Expanding Fish Health Capabilities with Quantitative Polymerase Chain Reaction (QPCR)

U. S. Fish and Wildlife Service
August 2002

Kimberly True
Assistant Project Leader
California-Nevada Fish Health Center

Expanding Fish Health Capabilities with Quantitative Polymerase Chain Reaction (QPCR)

Section TABLE OF CONTENTS Pg

Expanding Fish Health Capabilities with Quantitative Polymerase Chain Reaction (QPCR)

This report describes recent advancements in the field of biotechnology, specifically in the technique termed Quantitative Polymerase Chain Reaction (QPCR). The purpose of this report is to illustrate how this molecular tool could be employed by the U.S. Fish and Wildlife Service's Fish Health Centers to further advance scientific understanding and applied research in detecting, controlling and managing fish diseases in hatchery and wild fish populations.

> " PCR has transformed molecular biology through vastly extending the capability to identify, manipulate and reproduce DNA.
> It makes abundant what was once scarce – the genetic material required for experimentations."
>
> - Paul Rabinow Berkeley, California
> Making PCR, A Story of Biotechnology. Univ. of Chicago Press, 1996

Introduction: What is the Polymerase Chain Reaction or PCR?

Polymerase Chain Reaction (PCR) is a technique which amplifies a specific region, or sequence, of DNA in order to produce enough DNA to be detectable and/or further studied. PCR is commonly used in the fields of genetics and forensic science to identify animal species or individual humans by identifying their unique genetic code. In pharmacogenomic studies, PCR is used to discern normal gene function in the presence or absence of pharmaceutical compounds to determine the effects these compounds have on normal and abnormal cell functions. PCR is currently used in fish health, with a very high level of sensitivity and specificity, to detect disease-causing viruses, bacteria and/or parasites.

The PCR technique relies on a thermal-stable enzyme called DNA polymerase. Polymerase is a naturally occurring enzyme that catalyzes the formation and repair of DNA and RNA. The accurate replication of all living matter depends on the activity of this enzyme as DNA and/or RNA is the genetic blueprint for all subsequent structures and molecules that make up an individual organism.

In 1980 Kary Mullis at Cetus Corporation conceived of a way to start and stop a polymerase's action at specific points along a single strand of DNA, which allowed replication of a specific sequence of DNA, or 'target' DNA. When this step is repeated over and over again, millions of copies of the DNA can be formed. Mullis was awarded the Nobel Prize in Chemistry in 1993, however many others were instrumental in further developing the actual techniques which transformed the concept presented by Mullis into a feasible laboratory application.

When PCR and the polymerase enzyme that it employs were named "Molecule of the Year" in 1989, the editor of Science, Daniel Koshland Jr., provided a succinct explanation of PCR. He wrote:

> "The starting material for PCR, the 'target sequence' is a gene or segment of DNA. In a matter of hours, this target sequence can be amplified a million fold. The complementary strands of a double-stranded molecule of DNA are separated by heating. Two small pieces of synthetic DNA, each complementing a specific sequence at one end of the *target sequence*, serve as *primers*. Each primer binds to its complementary sequence. Polymerases start at each primer and copy the sequence of that strand. Within a short time, exact replicas of the target sequence have been produced. In subsequent cycles, double-stranded molecules of both the original DNA and the copies are separated; primers bind again to complementary sequences and the polymerase replicates them. At the end of many cycles, the pool is greatly enriched in the small pieces of DNA that have the target sequences, and this amplified genetic information is then available for further analysis."

Since the early inception and manipulations with PCR, scientists have learned to successfully manipulate DNA creating an immensely powerful tool that provides essentially unlimited quantities of the precise genetic material molecular biologists require to study genetics and a vast array of related fields such as molecular biology, immunology, epidemiology, pathology and pharmacology.

> "The versatility of the techniques is astounding and scientists have produced new uses with stunning regularity. In less than a decade, PCR has simultaneously become an absolute routine component of practically every molecular biological laboratory and a constantly changing tool whose potential has shown no signs of leveling off." – Paul Rabinow, 1998

How is PCR currently utilized in the field of Fish Health Management ?

PCR has been employed by the U.S. Fish and Wildlife Service's nine Fish Health Centers since the mid-90s. This technology was transferred to Fish Health Centers when they embarked on the National Wild Fish Health Survey (Survey) in 1994. The Survey was undertaken to determine the prevalence and distribution of fish pathogens in wild fish populations and gain a better understanding of disease processing in fish species. PCR was chosen as a collaborative testing technique because of the tremendous sensitivity this technique offered at that time.

