# Skantar Lab PCR and digest reaction SOPs

One small plant-parasitic nematode (i.e. Meloidogyne, etc) contains 10 picograms or less of DNA!

Note: All programs should have a lid pre-heat setting at 99 C!

Note: For degenerate primers, cycling conditions should have 68 degree extension times instead of the taq-optimal 72 degrees, to prevent the degenerate primers from "falling off".

**Protein inactivation cycle for all DNA extracts (add Proteinase K)**

Add 1 uL of working-concentration (1.2 mg/mL) Proteinase K to every 20uL of DNA extract/sample (total proteinase K concentration: 60 ug/mL).

**Program "60kill"**

1) 60 C – 60 minutes
2) 95 C – 15 minutes
3) 15 C – Hold

**Reagents list with product numbers:**

DreamTaq (hot-start) DNA polymerase (ThermoFisher): EP1701 (200 Units), EP1702 (500 Units)
Platinum Taq (hot-start) DNA polymerase (Invitrogen): 10966-018
PicoMaxx High Fidelity PCR System (Agilent): 600420
dNTP Mix, 10mM each (ThermoFisher): R0192
StrataClone PCR Cloning Kit (Agilent): 240205-5 (expiration ~6 months)
TOPO TA Cloning Kit, Dual Promoter, with One Shot™ TOP10 chemically competent E. coli cells and pCR™II-TOPO™ vector: K4600J10 (10 reactions), K460001 (25 rxns)
EcoRI restriction enzyme (New England Biolabs): R0101S

**NEW DreamTaq reactions for most genes except Hsp90 (which does better with PlatinumTaq?)**

---H20 (to 25 uL total reaction volume)

2.5 uL 10X DreamTaq buffer (includes MgCl2 at 2.0 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.2 mM final reaction concentration per dNTP)

0.75 uL primer 1 (0.3 uM final primer concentration)

0.75 uL primer 2 (0.3 uM final primer concentration)

0.125 uL DreamTaq (0.625 units)

## EcoRI digest (diagnostic for plasmid inserts)

2 uL 10X buffer (comes with EcoRI restriction enzyme)

0.5 uL EcoRI

16 uL H2O

18.5 uL above mix, add 1.5 uL plasmid DNA. Incubate at least 1 (optimum 2) hours at 37 C.

**Program "digest37c-2hr" for restriction enzyme EcoRI digests**

1) 37 C – 60 minutes
2) Repeat step 1
3) 15 C - Hold

**Hsp90 (1.0 – 1.4 Kb), 25 uL reaction volume**

16.55 uL H2O

2.5 uL 10X PCR buffer (comes with the Taq, contains no MgCl2)

0.5 uL dNTPs mix (10 mM each dNTP, 0.2 mM final reaction concentration per dNTP)

0.75 uL MgCl2 (50mM, 1.5 mM final reaction concentration)

0.75 uL U288 primer (10uM, 0.3 uM final reaction concentration)

0.75 uL L1110 primer (10uM, 0.3 uM final reaction concentration)

0.2 uL Taq (one unit; usually Invitrogen/Life technologies Platinum Taq polymerase)

To 22 uL of the above mix, add 3 uL template DNA (usually single-nematode extract from -80 freezer, in buffer with proteinase K, and cycled at 60 C for 60 minutes then 95 C for 15 minutes ["60-kill" cycle]).

**Program "Ramp2L" for Hsp90**

1) 94 C – 2 minutes
2) 94 C – 20 seconds
3) 65 C – 5 seconds
4) 60 C – 5 seconds
5) 55 C – 5 seconds
6) 50 C – 5 seconds
7) (45 C – 5 seconds – This additional step is in program "Ramp2lnew")
8) 68 C – 2 minutes (extended to 3 minutes in Ramp2LNew for "Ramp2L-pfu")
9) Repeat 44 times: steps 2 through 8

10) 68 C – 15 minutes
11) 15 C – Hold

**Hsp90 primers ~1.2Kb fragment**

U288-GAYACVGGVATYGGNATGACYAA (Handoo et al. 2005) Journal of Nematology 37(2):136–145. 2005. Morphological and Molecular Evaluation of a Meloidogyne hapla Population Damaging Coffee (Coffea arabica) in Maui, Hawaii.

