

Multiplex PCR Identification of Five Common Root-Knot Nematode Species in California  
(*Meloidogyne arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, and *M. javanica*)

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The sequence characterized amplified region (SCAR) is a PCR technique that is based on the sequence information from a specific DNA fragment of a target organism; it can produce a unique PCR band with very strong sensitivity and has been widely used for species identification. Several research papers have provided helpful SCAR information to the common species of root-knot nematodes (*Meloidogyne* spp.), e.g., *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, and *M. javanica*. SCARs for *M. exigua*, *M. fallax*, *M. mayaguensis*, *M. paranaensis* are also available in a few recent reports. Among these publications, however, most protocols provided single nematode species diagnostics with a species specific pair of primers using purified nematode DNA. The Nematology Laboratory at CDFA routinely conducts identification of nematode species detected in regulatory plant and soil samples. These samples may contain several unknown nematode species that require timely and accurate diagnoses. Soil samples processed by the lab often contain very few “unknown” root-knot nematode second stage juveniles (J2). Therefore, for all practical purposes consistently accurate and timely nematode diagnostic tasks require a strong and sensitive PCR protocol that can simultaneously test as many species as possible using a single or very few numbers of specimens per species.

A practical multiplex PCR protocol that can enhance and facilitate the identification of *Meloidogyne* spp. has been adapted at CDFA’s Nematode Diagnostics Laboratory. Target nematode species include the most common *Meloidogyne* spp. found in California. The tropical species *M. arenaria*, *M. incognita*, and *M. javanica* are common in California agriculture fields, *M. chitwoodi* and *M. hapla* are often found from potato and alfalfa fields respectively. In order to easily differentiate the nematode species based on a picture of the PCR products on a gel, the size of the SCAR-PCR products for the target species must be clearly distinct from one to another. Combinations of different primers from published information were tested to choose the appropriate multiplex primers for the five *Meloidogyne* spp. The more the number of primer pairs applied in the PCR reaction, the greater were the possibilities that primer dimers and nonspecific fragments would develop. One combination included the five target root-knot nematode species, and each species amplified a distinct sized product was found. The SCAR PCR primers are selected from published research papers (Dong, *et al.*, 2001, Zijlstra, *et al.*, 2000) and listed in Table 1.

Table 1. The species-specific primers for the five *Meloidogyne* spp. multiplex PCR.

Species	Sequence of primers	Size	References
<i>M. arenaria</i>	TCGAGGGCATCTAATAAAGG GGGCTGAATATTCAAAGGAA	950bp	Dong, 2001
<i>M. chitwoodi</i>	CGCTGATAATCAGAGCAAAC GCCAATTCATAAGTGTGTCTAG	600bp	Zijlstra, 2000
<i>M. hapla</i>	GCCTTCTTTGGATTCTCTCA GGCTCATCCTTGCTGTAAAT	420bp	Zijlstra, 2000
<i>M. incognita</i>	CTCTGCCCAATGAGCTGTCC CTCTGCCCTCACATTAAG	1200bp	Zijlstra, 2000
<i>M. javanica</i>	CCTTAATGTCAACACTAGAGCC GGCCTTAACCGACAATTAGA	1650bp	Dong, 2001

The primers were ordered from Integrated DNA Technologies, Inc., and the stock concentration of each primer was made to be 1 $\mu$ g/ $\mu$ l in the original tube. The primer pair of each nematode species (Table 1) was diluted by adding 10 $\mu$ l of the stock solution from each original tube into 480 $\mu$ l ddH<sub>2</sub>O. The five species primer solutions were then combined in a single tube to make the multiplex PCR primer reaction solution. The original primer stock solutions were kept at -80°C, and the reaction solutions at -20°C. The Taq PCR Core Kit from Qiagen Sciences, Inc. was chosen for this test, it seems the Q-Solution from this kit can improve the PCR quality. For each 25 $\mu$ l PCR reaction, the following recipe was used at the CDFa Nematology Laboratory (Table 2).

Table 2. The PCR reaction components.

Template DNA	10 $\mu$ l (purified nematode DNA or homogenized J2)
Tris pH8.0 (0.1M)	2.5 $\mu$ l
10x Buffer (Contains 15 mM MgCl <sub>2</sub> )	2.5 $\mu$ l (Qiagen, Inc.)
Q-Solution	5.0 $\mu$ l (Qiagen, Inc.)
Multiplex primer mix	4.0 $\mu$ l
dNTP	0.5 $\mu$ l (Qiagen, Inc.)
Taq	0.2 $\mu$ l (Qiagen, Inc.)
Total volume	~25 $\mu$ l

The annealing temperatures of these selected primers are 58-59°C. The parameters of the thermocycler were programmed as follows (Table 3).

