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A reverse transcriptase PCR assay for the identification of *Dirofilaria immitis* infective (L3) larvae in mosquitoes

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Dirofilariasis

- Worldwide distribution
- Mosquito-borne pathogen
- Caused by nematodes from genus *Dirofilaria*
  - *Dirofilaria immitis* – comsmopolitan distribution
  - *Dirofilaira repens* – Asia, Africa, Europe
- Canines and felines serve as primary reservoir
  - Can infect a wide range of mammalian orders
  - Serious veterinarian problem in many locales

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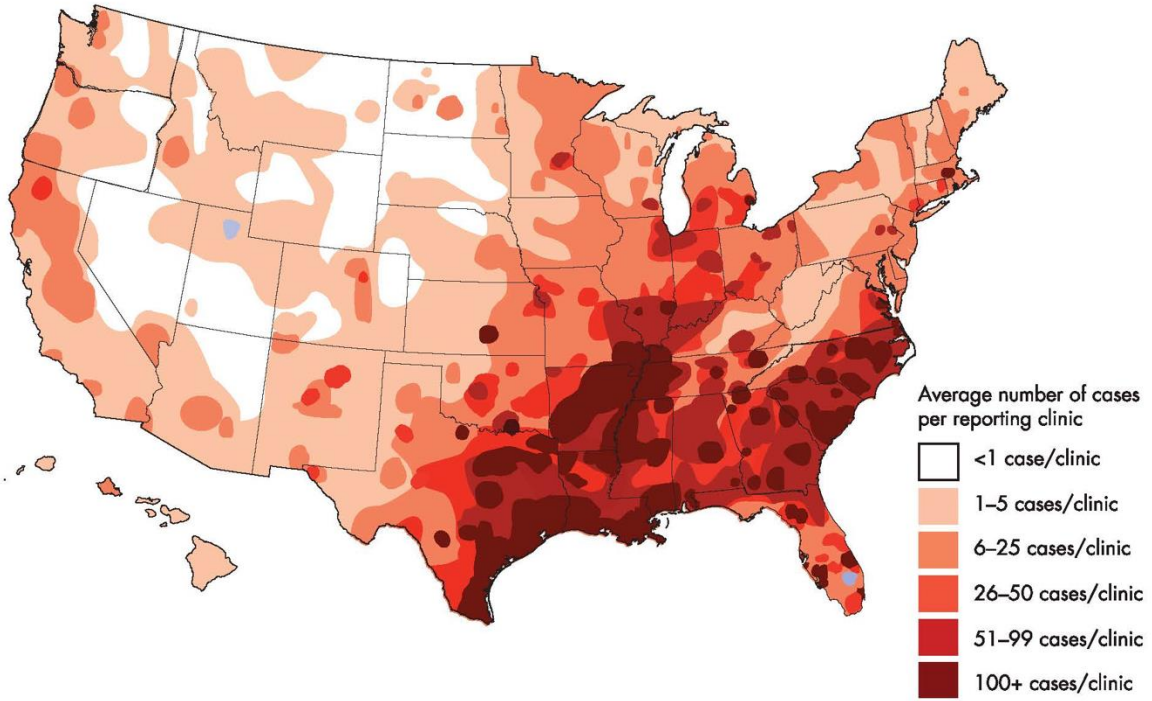
2022 HEARTWORM INCIDENCE

AMERICAN HEARTWORM SOCIETY™ EST.1974

 The severity of heartworm incidence as shown in this map is based on the average number of cases per reporting clinic. Some remote regions of the United States lack veterinary clinics, therefore we have no reported cases from these areas.



