PCR-RFLP Genotyping of Point Mutations in *Caenorhabditis elegans*

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[Abstract] This protocol describes the basic principle of PCR/restriction digest genotyping of point mutations in worms, based on the principle of Restriction Fragment Length Polymorphism (RFLP) analysis. This type of genotyping is particularly useful when phenotypic analysis of animals carrying point mutations is difficult (*e.g.,* in a complex genetic background).

I will illustrate the general procedures, using an example of *daf-2* gene, encoding the sole insulin/IGF-1 receptor of *C. elegans*. Gems *et al.* (1998) did a very elegant job and characterized a series of mutations of *daf-2*, including the following two temperature-sensitive hypomorphic alleles:

daf-2(e1370): Substitution C/T (wild type/mutant), amino acid change: Missense P to S.
Flanking sequences:
5'-CTCTATGAAATGGTTACACTCGGTGCTCAGCATATATTGGTTTGAGTAATGAGGTGT
Intracellular kinase domain, Class I, strong phenotype.

daf-2(e1368): Substitution C/T (wild type/mutant), amino acid change: Missense S to L.
Flanking sequences:
5'-TCCGGAATTTACGTATTGAGGCAAAGTACTGTTCAGAAATVTATATGCTATCACAGT
Extracellular ligand binding domain, Class II, weak phenotype.

Here I will show you how to design the primers for PCR-RFLP analysis.

daf-2(e1370): Designed by Seung-Jae Lee from the Kenyon lab.
Forward primer: 5'-CGGGATGAGACTGTCAAGATTGGAGATTTCGG-3'
Reverse primer: 5'-CAACACCTCATCATTACTCAAACCAATCCATG-3'
On the (-) strand, the nucleotide next to the 3' end of reverse primer is G in wild-type allele, which is mutated to T in *daf-2(e1370)*. Thus, by introducing another mutation (double C here, highlighted) into the reverse primer, it creates an *Nco I*-restriction site (*i.e.,* CCATGG) only for PCR products derived from wild-type but NOT *daf-2(1370)*.

daf-2(e1368)*:* Designed by Peichuan Zhang from the Kenyon lab.
Forward primer: 5'-GTTCCGGAATTTACGACGTATTGAGGCAACG-3'
Reverse primer: 5'-CTATCGGATCGAGTGGTATATTTAAC-3'
Similarly, on the (+) strand, the nucleotides next to the 3' end of forward primer is TC in wild-type allele, and TT in *daf-2(e1368)*. Thus, by introducing another mutation (C here, highlighted) into

the forward primer, a restriction site of *Acl* I (*i.e.,* AACGTT) is generated in the presence of *daf-2(1368)* point mutation.

The key is to introduce new mutation(s) at the 3' end of one of your primers. Since the difference of the sizes of digest products is just ~30-bp, the length of the primer, you have to pick the other primer to generate an amplicon of ~200-bp - 250-bp or so.

Here is a website that can help you design the primers with appropriate restriction site for genotyping: http://helix.wustl.edu/dcaps/dcaps.html (dCAPS Finder 2.0) (Neff *et al.,* 2002).

Materials and Reagents

1. PK lysis buffer
2. Proteinase K (Sigma-Aldrich, catalog number: P6556)
3. Common PCR reagent (*e.g.,* Invitrogen PCR kit – Life Technologies, Invitrogen™, catalog number: 10342-020; or home-made Taq and buffer)
4. Restriction enzymes (NEB)
5. Agarose gel
6. Ethidium bromide (Life Technologies, Invitrogen™, catalog number: 15585-011)

Equipment

1. MJ Research PTC-200 Thermo Cycler (MJ Research)
2. Thin-wall PCR tubes (USA Scientific, catalog numbers: 1402-2700 or 1405-8100)

Procedure

A. Isolate genomic DNA with proteinase K digest.

Tip 1. Typically, a large amount of PK lysis buffer is prepared (for the recipe, please refer to *Caenorhabditis elegans*/DNA/Single worm PCR) with supplement of proteinase K, and then small aliquots are stored (*e.g.,* 1 ml) at -20 °C. This robust enzyme works well at a range from 20 µg/ml to 100 µg/ml, and the key is to activate it at 60 °C for ~1 h and then kill it at 95 °C for ~15 min or so. You do not want to see proteinase K torture your Taq enzyme during the subsequent PCR reaction.

Tip 2. I prefer to pick reasonable numbers (*e.g.,* 10) of gravid adult animals and stick them into a PCR tube with PK lysis buffer (*e.g.,* 20 µl). It does not hurt to use more than 1 worm per PCR reaction (genomic DNA from ~1/2 worm works just fine for most robust PCR genotyping). For PCR-RFLP, it'd be better to use more than 1 worm per reaction (*e.g.,* 5 to 10). However, too much DNA template, in some cases, may inhibit your PCR reactions.

