AMPLIFICATION USING THE POLYMERASE CHAIN REACTION (PCR) OF A SINGLE COPY GENE (*Tph-1*) FROM GENOMIC *Brugia* DNA

1. In a 0.2 ml PCR tube, add the following:

30.5 μl	ddH_2O
8 μl	dNTPs mix
5 μl	10X PCR reaction buffer
3 μl	$MgCl_2$ (25 mM)
1 μl	*Tph* Primer F (10 pmoles/μl)
1 μl	*Tph* Primer R (10 pmoles/μl)
0.5 μl	*Taq* Gold DNA polymerase
49 μl final volume	

Mix by stirring with your pipet tip, add 1 μl template DNA (your *Brugia* genomic DNA).

2. Be sure to label this tube on the side with your group numbers and '*Tph* DNA PCR' (the temperature block will remove any writing on the cap).

3. Place your reaction tube in a chamber of the thermal cycler.

4. The PCR program has been set as follows:

   A. An initial denaturing step at 94°C for 12 minutes is performed to insure that all of the DNA sample is single-stranded and that the *Taq* Gold is activated.

   The following three steps (B-D) will be cycled 35 times:

   B. 94°C for 30 sec to denature the double-stranded DNA.

   C. 52°C for 30 sec to enable primers to anneal to template DNA.

   D. 72°C for 1 min to synthesize DNA from the primers (this is the

temperature at which *Taq* polymerase is most active).

E. After the 35 cycles, a final extension/annealing step at 72°C for 10 min is added to insure that all of the extensions are complete.

F. The program will hold the tubes at 8°C until the instrument is turned off.

We will run these on an agarose gel later with your RT-PCR samples.

RT-PCR: AMPLIFICATION OF THE *Tph* TRANSCRIPT FROM *Brugia* TOTAL RNA

An alternative to standard Northern blot analysis for the detection of a specific RNA sequence is the reverse transcriptase—polymerase chain reaction (RT-PCR) technique. This method is also referred to as RNA-PCR. With this technique, a sample of RNA is first subjected to reverse transcription to generate complementary DNA (cDNA) copies of the RNA sequence. This cDNA is then used as a template for a standard series of DNA PCR amplifications. The products of this reaction are then run on an agarose mini-gel and stained with ethidium bromide to visualize the amplified fragment.

RT-PCR affords several potential advantages that complement or replace those of standard Northern blot analysis. First, RT-PCR is very sensitive, so that only very small starting amounts of total RNA are required for the analysis (poly A^+ mRNA is usually not required). Second, because only a small subfragment of the entire mRNA transcript is usually amplified, RNA that is somewhat degraded can still provide a positive result. As with DNA PCR, however, the extreme sensitivity of the RT-PCR method renders this technique susceptible to false-positive results. Several controls that should be run routinely to verify the validity of any positive result will be discussed in lecture.

For this experiment, you will use 1 μl of your total RNA sample that you prepared yesterday. A cDNA copy of your RNA can be generated using random primers, oligo-dT primers, or a primer specific to the target RNA.

We will use the latter (a downstream specific primer).

After the RT reaction to generate a cDNA copy of each *Tph* mRNA transcript present in the sample, we will then run 40 DNA PCR cycles to amplify the desired fragment from the *Tph* cDNA template. The primers we will be using for the *Tph* cDNA will amplify a fragment about 400 base pairs in length. The two primers flank an intron in genomic DNA.

The amplified cDNA will then be run on an agarose gel for direct EtBr staining and visualization. NOTE: a protocol that uses DNase to eliminate any chance of DNA contamination can be obtained by contacting FR3. This is especially useful if you are working with a gene that has no introns.

1. We will use the Qiagen One-Step PCR Kit.

2. Mix together in a 0.2 ml microfuge tube:

29 μl RNase-free ddH_2O
10 μl 5X Qiagen OneStep PCR Buffer (contains 12.5 mM $MgCl_2$)
2 μl dNTP solution (10 mM each dNTP)
3 μl 10 pM/μl of the *Tph* Primer F (upstream specific)
3 μl 10 pM/μl of the *Tph* Primer R (downstream specific)
2 μl Enzyme mix containing *Taq* polymerase and Reverse Transcriptase

3. Add 1 μl of your Total RNA template last! This gives a total volume of 50 μl.

4. Place your tube into the PCR instrument. Once all of the groups have loaded their tubes, we will run one cycle to generate first strand cDNA and then 40 cycles of standard PCR to generate the double-stranded DNA PCR product.

The following parameters will be used for this PCR:

A. 50ºC for 30 min to anneal the *Tph* reverse primer (downstream specific primer) and do first-strand synthesis.

B. 95°C for 15 min to heat kill the reverse transcriptase enzyme

C. 52°C for 5 min (to anneal primers for PCR).

D. 40 cycles of: 72°C for 90 sec, 94°C for 45 sec, and 52 ^{o}C for 45 sec.

E. 72°C for 10 min (final extension).

F. Hold at 8°C.

In a real RT-PCR experiment, the following controls should be run:

1) A ‘no template’ control reaction in which 1 μl of water is added instead of the RNA template.

2) A ‘no RT’ control reaction in which 1 μl of water is added instead of reverse transcriptase enzyme.

3) A positive control should also normally be run. We will discuss options for positive controls in lecture.

Your RT-PCR products will be run on a gel with your genomic PCR products as will be described later.