Increasing evidence of human cases of Dirofilariasis

- *D. immitis*
  - Pulmonary infections
  - 119 recorded cases in the United States
  - 50+ reported cases from South America
  - Other infections: subcutaneous, ocular , male reproductive tissues
- *D. repens*
  - Subcutaneous and ocular infections
  - 1500+ human cases reported in medical literature
  - Human cases Europe/Asia

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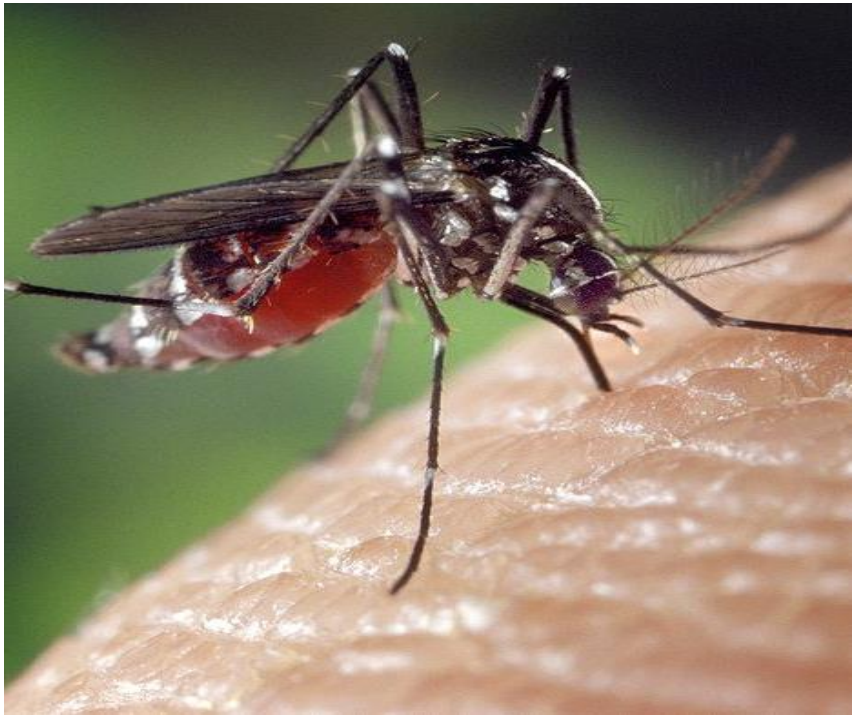
Mosquito vectors

- At least 60 species implicated as potential vectors
  - *Aedes*
  - *Anopheles*
  - *Culex*
  - *Ochlerotatus*



Aedes albopictus Courtesy: CDC

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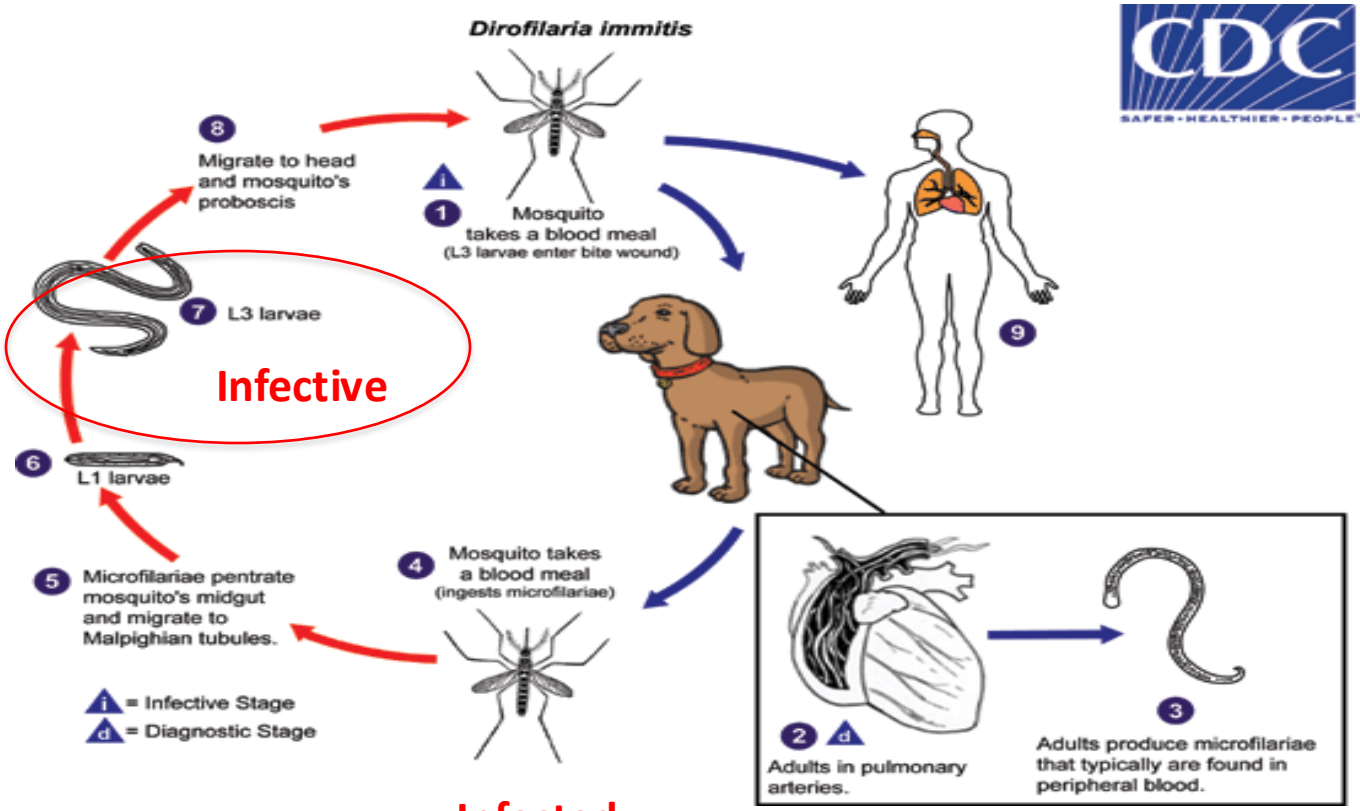
D. immitis life cycle