L1110-TCRCARTTVTCCATGATRAAVAC (Handoo et al. 2005) (see below, Skantar and Carta 2000 and 2004)

U831- AAYAARACMAAGCCNATYTGGAC ~450bp short-fragment, use instead of U288 with L1110 when that pair does not work. Skantar and Carta 2000, Biotechniques 2000 Dec:29(6):1182-4, 1186. Amplification of Hsp90 homologs from plant-parasitic nematodes using degenerate primers and ramped annealing PCR. Skantar and Carta 2004 J Nematol. 2004 Dec; 36(4): 466–480. Molecular Characterization and Phylogenetic Evaluation of the Hsp90 Gene from Selected Nematodes *Skantar and Carta 2000 and 2004 sequences omit the second-to-last A, which is necessary for the correct reading frame! Therefore, the above sequence is the correct one (2020).

**Hsp90 (1.0 – 1.4 Kb) with PicoMaxx system (including Pfu Taq), 25 uL reaction volume (for difficult template)**

16.80 uL H2O

2.5 uL 10X PicoMaxx Reaction Buffer (comes with the Taq, final reaction concentration of MgCl2 from buffer is 2 mM)

0.5 uL dNTPs mix (10 mM each dNTP, 0.2 mM final reaction concentration per dNTP)

0.75 uL U288 primer (10uM, 0.3 uM final reaction concentration)

0.75 uL L1110 primer (10uM, 0.3 uM final reaction concentration)

0.5 uL PicoMaxx Taq (1.25 units) from Agilent

0.2 uL Platinum Taq (one unit) from Life Technologies

To 22 uL of the above mix, add 3 uL template DNA (usually single-nematode extract from -80 freezer, in buffer with proteinase K, and cycled at 60 C for 60 minutes then 95 C for 15 minutes [“60-kill” cycle]).

**Program “Ramp2L-pfu” for Hsp90 with PicoMaxx**

1) 94 C – 2 minutes
2) 94 C – 20 seconds
3) 65 C – 5 seconds
4) 60 C – 5 seconds

5) 55 C – 5 seconds
6) 50 C – 5 seconds
7) 45 C – 5 seconds
8) 68 C – 3 minutes (increase to 4 minutes for Punctodera, 2kb fragment, "Ramp2LPstonei")
9) Repeat 44 times: steps 2 through 8
10) 68 C – 15 minutes
11) 15 C – Hold

**28S (D2A/D3B region, 800-1,000 bp), 25 uL reaction volume**

17.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.75 uL D2A primer (10 uM, 0.3 uM final reaction concentration)

0.75 uL D3B primer (10 uM, 0.3 uM final reaction concentration)

0.2 uL Taq (one unit)

To 23 uL of the above mix, add 2 uL template DNA.

**Program "D2D3KT" for 28S**

1) 94 C – 2 minutes
2) 94 C – 30 seconds
3) 55 C – 1 minute
4) 72 C – 2 minutes
5) Repeat 39 times: steps 2 through 4
6) 72 C – 7 minutes
7) 15 C – Hold

**28S primers**

D2A: ACAAGTACCGTGAGGGAAAGT De Ley et al 1999, Nunn 1992 (-TG on the end, De Ley et al. 2005)

D3B: TCGGAAGGAACCAGCTACTA D2A and D3B are for generating PCR product. De Ley et al 1999, Nunn 1992, De Ley et al 2005 (TGCGAAGGAACCAGCTACTA in Ye et al 2007, second and third bases are switched, Ye et al 2007 is incorrect.)

D3A-GACCCGTCTTGAAACACGGA Used with D3B for sequencing. Nunn et al. 1996, Baldwin et al. 1997, Duncan et al. 1999 (along with D3B). G.B. Nunn, B.F. Theisen, B. Christensen, P. Arctander. Simplicity-

correlated size growth of the nuclear 28S ribosomal RNA D3 expansion segment in the crustacean order Isopoda. J. Mol. Evol., 42 (1996), pp. 211-223. D2B in Ye et al. 2007 has the same sequence.

D2B-TCCGTGTTTCAAGACGGGTC (GTCGGGTTGCTTGAGAGTGC according to one paper?)

Use D3A and D3B for sequencing.

D3A and D2B are identical. I found a document online that also lists identical sequences for the two primers, although the sequences above are reverse-complements.

