PCR Testing

By Spike Cover
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When a koi is suspected of having koi herpes virus, the most sensitive way to confirm or refute the suspicion is to have a PCR test done on a sample of the fish's tissue (usually from the gills, liver and/or kidney).

So just what is a PCR test anyway? For this, we need some background basics before we get to the actual answer to this question.

Background:

The nucleus of each living cell contains long strands of DNA. DNA is deoxyribonucleic acid and resembles a long spiral ladder. The sides of the ladder are formed from sugar molecules. The rungs of the ladder are formed from another group of molecules, the nucleotide bases.

There are four bases, adenine (A), thymine (T), cytosine (C) and guanine (G). Each rung is made from two of the bases, one base attached to each side of the ladder. The two bases then meet in the middle and weakly bind to each other. The bases will only make rungs with specific combinations. The A bases will only join with T and C bases will only join with G. Each side of the ladder caries a long chain of bases (A,T,G,C) that form the code needed to build the organism. Because the bases will only form specific pairs in the rungs, each side of the ladder can be used as a sort of copy of the other side. If one side has the sequence T-G-C-A-G you know that the other side must be A-C-G-T-C (A only pairs with T and G only pairs with C).

Each type of life has nucleotides within the strands of DNA that are in a unique sequence. If a sufficient portion of this unique sequence can be determined (called "sequencing"), that sequence can be used to identify a particular species. In 2000, Ron Hedrick and Orin Gilad from the University of California at Davis were able to isolate the koi herpesvirus ("KHV") and later to locate and sequence a unique portion of it's DNA sufficient to be able to distinguish it from other DNA within the koi and that of other pathogens that invade koi. Since then, at least three other laboratories have developed tests for KHV that use similar methods.

The test:

PCR stands for Polymerase Chain Reaction and it is used to amplify specific regions of DNA. Another way to say this is that it is a test tube system for DNA copying that allows a "target" DNA sequence to be selectively amplified several million-fold within a short period of time (hours or minutes). The process is so sensitive that it can detect the presence of a single virus sequence in 100,000 cells.

During PCR, suspect DNA molecules are put into a small plastic tube with synthetic molecules of single stranded DNA (usually 20-30 nucleotides) called primers. Primers are designed to match with specific sequences in the DNA strand and two different primers are used to bracket the target region to be amplified. One primer is complementary to a DNA strand at the beginning

of the target region; a second primer is complementary to a sequence on the opposite DNA strand at the end of the target region. It is worth noting that the target region need not be sequenced, only the regions directly adjacent to it (the primer regions) which are used to define the target region.

Next, all the molecules needed to make copies of DNA molecules are added along with an enzyme that does the copying. The tube is placed into a PCR machine that heats the tube to about 95° C to make the double stranded DNA fall apart into 2 into single stranded molecules.

The tube is then cooled to 50-65° C so that the primers can bind to the target DNA. This process is termed "annealing."

Next the tube is heated to 72 degrees C so that the DNA synthesizing enzyme (DNA polymerase) can grab onto the primer sequences and copy the stretch of target DNA that runs between where the primers have attached. When this step is complete, there are 2 additional copies of the target DNA. The machine then heats the sample again to separate the new strands, cools so primers can bind, then warms so that the enzyme can make copies. The machine repeats the cycle as many as 32 times completing something like 1 billion copies of a single original molecule.

A simplified representation of this process is given below (thanks to Dr. Andy Goodwin).

Normal Double-Stranded DNA

----G-A-T-C-T-T-A-A-G-C-G-C-T-T-A-T-A-A-A-T-C-C------
----C-T-A-G-A-A-T-T-C-G-C-G-A-A-T-A-T-T-T-A-G-G-----

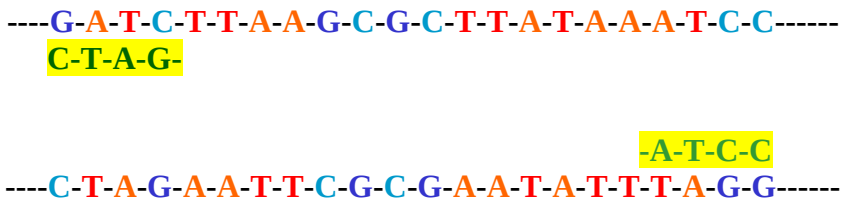
"Melting"

