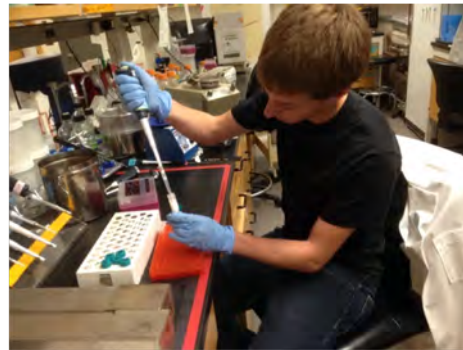
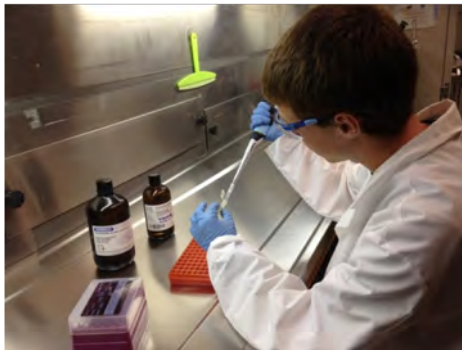


Quantitative PCR (qPCR)

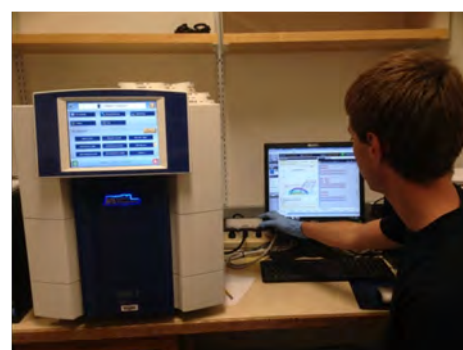
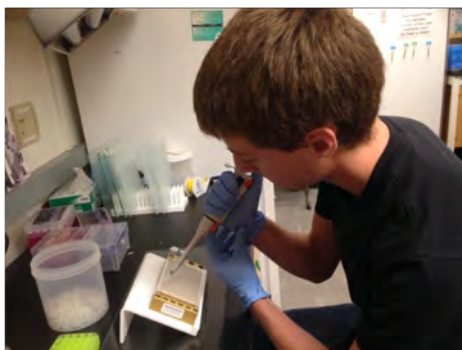
The polymerase chain reaction (PCR) is a method of making many copies of a specific segment of DNA. PCR makes it easy to rapidly and efficiently generate thousands or even millions of copies of a particular DNA sequence starting with as little as a single copy. PCR generally involves a series of thermal cycles during which the target DNA is repeatedly heated and cooled in small tubes in the presence of short single stranded fragments of DNA (primers) that are complementary to the ends of the region to be amplified, DNA polymerase, and an appropriate buffer. When using standard PCR, the copies of DNA made are only analyzed at the end of the process, usually by running them on an agarose gel (end point analysis). A detailed, clearly illustrated discussion of PCR can be found on Wikipedia (https://en.wikipedia.org/wiki/Polymerase_chain_reaction), and an excellent video illustrating the process is provided by the DNA Learning Center at <https://www.youtube.com/watch?v=2KoLnIwoZKU>.

The environmental DNA samples submitted to FishTracker are analyzed at Cornell using a variation of the standard polymerase chain reaction (PCR) known as quantitative PCR (qPCR). qPCR is more sensitive than standard PCR and allows analysis of the DNA copies being made as the reaction is actually going on (real time analysis). The reaction is monitored by incorporating a fluorescent dye into the newly made DNA product, which can then be measured on a special instrument that provides real time information about what DNA containing the dye is present in the reaction and how much. A more detailed overview of qPCR can be found here (<https://www.youtube.com/watch?v=1kvy17ugl4w>).

Pictures showing the processing of eDNA samples at Cornell and an example of a qPCR program and the kind of qPCR data generated is shown in below, along with a basic explanation of how to interpret the information provided by qPCR analysis.