**ITS (800-1,000 bp), 25 uL reaction volume**

17.05 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 (use 0.5 if DNA is robust?) uL AB28 primer (10 uM, 0.3 uM final reaction concentration)

0.75 (use 0.5 if DNA is robust) uL TW81 primer (10 uM, 0.3 uM final reaction concentration)

0.2 uL Taq (one unit)

To 22 uL of the above mix, add 3 uL template DNA.

**Program "ITS1and2" for ITS**

1) 95 C – 2 minutes
2) 95 C – 30 seconds
3) 55 C – 30 seconds
4) 72 C – 1:30
5) Repeat 34 times: steps 2 through 4
6) 72 C – 5 minutes
7) 15 C – Hold

**ITS primers**

AB28: ATATGCTTAAGTTCAGCGGGT Duncan et al. 1999.

TW81: GTTTCCGTAGGTGAACCTGC Duncan et al. 1999. Duncan, L. W., R. N. Inserra, W. K. Thomas, D. Dunn, I. Mustika, L. M. Frisse, M. L. Mendes, K. Morris, and D. Kaplan. 1999. Genetic and morphological relationships among isolates of Pratylenchus coffeae and closely related species. Nematropica 29:61–80.

Also : A.M. Skantar, Z.A. Handoo, G.N. Zanakis, and E.A. Tzortzakakis. Molecular and Morphological Characterization of the Corn Cyst Nematode, *Heterodera zeae*, from Greece. J Nematol. 2012 Mar; 44(1):58-66.

**18S (long fragment, ~1.8 Kb), 50 uL reaction volume**

(Double the usual reaction volume to result in sufficient DNA to send for sequencing with 4 primers)

35.1 uL H2O

5 uL 10X buffer (comes with the Taq)

1 uL dNTP mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

1.5 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

1.5 uL 18S-G18S4 primer (10 uM, 0.3 uM final reaction concentration)

1.5 uL 18S-18P primer (10 uM, 0.3 uM final reaction concentration)

0.4 uL Taq (one unit)

To 46 uL of the above mix, add 4 uL template DNA.

**Program "18Slongfragment" (formerly "Burs-Hali-18S) for 18S**

8) 94 C – 2 minutes
9) 94 C – 30 seconds
10) 50 C – 30 seconds
11) 68 C – 2 minutes
12) Repeat 39 times: steps 2 through 4
13) 68 C – 10 minutes
14) 15 C – Hold

**18S primers**

18S-G18S4: GCTTGTCTCAAAGATTAAGCC (Also SSU_F04)

18S-18P: TGATCCWKCYGCAGGTTCAC (Also SSU_R_81) (these two are used for generating long-fragment PCR) Blaxter et al. 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 1998 Mar 5;392(6671):71-5. ALSO De Ley et al. 2002. De Ley, I.T., De Ley, P., Vierstraete, A., Karssen, G., Moens, M., Vanfleteren, J., 2002. Phylogenetic analyses of Meloidogyne small subunit rDNA. J. Nematol. 34, 319–327.

550F: GGCAAGTCTGGTGCCAGCAGCC

1108R: CCACTCCTGGTGGTGCCCTTCC (These two are internal primers for sequencing long fragments generated by the above primer pair 18P and G18S4) Thomas 2011. Thomas, W. K. 2011. Molecular techniques, in International Seabed Authority (Eds), Marine benthic nematode molecular protocol handbook (nematode barcoding), Technical Study: No. 7, ISA Technical study series, 22–37.

Other 18S primers (smaller-fragment PCR):

18S1.2: GGCGATCAGATACCGCCCTAGTT Skantar et al. 2012

18Sr2b: TACAAAGGGCAGGGACGTAAT Skantar et al. 2012. A.M. Skantar, Z.A. Handoo, G.N. Zanakis, and E.A. Tzortzakakis. Molecular and Morphological Characterization of the Corn Cyst Nematode, *Heterodera zeae*, from Greece. J Nematol. 2012 Mar; 44(1):58-66.

**Program "18S1.2-r2b" (formerly "18SRKN") (~700bp band)**

1) 94 C – 2 minutes
2) 94 C – 20 seconds
3) 59 C – 30 seconds
4) 72 C – 30 seconds
5) Repeat 39 times: steps 2 through 4
6) 72 C – 5 minutes
7) 15 C – Hold

Recipe:

16.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL 18S1.2 primer (10 uM, 0.2 uM final reaction concentration)

0.75 uL 18Sr2b primer (10 uM, 0.2 uM final reaction concentration)

0.2 uL Taq (one unit)

To 22 uL of the above mix, add 3 uL template DNA.