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PCR and the conundrum of infective vs. infected mosquitoes

- PCR can be employed to detect mosquitoes infected with *D. immitis*
  - Cannot discriminate parasite stage
- Reverse transcriptase PCR based upon amplification of an infective L3-stage gene in *D. immitis*
  - Powerful tool to identify epidemiologically important mosquito vectors

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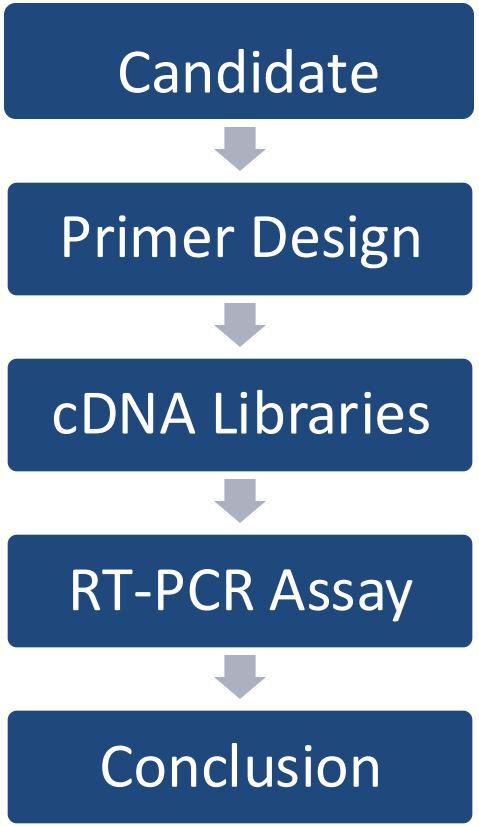
Stage Specific Reverse Transcriptase-PCR Assay

Objective: Using a bioinformatics approach to develop an assay which will amplify a gene expressed only in the infective stage (L3) in *D. immitis*

This approach was used successfully to develop similar assays for human filarial pararsites *Brugia malayi* and *Wuchereria bancrofti* (Laney et al. 2008; Laney et al., 2010)



Target selection

- We screened sequences from ESTs and cDNA clones from *Brugia malayi* and *Wuchereria bancrofti*

- We performed BLASTN analysis of these sequences against the *Dirofilaria immitis*, nuclear genome assembly 2.2

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- cDNA-to-genomic DNA using SPIDEY was performed
- Identifies splice junction sites
- At least one primer was designed to span an intron/exon junction