Traditionally, pathogens infecting fish have been detected by standard microbiological techniques such as direct observation with a microscope, culture of bacterial organisms on selective growth media, or culture of viruses in specialized cell cultures that support replication of viral agents. All of these methods are valid approaches to detecting pathogens, however the organisms must be present in fairly large numbers in the fish host in order to detect them with standard microbiological methods. These detection methods work well when fish are actively infected by a pathogen, however they simply do not offer the sensitivity needed to detect pathogens if they occur at very low numbers in the environment or the fish host. This often is the case in latent infections, or during the initial onset of disease. The ability to detect pathogens early in the infectious process is

important to effectively control and manage fish diseases in both hatchery settings and in wild fish populations.

Therefore PCR was selected as collaborative pathogen detection tool in the National Wild Fish Health Survey because it offered the greatest sensitivity (the ability to detect pathogens at very low levels) of all available detection methods. PCR also offered very high specificity – the ability to identify the unique genetic sequence of a particular pathogen. However, because PCR was a newly emerging technique at that time, and had not been validated as a primary detection method in the field of fish health, it was only employed to confirm the identification of an organism after it was detected by standard, or more traditional methods. Pathogens that are detected using standard assay methods are further tested with PCR to collaborate the fish health findings of the Survey.

The potential for PCR technology as an initial screening tool, or diagnostic test, has advanced significantly since the initial use in the National Wild Fish Health Survey. Several researchers, universities, and commercial laboratories are using PCR to detect fish pathogens with tremendous sensitivity and success. For example, PCR can detect bacteria, viruses or parasites when as few as 10-40 organisms exist in the fish sample. This technological advance in detection sensitivity has allowed users to detect bacterial, parasitic and viral pathogens that normally would go undetected by traditional testing methods.

Because fish can often be carriers of a pathogen such as a virus or bacteria, yet show no external or clinical signs of disease, PCR is also a valuable tool to detect "low level" or sub clinical infections in both hatchery and wild fish populations. The larger application of PCR in fish health detection and research has occurred with the myxosporidean parasite, *Myxobolus cerebralis*, which causes Whirling Disease in trout species in the Northwest and Intermountain states. PCR has also been very effective in detecting other significant fish pathogens, such as infectious hematopoietic necrosis virus (IHNV), and the bacteria *Renibacterium salmoninarum* which causes Bacterial Kidney Disease (BKD).

While PCR offers much greater sensitivity and specificity than traditional detection methods, the disadvantage of this molecular tool is the fact that it is a qualitative detection method only, meaning that the assay tells one whether or not the specific target DNA is present in the sample, but it does not provide any information about the quantity of the DNA or the infection level of the pathogen in the fish host. So the answer obtained with standard PCR is a "yes" or "no" to the question of pathogen presence.

In disease management, most often we need to understand the severity of an infection in order to provide an appropriate response that addresses the level of biological risk to the population. For example, the biological significance of a pathogen is related to several factors including the severity of infection, the health of the fish host and its ability to tolerate the infection, and the long-term effects on normal function and ultimately long-term survival. The fish health biologist needs to know the severity of the infection to assess the overall impacts to the health of individual fish or a population of fish. A detection method that is qualitative only, such as PCR, does not provide this information.

Recent advancements in PCR technologies and the systems used to perform PCR assays have now developed a quantitative capability. Quantitative PCR (QPCR) or "real-time" PCR has emerged as the next level of technological advancement in the field of biotechnology. With QPCR, fish health centers would be able to address these biological questions more thoroughly, assess the risks to populations more effectively, and address many current and future health management issues.

What are the recent technological advancements in PCR and what benefits do they provide?

In the standard PCR technique, an instrument called a thermocycler is used to amplify the target sequence of DNA. As explained previously, heat separates the double-stranded DNA and allows the primers to attach and then copy the target DNA sequence. In the first cycle 2 copies of DNA are made from each double-stranded DNA present in the sample. In the second cycle 4 copies are made, then 8, etc. so that the copying, or amplification, proceeds in an exponential manner. This amplification process creates millions of copies of the exact target DNA sequence in a matter of a few hours. When the amplification process is complete, the vast majority of DNA in the sample is the target DNA and therefore the same size or base pair length.

Once the DNA is synthesized, it is visualized by loading a sample onto an agarose gel and an electric current is applied. DNA moves through the semi-solid gel matrix based on the size of the DNA sequence. On the electrophoretic gel, the DNA migrates according to its sequence size and is then stained with a dye so that the DNA bands are visible under ultra-violet light. Size standards (DNA markers) are run on the gel alongside the test sample to provide references to compare the size of the target DNA produced during amplification.