**18S short fragment primers:**

18S1.2: GGCGATCAGATACCGCCCTAGTT (See above for publication reference)

18Sr2b: TACAAAGGGCAGGGACGTAAT

**Mitochondrial (Intergenic Spacer?), 25 uL reaction volume**

16.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.75 uL C2F3/COIIR primer (10 uM, 0.3 uM final reaction concentration)

0.75 uL 1108/1RNAF primer (10 uM, 0.3 uM final reaction concentration)

0.2 uL Taq (one unit)

To 22 uL of the above mix, add 3 uL template DNA.

**Program "Mitonaria" for Mitochondrial (Intergenic Spacer?) (~1200bp band)**

1) 94 C – 2 minutes
2) 94 C – 30 seconds
3) 48 C – 30 seconds
4) 72 C – 2 minutes (changed from 68 C 7/25/2019) (increase to 3 minutes for "pfu" program)
5) Repeat 44 times: steps 2 through 4
6) 72 C – 5 minutes (changed from 68 C 7/25/2019)
7) 15 C – Hold

**Mitochondrial primers**

1RNA(also 1108): TACCTTTGACCAATCACGCT Powers and Harris 1993

COII(also C2F3): GGTCAATGTTCAGAAATTTGTGG Powers and Harris 1993. T O Powers and T S Harris. A polymerase chain reaction method for identification of five major Meloidogyne species. J Nematol. 1993 Mar:25(1):1-6.

**Program "IGS1and2" (~750-900bp band)**

1) 94 C – 2 minutes
2) 94 C – 30 seconds
3) 53 C – 30 seconds
4) 72 C – 1.5 minutes (increase to 2 minutes for "pfu" program)
5) Repeat 44 times: steps 2 through 4
6) 72 C – 7 minutes
7) 15 C – Hold

Recipe:

17.375 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL 18S-IGS primer (10 uM, 0.3 uM final reaction concentration)

0.75 uL 5S-IGS primer (10 uM, 0.3 uM final reaction concentration)

0.125 uL Dream Taq (one unit)

To 22 uL of the above mix, add 3 uL template DNA.

**IGS Primers:**
5S-IGS (194): TTAACTTGCCAGATCGGACG Blok et al. 1997
18S-IGS (195): TCTAATGAGCCGTACGC Blok et al. 1997. V.C. Blok, M.S. Phillips, and M. Fargette. Comparison of Sequences from the Ribosomal DNA Intergenic Region of Meloidogyne mayaguensis and Other Major Tropical Root-knot Nematodes. J. Nematol. 29(1):16-22. 1997.

**Program "Hsp90 arenaria" (~1Kb band)**

1) 94 C – 2 minutes
2) 94 C – 30 seconds
3) 55 C – 20 seconds
4) 68 C – 1.5 minutes
5) Repeat 39 times: steps 2 through 4
6) 68 C – 5 minutes
7) 15 C – Hold

Recipe:

16.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL MgCl2 (50mM, 1.5 mM final reaction concentration)

0.75 uL RKN-d1F primer (10 uM, 0.3 uM final reaction concentration)

0.75 uL RKN-5R primer (10 uM, 0.3 uM final reaction concentration)

0.2 uL Platinum Taq (one unit)

To 22 uL of the above mix, add 3 uL template DNA.

RKN Hsp90 primers:

RKN-d1F (Hsp90): GCYGATCTTGTYAACAACCYTGGAAC Nischwitz et al 2013
RKN-5R (Hsp90): TCGAACATGTCAAAAGGAGC Nischwitz et al 2013. Occurrence of Meloidogyne fallax in North America, and Molecular Characterization of M. fallax and M. minor from U.S. Golf Course GreensPlant Disease 97(11):1424-1430 · November 2013

**Program "Cox1and2"**

1) 94 C – 2 minutes
2) 94 C – 1 minute
3) 45 C – 1 minute (changed from 50 C 7/25/2019 to reflect publication conditions) !!Should be changed to 55 C per Jannsen et al 2016! (11-2019)
4) 72 C – 1.5 minutes (increase to 2 minutes for "pfu" program)
5) Repeat 39 times: steps 2 through 4
6) 72 C – 10 minutes
7) 15 C – Hold

Recipe:

18.375 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL Cox1F (or Cox2F) primer (10 uM, 0.3 uM final reaction concentration)

0.75 uL Cox1R (or Cox2R) primer (10 uM, 0.3 uM final reaction concentration)

0.125 uL DreamTaq (one unit)

To 23 uL of the above mix, add 2 uL template DNA.