B. Perform a standard PCR, 20 µl per reaction.

1. Set up the PCR, by adding the following component into a thin-wall PCR tube on ice in this order:

DNAase-free ddH_2O	11.0 µl
dNTP mix (10 mM each)	0.4 µl (final, 200 µM each)
Forward primer (10 µM)	0.4 µl (final, 0.2 µM each)
Reverse primer (10 µM)	0.4 µl (final, 0.2 µM each)
PCR buffer (10x)	2.0 µl (final, 1x)
$MgCl_2$ (50 mM)	0.8 µl (final, 2.0 mM)
Worm lysates (20 µl)	2.0 µl
Taq (5 U/µl)	0.1 µl (final, 0.5 U per reaction)

2. Run PCR (put the tube on the block when it is hot):

1 cycle	94 °C, 3 min
30 cycles	94 °C, 10 sec; 58 °C, 30 sec; 72 °C, 30 sec
1 cycle	72 °C, 10 min

C. Digest with respective enzymes, 37 °C, O/N. Prepare multiplex (N+2) for N reactions:

ddH_2O	2.5 µl
10x buffer	2.5 µl
Enzyme (5 U/µl to 20 U/µl)	0.2 µl

Aliquot 5.0 µl into each PCR tube.

D. Resolve O/N digest of PCR products on 2.0%-2.5% agarose gel.

For *daf-2(1370)*:

Bands expected from *Nco* I-digest (NEB buffer 3): Wild-type, 202-bp; mutation, 234-bp.

For *daf-2(1368)*:

Bands expected from *Acl* I-digest (NEB buffer 4 + BSA): Wild-type, 215-bp; mutation, 186-bp.

Tip 3. Due to the small difference between the sizes of products, it is recommended to run the gel for a long time. Always add wild-type and mutants with known genotypes as controls. Ethidium bromide migrates toward cathode (-), just the opposite to the direction of DNA migration. To help subsequent visualization of DNA under UV light, it would be advised to add a few microliter of ethidium bromide (10 mg/ml) into the electrophoresis buffer near the anode (+).

Representative data

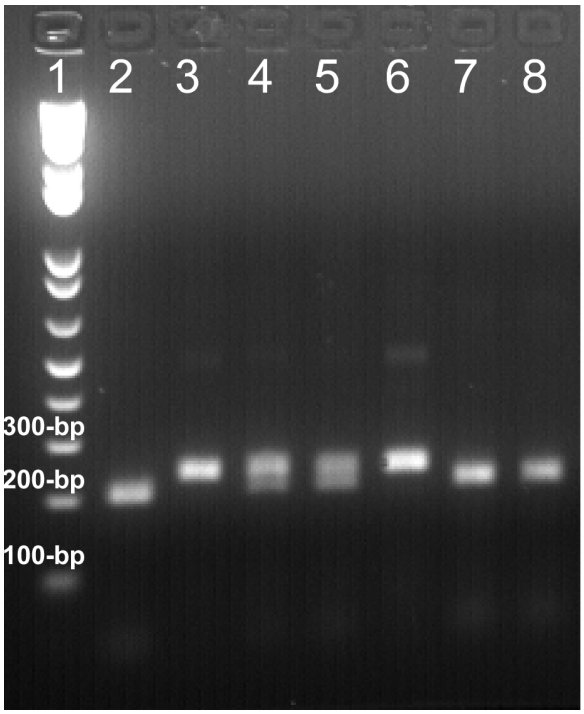

Figure 1. Representative data of PCR genotyping are shown here. 20 l of *daf-2(e1370)* allele-genotyping PCR products were digested with *Nco* I at 37 °C overnight. The digested DNA fragments were resolved on a 2.0% agarose gel. Expected sizes of DNA bands: wild-type, 202-bp; *daf-2(e1370)* mutation, 234-bp. Lane 1: 1 Kb Plus DNA Ladder (Life Technologies, Invitrogen™, catalog number: 10787-018). Lane 2, 7, 8: *daf-2(e1370)*$^{+/+}$; Lane 4, 5: *daf-2(e1370)*$^{+/-}$; Lane 3, 6: *daf-2(e1370)*$^{-/-}$. The results are highly reproducible, and necessary controls should always be included to assure the results.

Recipes

1. PK lysis buffer
   10 mM Tris-HCl (pH 8.0)
   50 mM KCl
   2.5 mM $MgCl_2$
   0.45% Tween-20
   0.05% gelatin
   20 g/ml proteinase K
   Prepare and store small aliquots at -20 °C

Acknowledgments

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References

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2. Neff, M. M., Turk, E. and Kalishman, M. (2002). Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet* 18(12): 613-615.