Spidey Home

Genomic: lcl|nDi.2.2.scaf00136 No definition line found

mRNA: gi|45243457|gb|CK854857.1|CK854857 SWWbL3CAW30C09SK Wuchereria bancrofti L3 cDNA (SAW96MLW-WbL3) Wuchereria bancrofti cDNA clone SWWbL3CAW30C09 5', mRNA sequence

Alignment is on minus strand of genomic sequence and on plus strand of mRNA sequence
mRNA coverage: 84%
Overall percent identity: 85.7%

	Genomic coordinates	mRNA coordinates	length	identity	mismatches	gaps	Donor site	Acc. site
Exon 1	102335-102573	16-254	239	87.9%	29	0		
Exon 2	102006-102018	278-290	13	100.0%	0	0		
Exon 3	101852-101991	311-450	140	80.7%	27	0		

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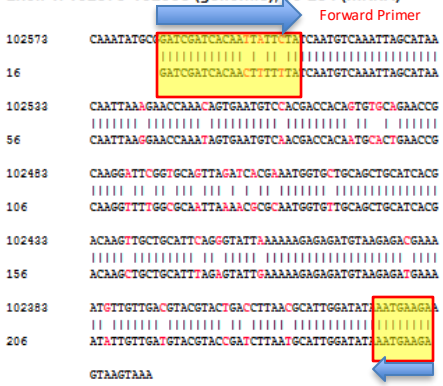
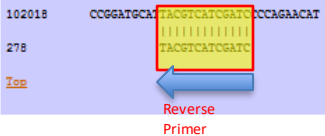
Exon 1: 102573-102335 (genomic); 16-254 (mRNA)

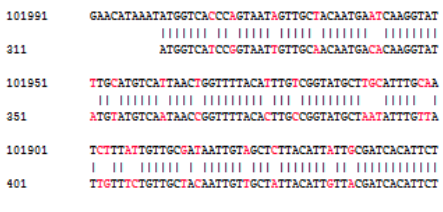


Top

Exon 2: 102018-102006 (genomic); 278-290 (mRNA)



Exon 3: 101991-101852 (genomic); 311-450 (mRNA)



One primer should span the intron-exon boundary

This should prevent amplification of genomic DNA

Selection of candidate genes for primer design

- 5 Candidate Genes
- 11 Potential Target Sequences
  - 7 Collagen (1 *B. malayai* and 6 *W. bancrofti*)
  - 2 *B. malayai* Pyruvate dehydorgenase genes
  - 2 *W. bancrofti* Cuticlin
- Constitutively expressed gene, *Ditph,* that is present in all larval stages

Table 1. Candidate Gene Primer Sequences

Gene Identifier	Direction	Sequence 5'→3'
H98312	F	GAAGCACGAGAAAAAGCTTATAC
H98312	R	TCAGATGGTGGACGGTG
CB338725A	F	GTTCGAGAAACAATTCGATGGTC
CB338725A	R	CGGCTGCATCAACCTCTTTC
CB338725B	F	CTATGTCAGTTATCGATCTCGCG
CB338725B	R	CGGCTGCATCAACCTCTTTC
CK8500976	F	GCGATGATAATCAAGCTTTGCC
CK8500976	R	TTGGACGAAATTTAAACGAAATGG
CK855471A	F	GAGAACGCGCATACAAGGC
CK855471A	R	GGTTTGCAACAACTATCACAGGC
CK855471B	F	GGAGCCTGTGATAGTTGTTGC
CK855471B	R	CTGGTTCGCCCGGTTG
CK855471C	F	GGAAACAGCGGTAGTGCTG
CK855471C	R	CTGGTGGTCCATTTGGTCC
CK855471D	F	GAGAACGCGCATACAAGGC
CK855471D	R	CAGCACTACCGCTGTTTCC
CK855471E	F	CTTCAATTTTGCAAATCCTCAGC
CK855471E	R	CGCTGTTTCCTGGTCTGC
CK855471F	F	GGAGCCTGTGATAGTTGTTGC
CK855471F	R	CTGGTGGTCCATTTGGTCC
CK854857	F	CAGTGAATGTCCACGACCAC
CK854857	R	CTGGGTGACCATGATCGATG