While some techniques do exist to remove and quantify the amount of DNA from a gel, they are very labor intensive, not always accurate, and not practical to employ for any significant number of samples. As discussed above, the information provided by PCR on an agarose gel is qualitative only, meaning the test detects the target DNA if it is present in the sample, i.e., we visualize a band of the appropriate molecular weight to confirm that the specific DNA was produced in the PCR process. However, standard PCR does not tell us how much target DNA was present in the fish tissue.

In Quantitative PCR, on the other hand, the amplification and quantitation of the DNA is performed in a single instrument in either a plate format or in a small tube. This eliminates the need for electrophoretic gels entirely and more importantly provides an accurate measure of the amount of target DNA that is present in the fish sample. In terms of the biological significance of the pathogen infection, this very important and obviously much more useful than the information obtained with standard PCR. QPCR not only tells us that the pathogen is present, it tells us exactly how much of the pathogen is present in an individual fish (such as important broodfish), or in a stock of fish such as a hatchery or wild fish population.

What are the molecular mechanisms and technology used in Quantitative PCR ?

There have been various approaches used by different biotechnology companies in developing Real-Time or Quantitative PCR, however only the two predominant technologies will be described in this report.
In general, the newest generation of PCR instruments and reagents allows the simultaneous amplification and quantification of specific DNA sequences in a single instrument. These instruments detect the DNA generated within the PCR process on a cycle-by-cycle basis. The fewer cycles it takes to reach a detectable threshold level of DNA, the more target DNA there was in the test sample to begin with. This technique and the concurrent analysis are referred to as "real-time" or "kinetic" PCR because the reactions can be monitored as they occur and the number or copies of the target DNA are reported instantaneously.

Other types of competitive PCR assays have been developed and are described in the literature as quantitative PCR because they measure the amount of DNA produced. They accomplish this by referencing another similar target that is produced simultaneously. However for the purpose of this report, QPCR or real-time PCR are used interchangeably and refer specifically to the protocols used in the newly developed instruments that are fluorescence-detecting thermocyclers. These machines amplify DNA in the same manner as a standard thermocycler, but also allow quantification of the DNA using fluorescent probes. It is the development of these instruments and the fluorescent probes that they employ that have made quantitative PCR possible in the clinical laboratory. The QPCR assay is highly sensitivity, specificity and efficient as it quantitatively analyzes multiple samples.

More specifically, in real-time PCR a signal (generally fluorescence) is monitored as it is generated, then tracked as it rises above background levels until the reaction reaches a threshold. Initial template levels can be calculated by analyzing the shape of the curve or by determining when the signal rises above the threshold value. Several different fluorescent probes are used in real-time PCR, some bind to double-stranded DNA while others use target sequence-specific reagents such as exonucleases, hybridization probes, or molecular beacons (hairpin probes).

Although more expensive, probes that are sequence-specific add specificity to the assay and also enable multiplexing applications. Multiplexing is the ability to track and measure more than one fluorescent probe simultaneously, thus allowing more than one DNA sequence to be monitored and quantified concurrently. Only sequence-specific probes will be discussed as they are the most specific and commonly used fluorescent probes. A brief synopsis of the two technological advances in instrumentation and systems, and the fluorescent probes they use are described below.

The first commercially available fluorescence-detecting thermocycler was the Prism 7700™ (Perkin-Elmer/Applied Biosystems). This instrument transmits laser light to each well of a 96-well plate and detects the fluorescence emitted by each well in return. The fluorescence is spectrally analyzed using a computer to quantify the amount of DNA based on the amount of fluorescence detected.

The Prism 7700™ system is optimized for use of the fluorogenic 5' exonuclease assay and requisite reagents known as the TaqMan™ product line. The fluorogenic probe is complementary to the target sequence, and initially contains both reporter and quencher moieties. When the probe is not bound to target DNA, its reporter and quencher dyes are in close proximity, and the reporter's fluorescent emission is quenched or inhibited. The probe is designed to anneal specifically between the forward and reverse primers sites of the target sequence. The probe anneals to the target DNA if it is present and during PCR the nuclease activity of Taq polymerase cleaves the reporter dye from the probe. The reporter dye, now separated from the quencher dye, emits a fluorescent signal. The fluorescent signal is emitted from the probe only after it binds to the target DNA and is cleaved during the course of PCR.

The fluorescent signal increases with each cycle, if the target DNA is present, as more probes are released with each additional copy of target DNA is synthesized. After the signal rises above background level, the rate of signal increase is tracked during a number of linear cycles. QPCR eventually reaches a plateau due to limited enzyme activity of the polymerase enzyme and the DNA building blocks (nucleotides) present in the reaction mixture. Therefore, the calculations of DNA are based on the linear area of the exponential curve before the QPCR reaches a plateau. The slope data of the curve is then used to calculate the initial target DNA levels in the sample.