Table 3. The parameters of thermocycles

Step #1: (hot-start)	94°C for 4 min.
Step #2: (amplification)	94°C for 1 min. (denaturation) 55°C for 1.5 min. (annealing) 72°C for 2 min. (extension) for 35 cycles
Step #3: (final extension)	72°C for 10 min.
Step #4: (hold)	4°C forever.

The multiplex PCR reactions were developed and tested with purified nematode DNA from the five target nematode species, and further applied to the homogenized second stage juveniles (J2). The multiplex PCR results are stable for all tested nematode species (Figure 1).

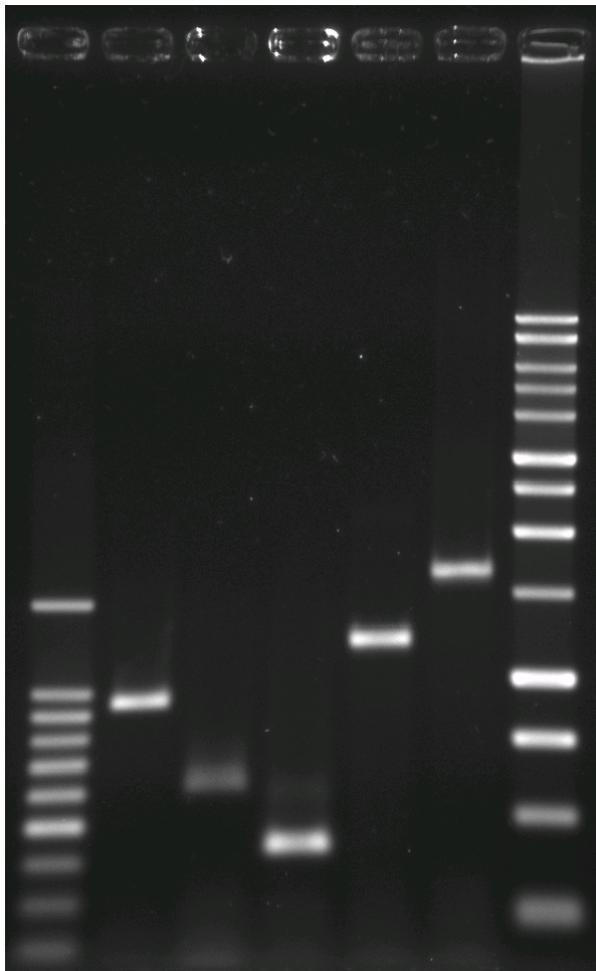


Figure 1. The amplified products of the multiplex SCAR-PCR from left to right, lane 1 and lane 7: DNA size standards; lane 2: *Meloidogyne arenaria* (950bp); lane 3: *M. chitwoodi* (600bp); lane 4: *M. hapla* (420bp); lane 5: *M. incognita* (1200bp); and lane 6: *M. javanica* (1650bp).

The SCAR-PCR products from the five *Meloidogyne* species can be clearly separated from this protocol. *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, and *M. javanica* produce 950bp, 600bp, 420bp, 1200bp, and 1650bp fragments respectively. Primer dimers and nonspecific PCR products are not developed in this multiplex combination (Figure 1). This multiplex SCAR-PCR provides a very strong sensitivity to detect homogenized nematode juveniles (J2) in the reactions, and it has greatly improved our practical diagnostic work at CDFA.

Other *Meloidogyne* species also of great concern to CDFA, include those that are invasive and bear resistance-breaking genes, such as, *M. enterolobii* (syn. *M. mayaguensis*) and *M. floridensis*. Early detection of these species is critical for an efficient and timely administration of CDFA's nematode regulatory program. *M. mayaguensis* has not been found in CA, the SCAR primers for *M. mayaguensis* are available but not included in this protocol. DNA sample from *M. floridensis* was tested with this multiplex PCR test, no DNA fragments were amplified from both *M. enterolobii* and *M. floridensis* with this multiplex primer combination.

## References

- Dong, K., Dean, R. A., Fortnum, B. A., and Lewis, S. A. 2001. Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*. *Nematropica* 31:273-282.
- Zijlstra, C. 2000. Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *European Journal of Plant Pathology* 106: 283-290.
- Zijlstra, C., Donkers-Venne, D. THM., and Fargette, M. 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterized amplified region (SCAR) based PCR assays. *Nematology* 2: 847-853.