Gene Identifier = Genebank Accession number available at http://www.ncbi.nlm.nih.gov/.
F= Forward Primer, R=Reverse Primer

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Preliminary screening of primer sets

- Screened all primers against two cDNA libraries
  - *D. immitis* mf library
  - *D. immitis* L3 library

- Screened each cDNA library with primers for *Ditph* – expressed in all stages

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Results of cDNA library screening

Table 2. cDNA Library PCR Screening of Gene Candidates

Gene Identifier	Putative Identification based on Protein similarity matches	DiMf cDNA Library	DiL3 cDNA Library
H98312	BM Collagen	-	-
CB338725A	BM Pyruvate	+	+
CB338725B	BM Pyruvate	-	+
CK850096	WB Cuticlin 1.0	-	+
CK855471A	WB Collagen	-	+
CK855471B	WB Collagen	-	-
CK855471C	WB Collagen	-	+
CK855471D	WB Collagen	-	-
CK855471E	WB Collagen	-	-
CK855471F	WB Collagen	-	+
CK854857	WB Cuticlin 2.0	-	-

Gene Identifier = Genebank Accession number available at http://www.ncbi.nlm.nih.gov/.
+ = PCR product detected, - = no PCR product detected
D. immitis cDNA library abbreviations: DiMf= microfilarial stage, DiL3 = infective L3 stage

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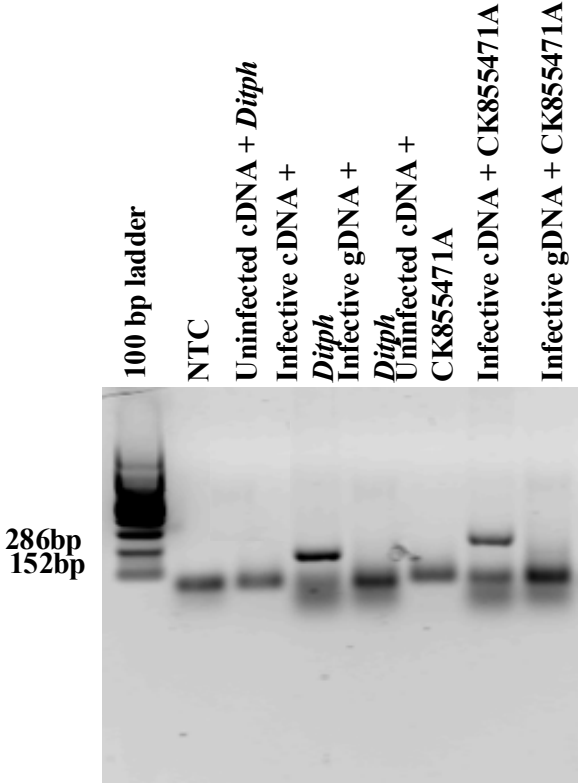
RT-PCR of candidate genes against mosquito pools

CONVENTIONAL RT-PCR



Tested five candidate genes against uninfected and, infective (14 DPI) *Ae. aegypti* black-eyed mosquitoes

RNA extractions were performed by Trizol extraction and cDNA synthesis was performed using the SuperScript IV First Strand Synthesis kit

Table 3. RT-PCR of Candidate Genes against Mosquito Pools.

Gene Identifier	Infective mosquito cDNA	Uninfected mosquito cDNA	Infective gDNA
CB338725B	-	-	-
CK850096	-	-	-
CK855471A	+	-	-
CK855471C	-	-	-
CK855471F	-	-	-

Gene Identifier = Genebank Accession number available at http://www.ncbi.nlm.nih.gov/.
+ = PCR product detected, - = no PCR product detected