**Cox1 primers (Janssen et al 2016) (~1000 bp fragment):**

Cox1F: ATCCTCCTTTGATGATTGATGG

Cox1R: AACTCAATAAAGAACCAATAGAAG

**Cox2 primers (Janssen et al 2016) (~450 bp fragment?):**

Cox2F: TTGAATTTAAGTGTTGTTTATTAC

Cox2R: GATTAATACCACAAATCTCTGAAC

**Program "Hetcox1" (~550 bp fragment)**

1) 94 C – 4 minutes
2) 94 C – 1 minute
3) 45 C – 1 minute
4) 72 C – 1.5 minutes
5) Repeat 39 times: steps 2 through 4
6) 72 C – 10 minutes
7) 15 C – Hold

Use same recipe for ITS: 3 uL template DNA, 22 uL PCR mix (Dream taq)

**Heterodera COXI primers (~430 bp fragment) Subbotin et al. 2017, Journal of Nematology.** Subbotin, S. A., Akanwari, J., Nguyen, C. N., Cid del Prado Vera, I., Chihtambar, J. J., Inserra, R. N. and Chizhov, V. N. 2017. Molecular characterization and phylogenetic relationships of cystoid nematodes of the family Heteroderidae (Nematoda: Tylenchida). Nematology 19:1065–1081

Het-coxiF: TAGTTGATCGTAATTTTAATGG

Het-coxiR: CCTAAAACATAATGAAAATGWGC

**Program "Cox1and2Cactodera" (~450 bp fragment)**

1) 94 C – 4 minutes
2) 94 C – 1 minute
3) 41 C – 1 minute
4) 72 C – 1.5 minutes
5) Repeat 39 times: steps 2 through 4
6) 72 C – 10 minutes
7) 15 C – Hold

2 uL template DNA, 23 uL PCR mix (Dream taq)

JB3: TTTTTTGGGCATCCTGAGGTTTAT (F)

JB4.5: TAAAGAAAGAACATAATGAAAATG (R)

(Cactodera COI mtDNA Bowles et al., 1992) Bowles J, Blair D, McManus DP. Genetic variants within the genus Echinococcus identified by mitochondrial DNA sequencing. Molecular and Biochemical Parasitology. 1992;54:165–174.

**Program "Cox1Pratylenchus" (~730 bp fragment)**

1) 94 C – 5 minutes
2) 94 C – 30 seconds
3) 50 C – 30 seconds (originally 54 C from Derycke 2005)
4) 72 C – 1.5 minutes
5) Repeat 44 times: steps 2 through 4
6) 72 C – 5 minutes
7) 15 C – Hold

2 uL template DNA, 23 uL PCR mix (Dream taq)

CO1- F7bP: GGDTGRACWTTHTAYCCNCC (F)

CO1-JB5: AGCACCTAAACTTAAAACATAATGAAAATG (R ) Derycke et al. (2005), Mehmet Ozbayrak 2019

JB3: TTTTTTGGGCATCCTGAGGTTTAT (F) Derycke et al. (2005), Bowles et al. 1992

Mehmet Ozbayrak, 5-2019

The cytochrome c oxidase subunit 1(CO1) gene region of mitochondrial DNA was amplified by PCR using primer sets of CO1- F7bP (5'-GGDTGRACWTTHTAYCCNCC-3'), and CO1-JB5 (5'-AGCACCTAAACTTAAAACATAATGAAAATG-3') Derycke et al. (2005) that resulted in 727-739bp of sequence for genetic analysis after trimming the primers from the amplified product. On occasion, forward primer JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') of Derycke et al. (2005) was used in a combination with the JB5 primer.

**Program "Cox1RootKnot" (~430 bp fragment) created 9-17-2020**

1) 94 C – 5 minutes
2) 94 C – 30 seconds
3) 54 C – 30 seconds
4) 72 C – 1 minute
5) Repeat 5 times: steps 2 through 4, decreasing annealing temperature by 1 C for each cycle
6) 94 C – 30 seconds
7) 50 C – 30 seconds (originally 54 C from Derycke 2005)
8) 72 C – 1 minute
9) Repeat 34 times: steps 6 through 8
10) 72 C – 5 minutes

11) 15 C – Hold

2 uL template DNA, 23 uL PCR mix (Dream taq)

CO1-JB5: AGCACCTAAACTTAAAACATAATGAAAATG Derycke et al. (2010)

JB3: TTTTTTGGGCATCCTGAGGTTTAT (F) Derycke et al. (2010), both primers also from above publications. I increased the extension time from 30s to 1 minute.