----G-A-T-C-T-T-A-A-G-C-G-C-T-T-A-T-A-A-A-T-C-C------

Heat

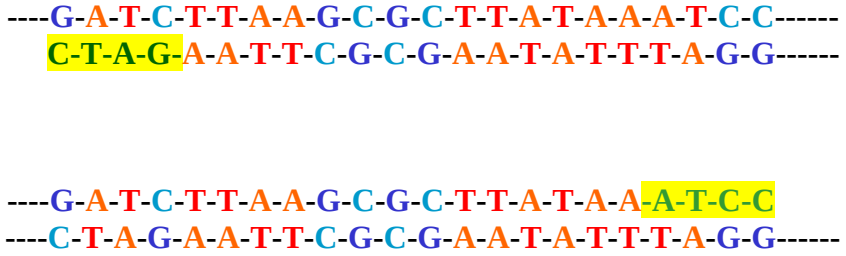
----C-T-A-G-A-A-T-T-C-G-C-G-A-A-T-A-T-T-T-A-G-G-----

Primer Binding (Annealing)



Primers shown in yellow highlight

Completing the copies (Extension)



This same cycle is repeated numerous times until the “target” regions (plus the primer regions) are replicated many times. As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty-two such cycles, a theoretical amplification factor of one billion is attained (the first two cycles are not counted but that a bit more complicated).

Cycles	Copies
1.	2
2.	4
3.	8
4.	16
5.	32
6.	64
7.	128
8.	256
9.	512
10.	1,024
11.	2,048
12.	4,096
13.	8,192
14.	16,384
15.	32,768

Cycles	Copies
16.	64,000
17.	128,000
18.	256,000
19.	512,000
20.	1,024,000
21.	2,000,000
22.	4,000,000
23.	8,000,000
24.	16,000,000
25.	32,000,000
26.	62,000,000
27.	128,000,000
28.	256,000,000
29.	512,000,000
30.	1,024,000,000

High temperature polymerase makes the reaction simpler and better

The original polymerase use in the PCR was from *E. coli*. This enzyme is sensitive to heat and is destroyed at the DNA denaturing ("melting") temperature. Thus new polymerase needed to be added to each cycle making for a tedious process.

A significant improvement came with the discovery and use of polymerase from a heat-tolerant bacterium isolated from the waters of hot springs. The bacterium *Thermus aquaticus* (*Taq*) lives in hot water (75º C), has polymerase works best at 72º C and is relatively stable even at 94º C. *Taq* polymerase added at the start of the PCR process remains active thru all the cycles. This allowed the process to be automated.

After the PCR process is completed, the products of the reaction are put in an agar gel and subjected to an electrical field. The molecules will migrate various distances through the gel based on size, shape and electrical charge. The gel is then stained with a fluorescent DNA stain so that the molecules can be seen. Each PCR reaction is designed to produce DNA molecule copies that will all be of the same specific size and that size can easily be determined from the gel. If the PCR reaction produces DNA molecules of the correct size, the test is positive.

Below is a photograph of an electrophoresis gel showing the test and controls (positive, negative and molecular weight).