TAQMAN® – the Flurogenic Probe Detection System

Quantitative real-time PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. The chemistry is the key to the detection system. A probe (i.e., TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome (usually 6-carboxyfluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) added at any T position or at the 3' end. The probe is designed to have a higher T_m than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

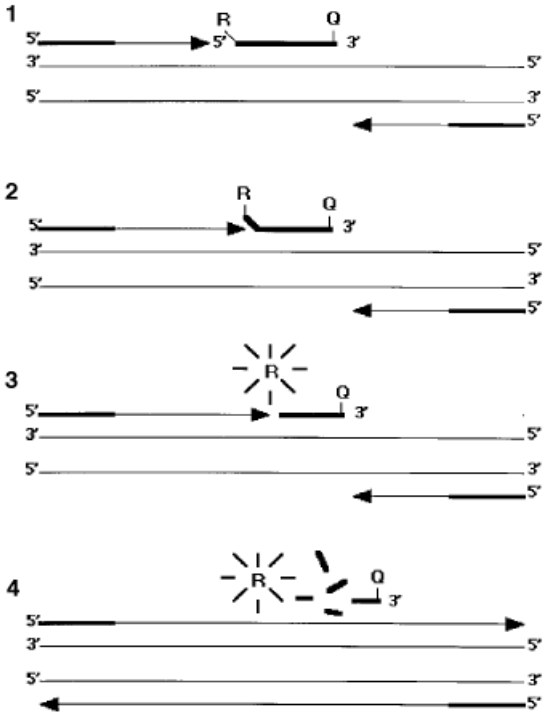

FIGURE 1. Fluorogenic 5' nuclease chemistry. **(1)** Forward and reverse primers are extended with *Taq* polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached anneals to the gene sequence between the two primers. **(2)** As the polymerase extends the primer, the probe is displaced. **(3)** An inherent nuclease activity in the polymerase cleaves the reporter dye (R) from the probe. **(4)** After release of the reporter dye from the quencher (Q), a fluorescent signal is generated.

FIGURE 2. The Perkin-Elmer/Applied Biosystems 7000 System. A compact fluorescent thermocylcer designed for low to moderate laboratory through put. The 7000 system utilizes the same fluorogenic probe system as the 7700 model but is more compact, and contains a tungsten-halogen light source rather than the laser light source.

The 7000 comes with a laptop computer and Primer Express™ software to optimize target sequences. The software assists users in the transition from primer sequences used for standard PCR applications to the Taqman®, or fluorogenic probe systems available for Quantitative PCR.

Photo courtesy of Kathy Clemens, Idaho Fish Health Laboratory.

Another approach to QPCR is utilized in the LightCycler™ technology produced by Roche Molecular Biochemicals. The LightCycler™ performs PCR in a small volume glass capillary tube that is heated and cooled in an airstream. This technology allows for extremely rapid (as little as 10 second) cycle times allowing PCR experiments to be completed in less than 30 minutes. Fluorescence is measured by moving a carousel carrying the capillary tubes past a detection station consisting of one light-emitting diode and three photo detection diodes. Each diode measures a different wavelength of light allowing the use of spectrally distinct fluorescent probes, or multiplexing. The LightCycler supports two fluorescence-based methods for the detection of amplified DNA: the general DNA stain SYBR Green or sequence-specific hybridization probe pairs.

SYBR Green™ exhibits very little fluorescence when free in solution however emission is greatly enhanced when it binds to double-stranded DNA. Prior to amplification, the reaction mixture contains the denatured DNA, the primer and the dye. When the primers attach to the target DNA, a few dye molecules can bind to the sequence of double stranded DNA. During elongation, the copying of the DNA sequence, more and more dye molecules bind to the newly synthesized DNA resulting in a dramatic increase in light emission.. During the next heating cycle when each DNA strand is separating into single strands (denatured), the dye molecules are released and the fluorescent signal falls. The reaction is monitored continuously and the increase in fluorescence is measured for each cycle and the amount of DNA copied is calculated from this data.