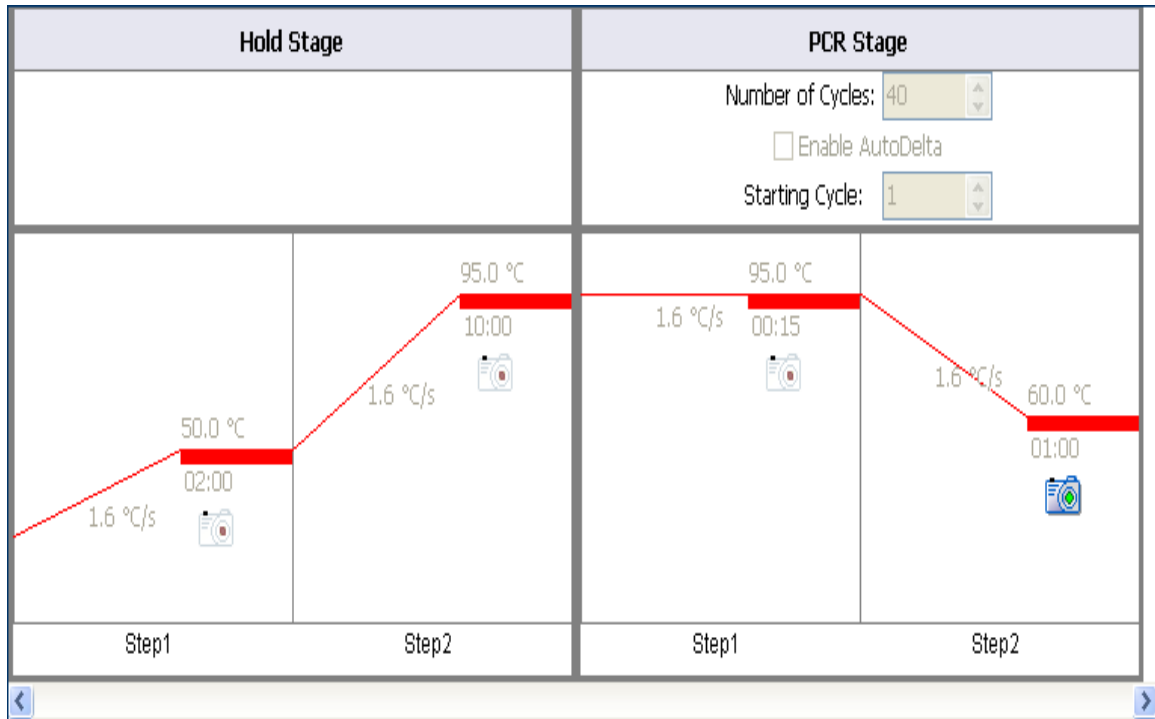


Extracting eDNA



Setting up the qPCR plate and running the qPCR machine

2-step qPCR program

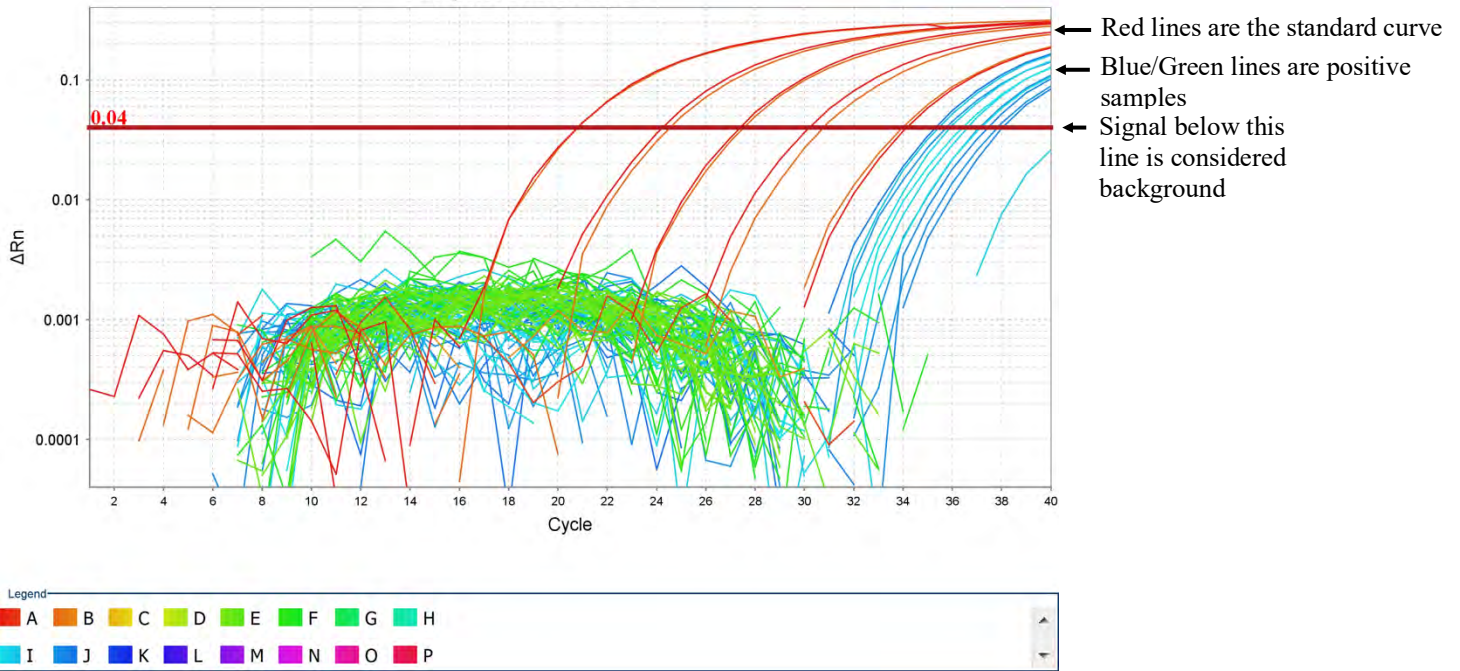


This is the qPCR program used in your experiment. There are 2 stages. The Hold stage has 2 steps. Step 1 of the Hold Stage (the 2-min, 50 °C step) is required for optimal AmpErase® uracil-N-glycosylase (UNG) activity. UNG is a component of the TaqMan Universal PCR Master Mix that helps to prevent reamplification of carryover-PCR products and greatly reduces the possibility of cross contamination. Step 2 of the hold stage (the 10-min, 95 °C step) is required to activate the AmpliTaq Gold enzyme used in the qPCR reaction, to cleave the dU-containing PCR product generated in the low temperature (18 to 50 °C) incubation, to substantially reduce AmpErase UNG activity, and to denature the native DNA in the experimental sample.

The 40 cycle PCR stage is made up of 2 steps, a 15 second 95°C denaturation step and a 1 minute 60°C combined annealing and extension step (most standard PCR protocols separate annealing and extension into 2 separate steps at 2 different temperatures). During these cycles the PCR product is amplified. An example of a graph of the results of amplification are shown in the Amplification Plot below.

