Optimizing Filarial Diagnostic RT-PCR Assays

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In order to monitor the progress of lymphatic filariasis elimination programs around the world, quantitative reverse transcriptase-PCR assays have been developed to detect infective stages of the disease-causing filarial parasites in mosquitoes. My work has focused on improving these assays by optimizing the preservation and extraction methods they require and by improving the species-specificity of one of the assays.

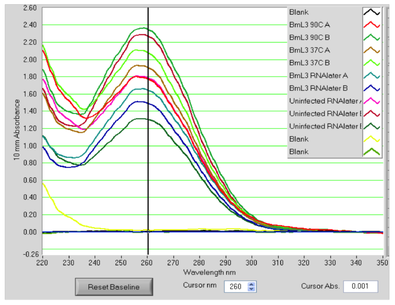
Comparison of Sample Preservation Methods

Current protocol requires preservation of mosquitoes in RNA*later*. Drying the mosquitoes rather than using RNA*later* would both reduce costs and simplify field collection. These different methods of preservation were compared to determine whether drying mosquitoes preserves parasitic RNA as well as using RNA*later* does.

RNA was extracted from *B. malayi*-infected mosquitoes preserved in RNA*later*, mosquitoes slow dried at 37°C for 1 hour (equivalent to sun drying), and mosquitoes fast dried at 90°C for 15 minutes. The yield and quality of the RNA extractions were measured using a nanodrop spectrophotometer.

Drying vs. RNAlater preservation		
Sample	**ng/µl**	**260/280**
BmL3 90C A	88.78	2.02
BmL3 90C B	117.58	1.94
BmL3 37C A	95.47	2.04
BmL3 37C B	104.14	2.02
BmL3 RL A	82.3	1.96
BmL3 RL B	74.66	2.02
Uninfected RL A	89.5	1.99
Uninfected RL B	65.18	1.99

BmL3 90C=fast-dired, BmL3 37C=slow-dried, BmL3 RL=RNA*later*

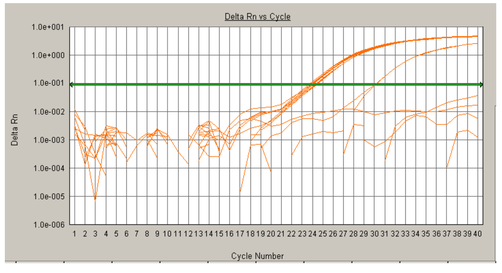


Nanospec results: all samples contain good quality RNA

Results:

• The quality and quantity of RNA extracted from dried mosquitoes is comparable to that of the RNA extracted from RNA*later* preserved mosquitoes.

A qRT-PCR assay was run to determine if the samples contained amplifiable parasite RNA. The *tph-1* assay (shown below in orange) tested for the presence of filarial parasites at any developmental stage.



qRT-PCR results: Slow-dried samples have a Ct value of about 30 cycles, fast-dried and RNA*later* samples have a Ct value of about 24 cycles

Results:

• All preservation methods yield amplifiable parasite RNA
• Ct values suggest RNA*later* and rapid drying are equally effective at preserving RNA
• Ct values suggest slow-drying is less effective at preserving RNA

Conclusion: Drying mosquitoes at 90°C for 15 minutes preserves parasite RNA as effectively as storage in RNA*later*. Drying at 37°C for 1 hour is less effective.

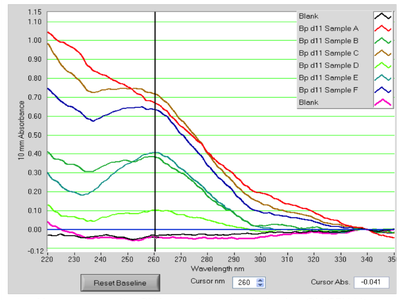
Comparison of RNA Extraction Methods

The current RNA extraction method uses a toxic TRI reagent, and is fairly cumbersome. The RNeasy Lipid Tissue Mini kit from QIAGEN is an easy to use, column-based RNA extraction protocol. These two methods were compared to determine if the QIAGEN kit is an efficient and reliable method of isolating parasitic RNA from mosquitoes and can be used instead of the TRI reagent protocol.

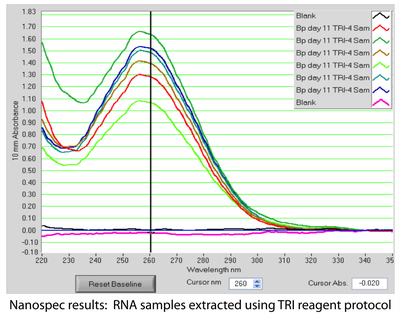
RNA was extracted from *B. pahangi*-infected mosquitoes using each protocol. The yield and quality of these extractions were measured using a nanodrop spectrophotometer.