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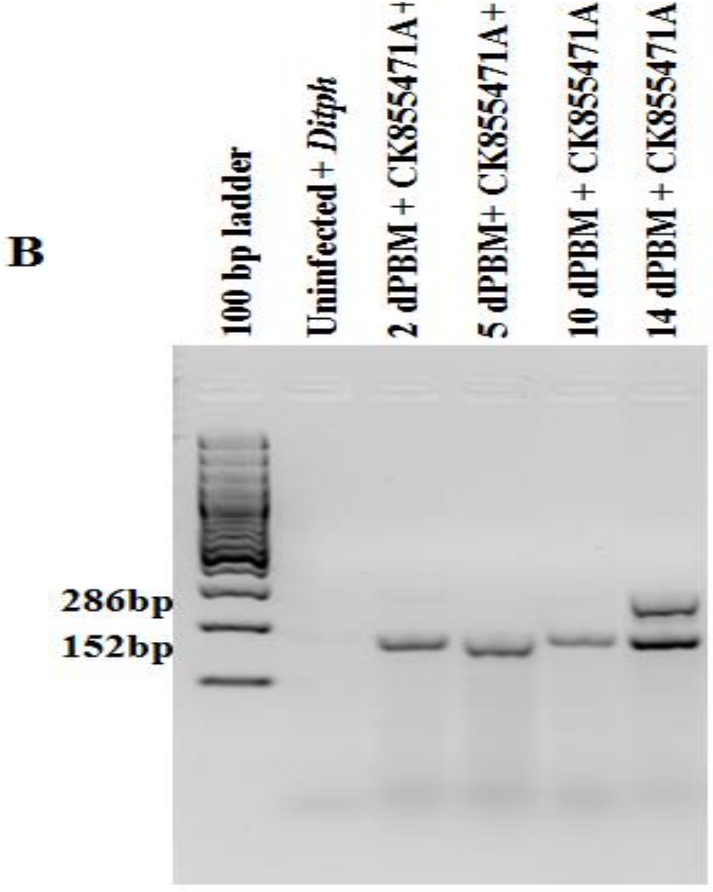
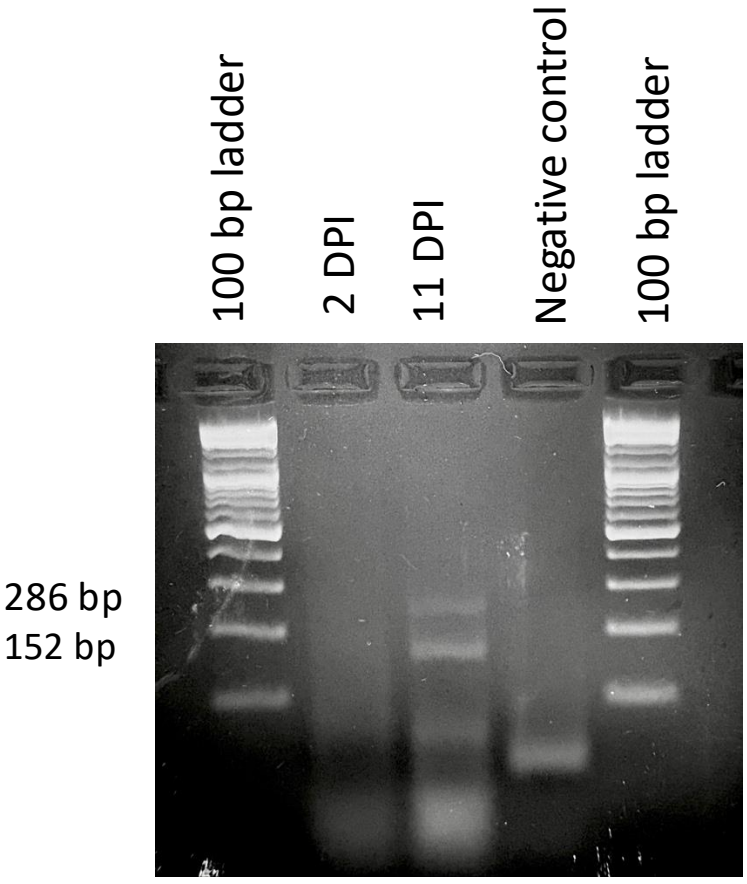
Time-course study