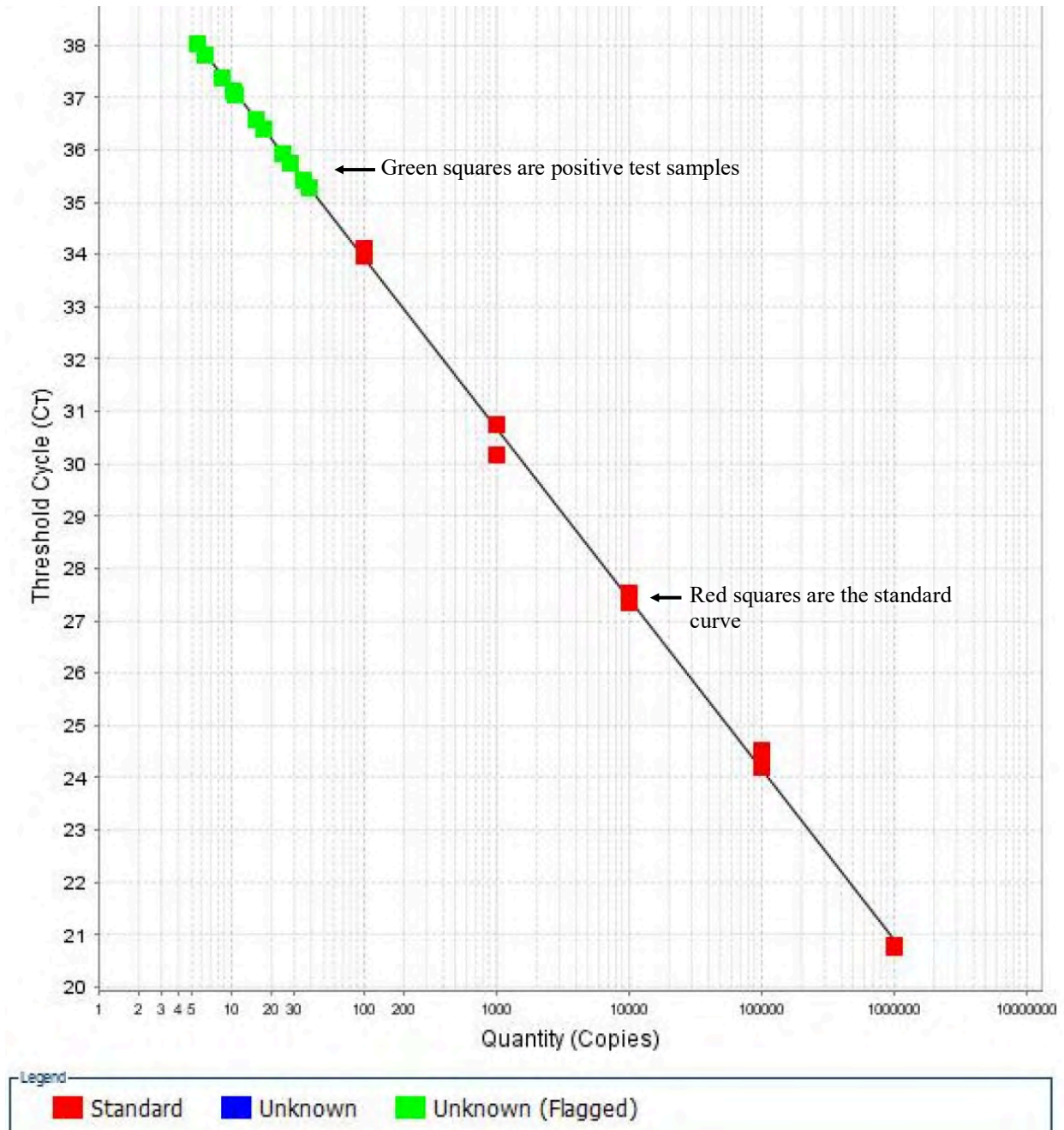
Real Time quantitative PCR (qPCR) is very much like standard PCR. The biggest difference is that in qPCR the amount of PCR product is measured after each round of amplification, while in standard PCR the PCR product is only measured at the end of amplification. The qPCR amplification products are measured as they are produced using a fluorescent label. A fluorescent dye binds to the accumulating DNA molecules during amplification, either directly or indirectly via a labeled hybridizing probe. The fluorescence is recorded during each cycle of the amplification process. The fluorescence signal is directly proportional to DNA concentration over a wide range. The linear correlation between the amount of PCR product and the fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction.

Amplification Plot



This is the output produced by the qPCR machine. For each reaction, standard template DNA of different concentrations is run (shown in red) along with samples to be tested (green lines). Our qPCR reactions use primers and probes that are very specific to the invasive fish species we are looking for. No other DNA will not produce a signal. The DNA probe is tagged with a fluorescent reporter dye. If DNA from the invasive species is present in the sample being tested, the qPCR reaction will result in the production of a fluorescent signal that is detected by the qPCR machine. As the qPCR reaction proceeds, at each cycle the accumulation of PCR products is detected directly by monitoring the increase in fluorescence in the reaction. The more template present, the earlier (lower cycle number) the fluorescent signal will be detected and shown on the graph. For example, the most concentrated control standard sample is indicated by the leftmost red line and the least concentrated standard sample is indicated by the rightmost red line. Multiple samples, positive and negative, tested at the same time, are shown in this plot.

Standard Curve



This graph shows an example of the standard curve and sample readings. This curve provides an approximate number of copies of the DNA template in the very tiny (1 ul) sample of water tested. Each sample tested is a separate mark on the graph. Positive samples (green boxes) are each done in duplicate. Red squares are the control samples of known DNA copy number.