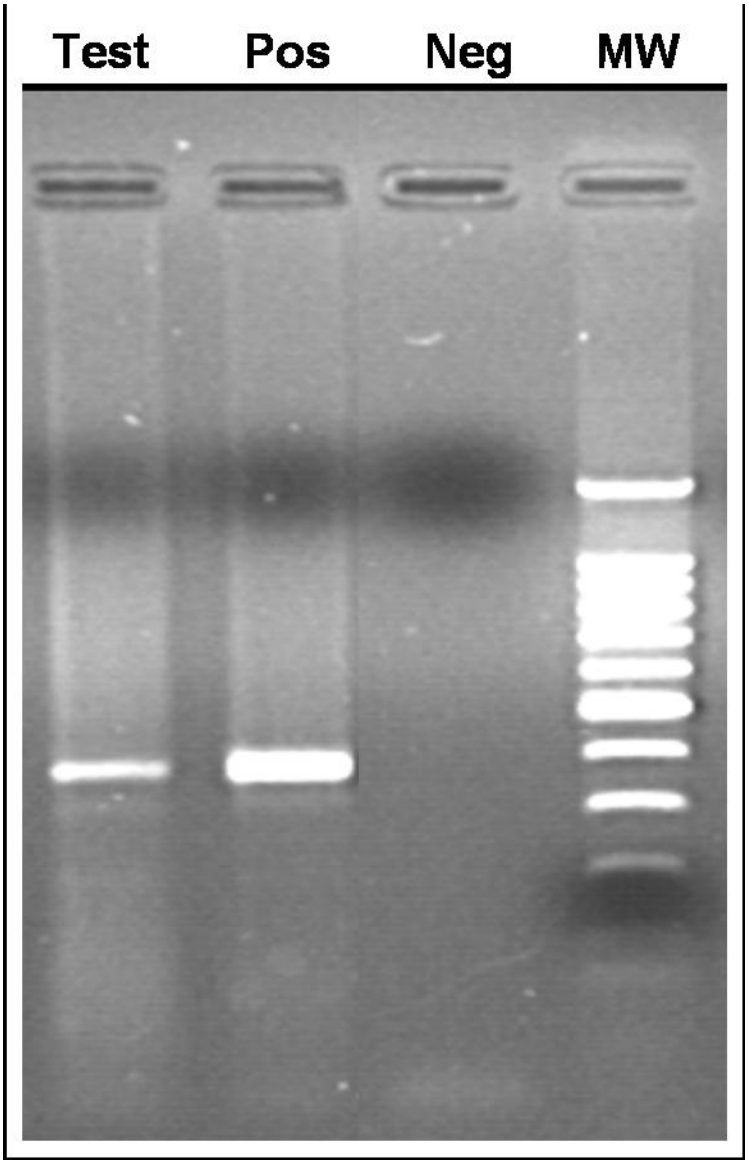


Photo courtesy of A. Goodwin, Univ. of AR at Pine Bluff

A new technique that precludes the use of electrophoresis includes the addition of a special dye in the tubes at the start of the test. If the target material is found and copied, the dye fluoresces and is detected by a sophisticated new generation of PCR machines.

Typically research equipment for traditional PCR testing is relative inexpensive and can be had for about $5000 (but the primers and Taq are relatively expensive). The problem is that these units are rather slow and 30+ cycles may take several hours. So researchers usually set up tests upon going home and read the results in the morning. The new assays that use fluorescent molecules to detect the products of the PCR test while the test is underway are much quicker and may produce results in under an hour. The first test of this type was also developed by Gilad and Hedrick at UC Davis. This assay is very rapid and more sensitive than traditional tests, however, the special PCR machine needed for this assay costs from 25-50 thousand dollars and the

chemicals used to run the test are also more expensive. This new technology is available in several fish disease diagnostic laboratories, but the current consensus is that for KHV this technology is most appropriate for research rather than diagnostic use.

Interpreting the results of PCR tests

The use of PCR tests in fish health has a controversial history but that's a subject for another time. Suffice it to say that if a fish or group of fish exhibits symptoms consistent with KHV, the environmental conditions are "right" (temperature, potential exposure, etc.) and a positive PCR result is obtained from suspect fish tissue, the likelihood that the fish was infected with KHV is virtually assured.

Glossary

anneal
a process by which the primers hybridize (by way of hydrogen bonds) to their complementary sequences on either side of the target sequence

amino acid
the building block of proteins

base pair (bp)
the complex of two nucleic acid bases

DNA (deoxyribonucleic acid)
ladder-like molecule that stores genetic information, including information needed to build proteins

electrophoresis
The movement of particles in an electric field toward an electric pole (anode or cathode); used to separate and purify biomolecules.

enzyme
a protein that helps control chemical reactions

nucleotide
the basic unit of DNA consisting of a base (A, C, T, or G), a sugar, and a phosphate

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