

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!

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?)

---H2O (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

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 (TGGGAAGGAACCAGCTACTA 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: GTTCCGTAGGTGAACCTGC 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 zae*, 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: TGATCCWKC YGCAGGTTAC (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 zaeae*, 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

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 Janssen 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: TTTTGGGCATCCTGAGGTTTAT (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: GGD TGRACWTTHTAYCCNCC (F)

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

JB3: TTTT TGGGCATCCTGAGGTTTAT (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'-GGD TGRACWTTHTAYCCNCC-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'-TTTT TGGGCATCCTGAGGTTTAT-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: TTTT TGGGCATCCTGAGGTTTAT (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)

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: AATWTWGGTGTGGAACCTCTGAAC

COIR9: CTAAAAACATAATGAAATGWGCWACWACATAATAAGTATC

0.2 uL Taq (one unit)

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