With the hybridization probe format, two specially designed, sequence-specific primers are labeled with fluorescent dye. One probe carries a fluorescein label and the other carries a different label (usually LC Red 6340 or CL Red 705). **The chemical nature of the hybridization probes**

prevents their extension: one probe contains fluorescein at the 3' end; whereas 5' labeled probe contains a 3' phosphate moiety. The sequences are designed so they orient to each other in a head-to-tail manner so that the dyes are positioned in close proximity to the each other. The first dye (fluorescein) is excited by the LightCycler's light emitting diode (LED) and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the emitted energy excites the dye attached to the second hybridization probe, which subsequently emits red fluorescent light at an even longer wavelength. The energy transfer, referred to as fluorescence resonance energy transfer (FRET) occurs efficiently only when the dyes are in close proximity . So in this type of assay, fluorescent intensity measurements are made after the annealing steps. The increasing amount of emitted fluorescence is proportional to the increasing amount of DNA generated during the linear phase on the ongoing PCR.

Applications of QPCR in Fish Health Management

What are the advantages and benefits of using QPCR in the Fish Health Laboratory ?

The first, and foremost advantage offered by QPCR is the extremely **high level of sensitivity** that allows detection of fish pathogens at very low DNA copy numbers. Manufacturer specifications claim a limit of detection of fifty copies of target DNA molecule and a linear dynamic range of five orders of magnitude. Research reports suggest single copy detection is possible and that ten copy detection is routine. This level of sensitivity allows the fish health biologist to detect pathogens early in the infection process prior to the progression to a full blown disease state. Early detection can prevent advanced disease and the associated long-term adverse impacts disease poses to overall fish health and population survival. With this level of sensitivity it is also possible to monitor pathogens in water supplies and natural environments that normally could not be detected with conventional microbiological detection methods.

Secondly, QPCR offers an extremely efficient and rapid method to detect pathogens and diagnose disease in the hatchery setting. Under normal fish culture conditions, fish health biologists are notified of a fish health problem within a few days of health problem and tissue samples are promptly collected for microbiological analysis. The laboratory time required to process and positively identify a bacterial or viral infection is generally is 4-7 days. During this period, the tissues are processed, assay results are interpreted and confirmation tests are completed. QPCR would allow **diagnosis of significant fish pathogens in 3-4 hours**. Conventional testing methods could still be employed to conduct further testing of the pathogen such as drug sensitivity in the case of bacterial pathogens. However the ability to diagnose diseases within 3-4 hours would be tremendous benefit to the hatchery program by allowing detection of significant fish pathogens in the earliest stages of infection. Early treatments such as antibiotic or chemical therapies are much more effective when initiated early in the disease process. Early treatment is very important in effectively controlling certain fish diseases in the hatchery setting.

Equally important as the sensitivity and rapid diagnosis provided by QPCR is the capability to **quantify the number of organisms** present in individual fish or populations. The amount of pathogen DNA present correlates with the amount of specific bacterial, parasitic, or viral agents present in the fish host. Therefore quantification of the pathogen DNA, and indirect quantification of the pathogen itself would provide critical information to the fish health biologist. This capability to monitor existing and newly emerging diseases with such sensitivity, as well as quantitatively, is a major advancement in fish health management. With this ability, fish health programs could improve their ability to monitor disease status and quantify infections and thereby more effectively prevent the transmission of diseases within facilities or geographical regions.

In summary, extreme sensitivity, rapid diagnosis, and a better understanding of the biological significance of pathogen infections in hatchery and natural fish populations can be achieved with QPCR technology. This tool will assist in the prevention, control and eradication of significant fish

pathogens in a variety of ways that are best illustrated by specific examples. The following section lists applications in current fish health programs that could benefit species across the country immediately. Future applications of QPCR and the potential of this technological advance in natural resource management is discussed briefly as well.

Major Advantages of QPCR:

- Extreme Sensitivity – ability to detect early (sub-clinical) and carrier infections
- Rapid diagnostics – allows identification of pathogens in a single day.
- Quantification of pathogens – ability to discern biological significance of infection and assess fish health risks with accuracy.

What are the Immediate Applications for the Fish Health Program ?

1. Detection and Management of Vertically Transmitted Diseases:

   Vertical transmission is reported in the literature for several pathogens, however for some pathogens such as IHNV there is still controversy about whether or not this virus is truly passed via the ova to the progeny. QPCR would allow early testing of progeny where traditional detection methods have lacked the sensitivity or specificity to ascertain whether vertical transmission is occurring. Early detection and on-going monitoring of certain pathogens such as IHNV and Cold Water Disease (*Flavobacterium psychrophilum*) would greatly assist our ability to manage these pathogens and gain a better understanding of disease transmission and progression of disease in juvenile fish populations.

