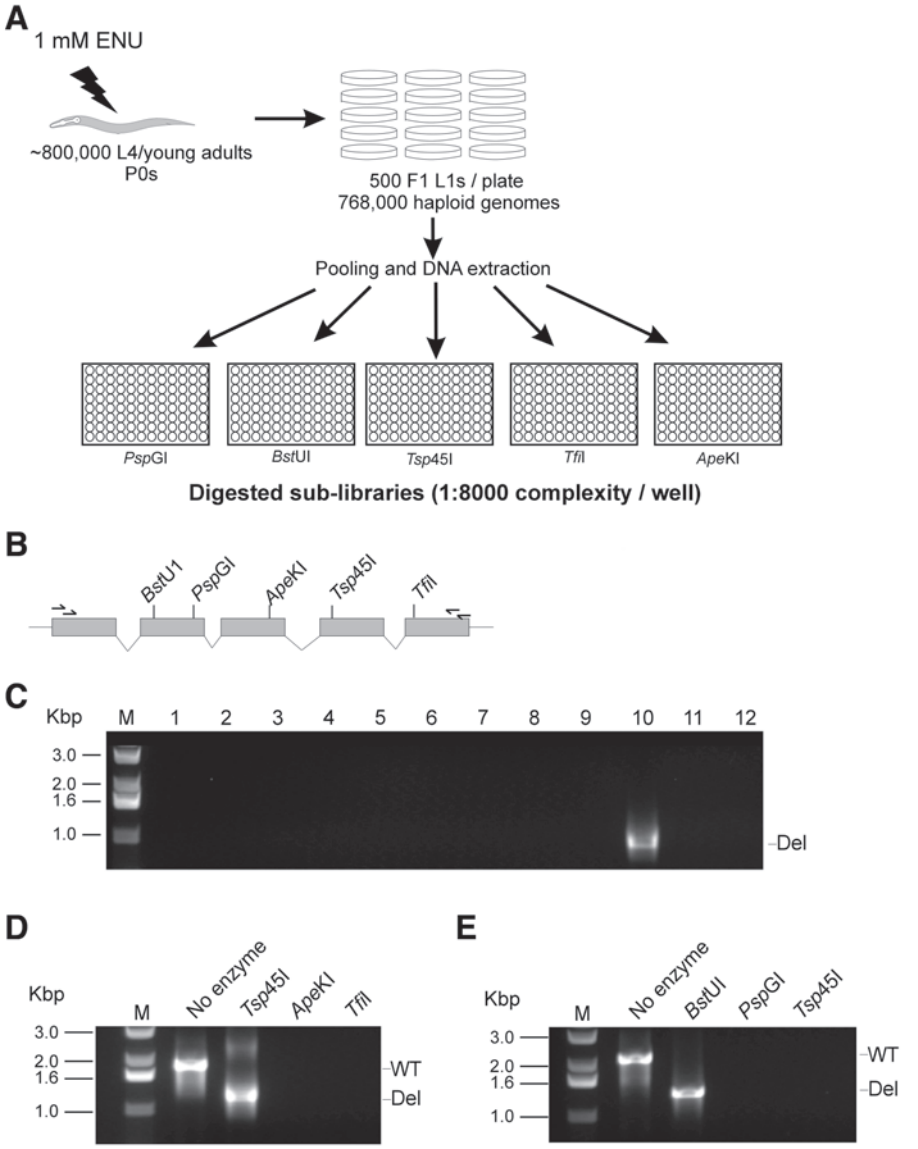

Fig. 1. Isolation of deletion mutants in *Caenorhabditis elegans*. **(A)** Mutagenesis and pooling strategy for deletion screening. Wild-type (WT) animals are mutagenized with 1 m*M* ethylnitrosourea (ENU) using standard methods ***(6)***. Approximately 500 mutagenized F1 worms are placed onto each of 768 6-cm nematode growth media (NGM) *(7)* 1.5 % agarose plates containing 0.75% peptone and seeded with OP50 bacteria. After 5 to 7 d, approx 25% of the worms on each plate are placed into one well of a 96-well plate. Crude DNA lysates *(5)* are pooled into one 8-well column (1:12,000 complexity) and one 12-well row (1:8000 complexity). All row pools are placed into a master plate, which is then divided into five 96-well plates and digested with one of five thermostable restriction enzymes. Each digested sublibrary is used as a template for nested polymerase chain reactions (PCRs), which each contain 0.2 units of the appropriate restriction enzyme. Positive deletion candidates are reconfirmed by screening the 8-well column pools, followed by the corresponding single-well sample. Single mutant animals are isolated through three rounds of sib selection using previously described methods *(5)*. **(B)** PCR screening strategy. Gene-specific nested primers are designed to encompass all available thermostable restriction enzyme sites while maintaining a distance of ≥200 bp between the end of the nested primers and the closest restriction enzyme site. A hypothetical gene structure is shown. **(C)** Initial screening of a 12-well row pool digested with *Tsp*45I using primers to *aqp-5*. One deletion positive was detected (lane 10). No WT product (~2.0 kb) is visible. **(D)** A DNA pool containing an *aqp-5* deletion was digested with *Tsp*45I, *Ape*KI, or *Tfi*I. WT product is approx 2.0 kb; deletion mutant is approx 1.3 kb. **(E)** A DNA pool containing an *aqp-2* deletion was digested with *Bst*UI, *Psp*GI, or *Tsp*45I. WT product is approx 2.5 kb; deletion mutant is approx 1.4 kb.

Table 1
Summary of Deletion Screen

Genes screened	Wild type product (kb)	Restriction enzyme sites in target region	Deletions identified in initial screen	Deletions recovered after three rounds of sib selection	Deletion size (bp); restriction site deleted
gpdh-2	2.0	*Psp*GI, *Tsp*45I	1	1	758; *Psp*GI
aqp-1	2.3	*Psp*GI, *Tsp*45I, *Ape*KI	0	0	
aqp-2	2.5	*Psp*GI, *Bst*UI, *Tsp*45I	2	1	843; *Bst*UI
aqp-4	1.3	*Psp*GI, *Bst*UI	1	1	748; *Psp*GI
aqp-5	2.0	*Tsp*45I, *Ape*KI, *Tfi*I	4	2	660; *Tsp*45I 1040; *Ape*KI
aqp-7	1.5	*Ape*KI, *Bst*UI	0	0	
aqp-8	1.4	*Psp*GI	3	3	330; *Psp*GI 348; *Psp*GI 749; *Psp*GI
aqp-10	1.2	*Psp*GI, *Tsp*45I	1	0	
aqp-11	1.5	*Psp*GI, *Bst*UI	1	1	894; *Bst*UI

from using *Psp*GI alone. Although our approach allows at least five different library digestions to be screened, it only requires the use of one set of primers per gene, which can have significant time and cost savings. With the commercial availability of more thermostable restriction enzymes, the majority of all genes in *C. elegans* should be amenable to deletion mutagenesis using this approach. Importantly, the efficiency of this method should make it possible for small academic laboratories to rapidly isolate deletions in their genes of interest.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grants DK51610 and DK61168 and a pilot project grant supported by the Vanderbilt O'Brien Center (DK39261) to K.S., by NIH grants HL33991, HL48268, and EY11239 to P.A., and by a research fellowship from the National Kidney Foundation to T.L. The authors declare no competing interests in this work.

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