RNeasy Lipid Tissue Mini Kit		
Sample	**ng/µl**	**260/280**
Bp d11 A	33.54	1.64
Bp d11 B	19.22	2.19
Bp d11 C	35.87	1.78
Bp d11 D	5.13	2.09
Bp d11E	20.34	2.06
Bp d11F	31.68	1.87

TRI-4 Mosquito RNA extraction protocol		
Sample	**ng/µl**	**260/280**
Bp d11 G	64.19	2.05
Bp d11 H	81.91	2.05
Bp d11 I	69.75	2.03
Bp d11 J	53.32	1.98
Bp d11 K	74.23	2.01
Bp d11 L	76.03	1.99



Nanospec results: RNA samples extracted using QIAGEN kit

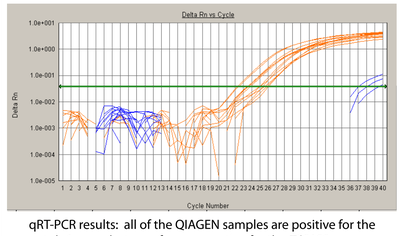


Nanospec results: RNA samples extracted using TRI reagent protocol

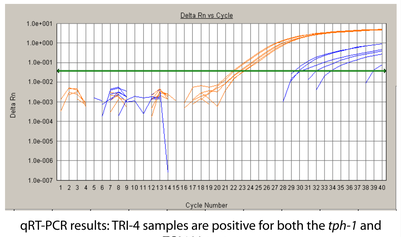
Results:

• QIAGEN kit yields RNA samples of variable quality and relatively low quantity
• TRI-4 protocol consistently yields good quality RNA in relatively high quantities

A qRT-PCR assay was run using the QIAGEN and TRI-4 samples to confirm that the samples contained amplifiable parasite RNA. The *tph-1* assay (shown below in orange) tested for the presence of parasites at any developmental stage, while the *TC8100* assay (shown below in blue) tested specifically for L3-stage parasites.



qRT-PCR results: all of the QIAGEN samples are positive for the *tph-1* assay, but very few are positive for the *TC8100* assay



qRT-PCR results: TRI-4 samples are positive for both the *tph-1* and *TC8100* assays

Results:

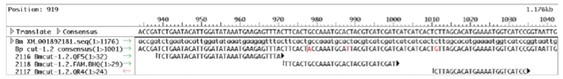
• Both protocols yield amplifiable parasite RNA
• Both protocols yield enough RNA for the *tph-1* assay
• QIAGEN kit does not consistently yield enough RNA for the *TC8100* assay

Conclusion: The QIAGEN RNeasy Lipid Tissue Mini Kit is less efficient, less consistent, and yields lower quality RNA than the TRI reagent method.

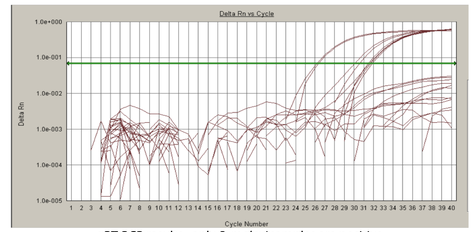
Making the *Brugia* L3 Detection Assay Specific to *B. malayi*

The current *Brugia* L3 detection assay is not species-specific. It detects both *B. malayi* parasites (which infect humans) and *B. pahangi* parasites (which infect animals). Two genes were investigated as possible targets of a species-specific assay.

A cuticlin gene, *cut-1.2*, which was identified as being L3-activated in *W. bancrofti* (1), was targeted as a possible candidate for the development of this assay. The *B. malayi* and *B. pahangi* orthologues were sequenced and compared for differences. A primer/probe set was designed to exploit single nucleotide polymorphisms (SNPs) between the species in order to make the assay specific to *B. malayi*.



Alignment of *B. malayi* and *B. pahangi cut-1.2* sequences with primers and probe: the reverse primer (2217) and probe (2218) both contain SNPs that make them specific to the *B. malayi* sequence.

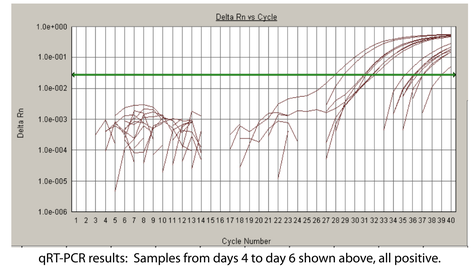


qRT-PCR results: only *B. malayi* samples are positive

Results:

• Assay detects only *B. malayi* RNA, not *B. pahangi, W. bancrofti* or *D. immitis* RNA
• Primers and probe (#2116-2118) are species-specific

An L3 detection assay requires the onset of expression of the target transcript to correlate with the development of the L3 stage of the parasite in mosquitoes. A developmental timecourse was tested using the species-specific primers and probe to determine if *cut-1.2* is L3-activated in *B. malayi*.



qRT-PCR results: Samples from days 4 to day 6 shown above, all positive.

Results:

• *cut-1.2* is expressed before the time point of L3 development in the mosquito (day 7)

Conclusion: Although the assay is species-specific, it cannot be used as a *B. malayi* L3 detection tool because *cut-1.2* is not L3-activated in *B. malayi*

Ongoing Research

Current research focuses on designing a *B. malayi*-specific assay using the *TC8100* gene, which is known to be L3 activated (2). Orthologues from both *B. malayi* and *B. pahangi* have been sequenced and compared. Several SNPs were identified that could potentially be exploited to develop an assay that detects only *B. malayi* RNA.

REFERENCES:
(1) Laney, S. J. et al. 2010. "Detection of *Wuchereria bancrofti* L3 Infective Larvae in Mosquitoes: A Reverse Transcriptase PCR Assay Evaluating Infection and Infectivity." PLoS Neglected Tropical Diseases, 4(2): e602
(2) Laney, S. J. et al. 2008. "A Reverse Transcriptase PCR Assay for Detecting Filarial Larvae in Mosquitoes." PLoS Neglected Tropical Diseases, 2(6): e251.