2. Early detection of pathogens in juvenile stocks:

   Several significant pathogens do not develop disease, or clinical signs until a later stage in the life history of salmonids. For example, Bacterial Kidney Disease may be passed from adult to progeny, but often the disease does not present itself until the juveniles are developed beyond the fingerling stage and approaching smolt status. Because of the small size of the kidney in juvenile fish, the standard detection method for BKD has been Fluorescent Antibody Technique (FAT), an insensitive method that often will not detect infections until they are clinical. Even the more sensitive ELISA assay is difficult to utilize for small fish because kidney tissues from several fish have to be pooled into a single sample to have adequate tissue quantities. Again, this approach lessens the sensitivity of the test, and does not provide an accurate picture of population prevalence. With QPCR, minimal tissue is required to conduct the assay. Therefore, juvenile fish can benefit from the extreme sensitivity of this assay, our ability to conduct early monitoring in the hatchery setting, and the more accurate prevalence data that could be generated when individual fish versus pooled sample sets are examined. With QPCR, early detection

and monitoring of juvenile fish will occur and allow more effective management of diseases like BKD prior to their progression to clinical disease.

3. Non-Lethal Sampling for Threatened and Endangered Species

Traditionally, non-lethal sampling methods have lacked the sensitivity to be fully utilized as screening tests for major fish pathogens. With the extreme sensitivity and quantitative capability of QPCR, non-lethal sampling could be a feasible approach to monitoring species that are too valuable to sacrifice for routine disease monitoring. T&E species and captive broodstock populations would benefit from our ability to discern disease status of a species or stock without negatively impacting large numbers of these extremely valuable and limited populations.

4. Ability to Detect Pathogens in Water Supplies and Natural Environments

QPCR would allow testing of hatchery water supplies and natural environments for the presence of fish pathogens in a sensitive and quantitative manner. Early detection would allow preventative measures to be taken (i.e., prophylactic antibiotic therapy) and help us learn more about the factors that contribute to water-born pathogen infections (i.e., temperature, flows, water quality).

5. Quantitative Validation of Existing Collection Methodologies and Detection Assays

QPCR could be an essential tool in validating the sensitivity and specificity of existing collection and screening methodologies. The FWS is actively working with the American Fisheries Society (AFS) to draft new protocols for hatchery inspections. The need to validate existing methodologies has already been identified as an essential need and area of further research. QPCR is the ideal tool to utilize for assay validation because of the extreme sensitivity and specificity of this tool, which will allow us to accurately measure and compare other methodologies.

For example:
QPCR could help quantify pathogen DNA in fish infected with *Myxobolus cerebralis*. Currently there is controversy over which tissue preparations (halved heads or spores from digest) are optimum for further confirmation of specific Myxobolid species present in the fish. Currently, tissues are processed and tested differently, depending on life stage of the fish and the confirmation method that will be used (histology and non-quantitative PCR). QPCR could determine the most appropriate method for various life stages and tissue types and possible provide a single approach for all life stages. Eventually, the QPCR assay itself could replace all existing methods with a more sensitive, specific and most importantly quantitative assay. This in turn would lead to better standardization among laboratories in the various states testing for Whirling Disease. Once assay comparisons were done, we would also have the ability to go back and compare previous methods more directly and accurately.

QPCR would also be very beneficial in validating the Enzyme linked Immunosorbent Assay (ELISA). While ELISA can be an effective tool for testing large numbers of fish such as adult broodstock or hatchery stocks, the test measures a protein that does not always directly correlate with active infection with the causative bacterium *Renibacterium salmoninarum*. QPCR could correlate the protein

values with actual infection levels (counts of bacterial cells) or eventually replace the ELISA entirely as a more direct and accurate measure of Bacterial Kidney Disease in fish populations.

6. Improved Quality Assurance / Quality Control

Another aspect of QPCR is the improvement in overall QA/QC in the laboratory. Because standard PCR generates large quantities of specific DNA or RNA, there is a major concern regarding the potential for laboratory contamination. In standard PCR, DNA is produced in large quantities, can be easily spread by non-visible aerosols and is difficult to disinfect with standard laboratory methods. DNA can persist on specimen tubes and laboratory surfaces and can easily spread between test samples and laboratory surfaces and equipment.

The most susceptible step in standard PCR process is during transfer of amplified DNA or RNA from one tube to another, or during loading of electrophoretic gels. With QPCR, all reactions occur within a closed tube or plate that is contained within the fluorescent thermocycler. Electrophoretic gels are completed eliminated from the assay. This greatly reduces the potential for laboratory contamination and cross-contamination of samples that can result in false positives or inaccurate test results.