**Program "HelicoCox1" (~600 bp fragment)**

1) 94 C – 3 minutes
2) 94 C – 30 seconds
3) 40 C – 30 seconds
4) 72 C – 1 minute
5) Repeat 44 (up to 54?) times: steps 2 through 4
6) 72 C – 5 minutes
7) 15 C – Hold

3 uL template DNA, 22 uL PCR mix (Dream taq)

M2F: ATTGGiGSTTTTGGTAATT

RH1R: CCAACAATGAATATATGATG

(Helicotylenchus COI mtDNA K. Rybarczyk-Mydlowska et al., 2019)

**Program "Nadh5"**

1) 94 C – 2 minutes
2) 94 C – 1 minute
3) 45 C – 1 minute
4) 72 C – 1.5 minutes
5) Repeat 39 times: steps 2 through 4
6) 72 C – 10 minutes
7) 15 C – Hold

**Program "Nadh5low"**

1) 94 C – 2 minutes
2) 94 C – 1 minute

3) 40 (or 35) C – 1 minute
4) 72 C – 1.5 minutes
5) Repeat 39 times: steps 2 through 4
6) 72 C – 10 minutes
7) 15 C – Hold

Recipe:

17.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL NAD5F2 primer (10 uM, 0.2 uM final reaction concentration)

0.75 uL NAD5R1 primer (10 uM, 0.2 uM final reaction concentration)

0.2 uL Taq (one unit)

To 23 uL of the above mix, add 2 uL template DNA.

**Nadh5 primers (Janssen et al 2016):**

NAD5F2: TATTTTTTGTTTGAGATATATTAG

NAD5R1: CGTGAATCTTGATTTTCCATTTTT

**Program "Scarenaria"**

1) 94 C – 2 minutes
2) 94 C – 30 seconds
3) 61 C – 30 seconds
4) 72 C – 2 minutes
5) Repeat 44 times: steps 2 through 4
6) 72 C – 5 minutes
7) 15 C – Hold

**Criconematidae Mitochondrial (Cytochrome Oxidase 1), 25 uL reaction volume**

10.05 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

3 uL COI-F5 primer (10 uM, 1.2 uM final reaction concentration)

3 uL COI-R9 primer (10 uM, 1.2 uM final reaction concentration)

0.2 uL Taq (one unit)

To 20 uL of the above mix, add 5 uL template DNA.

**Program "CriconemCOI" for Criconematidae Mitochondrial (Cytochrome Oxidase 1)**

1) 94 C – 5 minutes
2) 94 C – 30 seconds
3) 48 C – 30 seconds
4) 72 C – 1.5 minutes (ramping rate of 0.5 deg. C per second?)
5) Repeat 49 times: steps 2 through 4
6) 72 C – 5 minutes
7) 15 C – Hold

**Criconematidae Cytochrome Oxidase 1 (universal?) primers (Tom Powers [T.O. Powers] et al 2014)**

COI-F5: AATWTWGGTGTTGGAACTTCTTGAAC

COIR9: CTTAAAACATAATGRAAATGWGCWACWACATAATAAGTATC

**Other, infrequently-used programs**

**Program "Xiphcoi"**

1) 95 C – 10 minutes
2) 94 C – 30 seconds
3) 45 C – 40 seconds
4) 72 C – 1 minute
5) Repeat 5 times: steps 2 through 4
6) 94 C – 30 seconds
7) 37 C – 30 seconds

8) 72 C – 1 minute
9) Repeat 34 times: steps 6 through 8
10) 72 C – 10 minutes
11) 15 C – Hold

Recipe:

17.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.5 uL COIF primer (10 uM, 0.2 uM final reaction concentration)

0.5 uL XIPHR2 primer (10 uM, 0.2 uM final reaction concentration)

0.2 uL Taq (one unit)

To 22.5 uL of the above mix, add 2.5 uL template DNA.