Tested CK855471A against uninfected, 2, 5, 10, 11, and 14 (DPI) mosquitoes





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Primer-BLAST » JOB ID:V12I6SqlJw0AMz02MFYZBEpNCDZnXhMrZg

Primer-BLAST Results

Edit search NEW

Input PCR template none

Specificity of primers Target templates were found in selected database: Nucleotide collection (nt) (Organism limited to Filarioidea)

Other reports Search Summary

Detailed primer reports

Download primer pairs

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAGAACGCGCATACAAGGC	19	59.65	57.89	4.00	2.00
Reverse primer	GGTTTGCAACAACTATCACAGGC	23	60.86	47.83	6.00	2.00

Products on target templates

>XM_001897725.2 Brugia malayi Collagen protein 109, putative (Bma-col-109), partial mRNA

```
product length = 262
Forward primer  1   GAGAACGCGCATACAAGGC  19
Template        14  ...................  32

Reverse primer  1    GGTTTGCAACAACTATCACAGGC  23
Template        275  ......................  253
```

>XM_003149486.1 Loa loa hypothetical protein partial mRNA

```
product length = 262
Forward primer  1   GAGAACGCGCATACAAGGC  19
Template        14  ....G..............  32

Reverse primer  1    GGTTTGCAACAACTATCACAGGC  23
Template        275  ...A.A........G........  253
```

Screening the **CK855471A** primer set against all sequences deposited in GenBank from the Superfamily Filarioidea did yield hits against *Brugia malayi* and *Loa loa*

Key features of CK855471A

- Ortholog to a *Wuchereria bancrofti* L3 cDNA
- Spans identifiable intron-exon boundaries
  - No amplification of gDNA was seen
- No amplification of uninfected mosquitoes
- No expression in infected mosquito pools prior to 11 days post blood meal (pre-L3 stage)

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Significance of Study

- Allow for confirmation of mosquito vectors capable of transmission
- Template for other identifying larval stage expression in other members of *Dirofilaria*

➢ Ongoing work*:*
  - Assay Sensitivity – Determining the maximum number of mosquitoes that can be analyzed per pool
  - Sequencing of PCR products
  - Monitoring or surveillance tool in the field-screen mosquito species that have been shown to be positive for *D. immitis* in standard PCR assays

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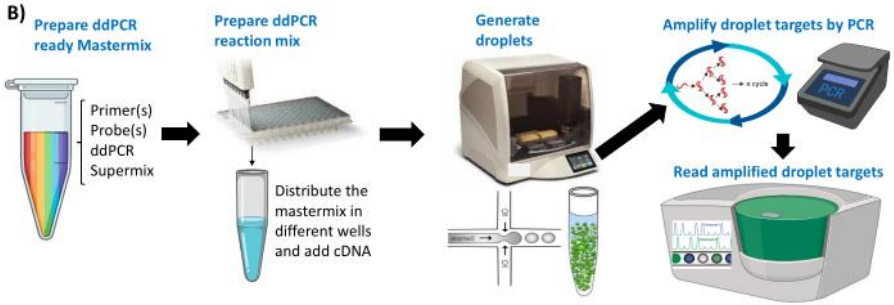
Table 1. Female mosquitoes collected March 2023 in Lowndes county, GA.

	An. crucians females	*An. quadrimaculatus* females
Barber	13	0
Baytree	12	0
Brown Rd.	4227	161
Bunche	23	96
Deloach	451	38
East Park	6	0
Hammock	139	20
Masonic Lodge	48	10
Old Clyattville	8766	137
Plantation	63	15
Public Works	2	0
Sheeley	36	7
Thomas	1587	163
Woodmen Cir.	221	7
Total	15594	654

Expanding the Study-VSU Biotechnology and Genomics Hub for South Georgia

University Investment

1. Sanger Sequencing
2. Droplet Digital PCR (ddPCR)



- ddPCR-superior sensitivity and precision over standard PCR
- Ideal platform for identifying mosquito pools with L3 expressed gene

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Acknowledgments

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  - Valdosta State University Faculty Seed Grant
  - Valdosta State University Graduate School

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