7. Efficacy Studies:

Fish populations are often treated with chemicals or antibiotics to eliminate parasites or bacterial infections. Following treatment, pathogens may still be present but not detected due to the low numbers of organisms remaining in the fish or water supply. QPCR could quantify the number of pathogens remaining in individual fish and assist in determining the true efficacy of the treatment. Using a quantitative approach to treatments would help improve our understanding of the efficacy of specific compounds, and allow us to determine the most effective route, dosage and duration for treatments. QPCR could be very beneficial in assisting with drug or chemical INAD submissions by demonstrating efficacy in a direct and quantitative manner rather than relying on indirect measures such as cumulative mortality.

8. Provide scientific robustness to the National Wild Fish Health Survey

The NWFHS currently relies on standard detection methods that often result in qualitative data only, in terms of presence or absence of fish pathogens in a geographical area. QPCR would provide quantitative data to the Survey providing a more meaningful measure of pathogen prevalence and distribution. More importantly, QPCR would provide an accurate measure of the biological risks associated with a specific pathogen based on the infection levels found in the fish populations. This information is important to fishery resource managers who need to be able to make a distinction between the presence of a pathogen and actual disease in a fish population or geographical region.

9. Applied Research

QPCR could provide a highly precise method of quantifying bacteria, virus, or parasites used in susceptibility studies by providing accurate challenge dosages and measured responses in experimental fish. Accuracy in the challenge dose, and the infective response of the test subjects is very important to understand the

physiological effects of pathogens at various infection levels. Standard methods require culture of the organism, and quantification by bacterial plate counts, dilution titers and/or filtration methods that are all very labor intensive and less accurate due to differences in sensitivity of each of these methods. QPCR could be used immediately to more accurately derive challenge doses, and then confirm the actual pathogen counts directly or validate the values obtained with the more traditional methods. QPCR would also be far more accurate to evaluate the disease response in challenged fish than existing methods. The quantization of disease or immune response could also allow researchers to compare data between various studies directly. The accurate and quantitative data analysis that is possible with QPCR is a critical component of research aimed at understanding disease processes and the factors that exacerbate or impede disease progression.

Specific Examples of Immediate Applications of QPCR for Fish Health Centers

REGION 1

California Nevada Fish Health Center

Rapid diagnostics for significant pathogens at Coleman NFH.

- Early monitoring and rapid diagnosis of IHNV and Flavobacterium columnare, allowing immediate measures to be taken by hatchery staff and the FHC to treat or control the spread of disease.

Broodstock management of the endangered Winter-run chinook at Livingston Stone NFH.

- Improved detection sensitivity and monitoring ability for *Renibacterium salmoninarum,* IHNV and the Rosette Agent utilizing non-lethal sampling methods (blood, mucous, intestine contents) from individually PIT tagged animals.
- Ability to more effectively segregate family groups with low BKD disease incidence and prevent horizontal transmission of pathogens during early rearing (juveniles) and extended rearing (captive broodstock).

Temperature Studies on the Physiological Effects on Chinook and Steelhead

- Ability to expand current measures between treatment groups to include physiological indicators of growth and stress (Growth Hormone, Insulin-like Factor 1 and cortisol). These hormones could be monitored much more rapidly and accurately with QPCR. Primers already exist for the several physiological significant proteins and hormones.

Broodstock management of the threatened Lahontan cutthroat trout at Lahontan NFH.

- Increased sensitivity in screening natural fish populations prior to transferring progeny to the hatchery and incorporating them into the captive broodstock program.
- Ability to identify the sex of immature adults allowing better manage of broodstock in holding facilities and during spawning operations.

Idaho Fish Health Center

Broodstock management of the endangered Columbia River Spring Chinook at Dworshak NFH.

- The Idaho Fish Health Center has acquired a QPCR system to monitor Columbia River Spring Chinook for BKD. With very small numbers of returning adults, it was deemed critical to determine the level of *Renibacterium salmoninarum* in the females and isolate the eggs and progeny from fish with high bacterial infections.

Lower-Columbia River Fish Health Center

Management of Cold Water Disease (*Flavobacterium psychrophilum)* in Coho salmon stocks.

- Coho salmon that are an integral component of the mitigation program for northwest Tribes of the Columbia River such as the Yakima Tribe and the Nez Perce Tribe. With QPCR, detection of *Flavobacterium psychrophilum* could occur early during the incubation period or as feeding fry. Early antibiotic therapy could commence prior to the progression of this bacterial disease, and assist in preventing the severity of skin and muscle necrosis that impairs swimming, causes skeletal deformities and increased mortality.