**Program "XiphHsp90ramp"**

1) 94 C – 2 minutes
2) 94 C – 30 seconds
3) 45 C – 30 seconds
4) ~~60 C – 10 seconds~~
5) ~~55 C – 10 seconds~~
6) ~~50 C – 10 seconds~~
7) 68 C – 2:30 minutes (change to 72 C since primers not degenerate?)
8) Repeat 44 times: steps 2 through 7
9) 68 C – 15 minutes (change to 72 C since primers not degenerate?)
10) 15 C – Hold

Recipe:

17.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.5 uL XL-3F primer (10 uM, 0.2 uM final reaction concentration)

0.5 uL XL-3R primer (10 uM, 0.2 uM final reaction concentration)

0.2 uL Taq (one unit)

To 22.5 uL of the above mix, add 2.5 uL template DNA.

**Xiphinema species-specific Hsp90 PCR primers**

XL-3F AAA TCA CGC CCA ACA AGA AC

XL-3R TCA ATC CAC TTC TTC CAT GCG

**Program "DitylenchHsp90"**

1) 94 C – 2 minutes
2) 94 C – 20 seconds
3) 50 C – 20 seconds
4) 68 C – 2 minutes
5) Repeat 44 times: steps 2 through 4
6) 68 C – 15 minutes
7) 15 C – Hold

Recipe

16.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.5 uL Dit3F primer (10 uM, 0.2 uM final reaction concentration)

0.5 uL Dit3R primer (10 uM, 0.2 uM final reaction concentration)

0.2 uL Taq (one unit)

To 22 uL of the above mix, add 3 uL template DNA.

**Program "hmedicaghsp90"**

1) 94 C – 2 minutes
2) 94 C – 20 seconds
3) 50 C – 1 minute
4) 72 C – 2 minutes

5) Repeat 44 times: steps 2 through 4
6) 72 C – 15 minutes
7) 15 C – Hold

Recipe

16.55 uL H2O

2.5 uL 10X buffer (comes with the Taq)

0.75 uL MgCl2 (50 mM, 1.5 mM final reaction concentration)

0.5 uL dNTPs mix (10 mM each dNTP, 0.5 mM final reaction concentration per dNTP)

0.5 uL forward primer (10 uM, 0.2 uM final reaction concentration)

0.5 uL reverse primer (10 uM, 0.2 uM final reaction concentration)

0.2 uL Taq (one unit)

To 22 uL of the above mix, add 3 uL template DNA.

Hmedicag90-1F GAT ACG GGG ATT GGG ATG ACT AA Tm=56 C (63 calculated)

Hmedicag90-2F GAC ACC GGA ATC GGC ATG ACC AA Tm=61.8 C (68 calculated)

Hmedicag90-1R TCG CAA TTG TCC ATG ATG AAA AC Tm=54.4 C (61 calculated)

The above two primer sets yielded only fragments far below expected, not nematode Hsp90.

Hmedicag90-66F TCAGGTACAGTTTTCTAGTCGC

Hmedicag90-1133R TTGTTCTCAAAGAGATCGAACG

The above two primers yielded no PCR product (4/2019)

**Program "CactHsp90" for Hsp90 (created 3-9-2020)**

1) 94 C – 2 minutes
2) 94 C – 20 seconds
3) 50 C – 30 seconds
4) 68 C – 2 minutes
5) Repeat 44 times: steps 2 through 8
6) 68 C – 15 minutes
7) 15 C – Hold

**Hsp90 primers**

CactHsp90-26F - CGGAYCTTGTCAACAACCTCG

CactHsp90-1302R- GCATTCTTGCTTTTCTTGTTCTCG (Skantar 2020)

**Miscellaneous primers:**

Primer order 5/27/2015

M. arenaria SCAR primers:

Far TCGGCGATAGAGGTAAATGAC

Rar TCGGCGATAGACACTACAAACT

Punc3Rev GTTCAGCTCTTCGTCCTCAATG

Punc1F (near U288) TCATGGAGGCATTGCAGGT

M. enterolobii specific SCAR primers (520 bp fragment):

MK7-F GATCAGAGGCGGGCGCATTGCGA

MK7-R CGAACTCGCTCGAACTCGAC

5 min at 94_C; 40 cycles of 30 s at 94_C, 30 s

at 62_C and 1 min at 72_C; plus a final extension step of

8 min at 72_C. (Tigano 2010 Plant Pathology)