- QPCR could also be employed to gain a better understanding of vertical transmission of Cold Water Disease from adults to young fry. Vertical transmission is suspected for this pathogen, and with the sensitivity of QPCR this could be proven or refuted once and for all. If vertical transmission occurs, eggs from positive females could be segregated, as is currently done for Bacterial Kidney Disease. Uninfected progeny could be reared separately, greatly reducing their risk of contracting the disease during early rearing, or at least minimizing the infection levels.

Monitoring and control of Bacterial Kidney Disease in Columbia River stocks.

- QPCR could eventually replace the Enzyme-linked Immunosorbent Assay (ELISA) which detects a soluble protein produced by *Renibacterium salmoninarum* (Rs) bacteria. Unfortunately, the protein can persist for long periods of time when bacteria are not present so this method is a direct measure of bacterial infection. Using QPCR, researchers have shown a strong relationship between the presence of Rs DNA in the fish host and the active transcription of mRNA. This indicates that when Rs DNA is present, the bacteria are alive and metabolically active. This important distinction gives a more accurate assessment of bacterial infections in the hatchery setting and allows more effective management of the disease.

Olympia Fish Health Center

Management of Cold Water Disease (*Flavobacterium psychrophilum)* in Coho at Quilcene NFH.

Current methods require culture and identification of the bacteria, which requires 3-5 days. QPCR could detect the bacteria and quantify the infection level in adults and the progeny in a single day. This information could be very valuable in evaluating prophylactic treatments of adults prior to spawning, improving incubation rearing methods to prevent or minimize

infections, and to segregate negative progeny to prevent horizontal transmission within the hatchery setting.

REGION 2

Pinetop Fish Health Center

Improved management of threatened and endangered species such as Gila trout and the Colorado River cyprinids (Colorado pikeminnow, humpback chub) and razorback sucker.

- Rapid and highly sensitive detection methods to detect and monitor significant fish pathogens.

REGION 3

LaCrosse Fish Health Center

Several existing projects would benefit from the application of QPCR including:

- QPCR would benefit a joint project with the University of Wisconsin, Lacrosse to determine the host-susceptibility of salmonids and coregonids (whitefish and chubs) to the microsporidean parasite Heretosporis sp. (primer sequences are now available for this newly detected parasite).
- Further development of monitoring program for *Myxobolus cerebralis* (Whirling Disease) in T&E species, such as coaster brook trout.
- Rapid assay to measure stress response of lake trout to handling and loading during stocking operations. Several hatcheries plan to employ fish pumps for loading lake trout into distribution trucks. QPCR would help discern if this approach, as well as other fish culture practices, lowers the stress induced during rearing and stocking operations.
- Further development of non-lethal sampling methods for detection of viruses in sturgeon and paddlefish.
- Valuable tool in species identification of juvenile freshwater mussels encountered in the field and to determine the genetic diversity of cultured endangered mussels.

REGION 4

Warm Springs Fish Health Center

- Largemouth Bass Virus (LMBV) monitoring in the southeastern United States. Since 1995, LMB virus has been found in lakes and impoundments in 17 states from Texas to the Chesapeake Bay area. Fish kills attributable to LMBV have been confirmed in more than two dozen locations. The extreme sensitivity and speed with which QPCR can be performed could greatly improve the monitoring and control program for this virus.
- Channel Catfish Virus – The increased sensitivity of QPCR would allow detection, quantification, and improved management of this important virus for the aquaculture industry. Currently the virus is difficult to detect due in the latent or carrier state, and is usually only detectable following stress mediated events such as spawning or seasonal changes in water quality.
- Swim Bladder Sarcoma Virus – QPCR could greatly benefit current research on SBSV and further our understanding of this disease.

- Improve monitoring and control strategies for Bacterial Kidney Disease caused by *Renibacterium salmoninarum* bacteria.

REGION 5

Lamar Fish Health Center

Infectious salmon anemia (ISA) virus is an emerging viral pathogen of farmed Atlantic salmon. The ISA disease was first reported in Norway in 1984, and has since been reported in New Brunswick, Canada and in Maine in 2001. In addition to the direct impacts the disease may have on Maine farmers, increased fish health regulations will affect the industry as well as current plans for the conservation and protection of native stocks of Atlantic salmon in Maine. QPCR will greatly assist in rapid diagnosis and subsequent containment of the ISAV in the northeastern United States.

- QPCR would provide quantification of virus to help elucidate the mechanisms involved with transmission between farmed and wild fish, which is likely an important mechanism for viral traffic through populations and geographical areas.
- Currently blood samples are collected from a subsample of returning adults to screen for ISAV. QPCR would allow thorough examination of entire populations by allowing rapid and efficient testing of large populations of returning adults.
- QPCR would assist in managing broodstock, which are often held for several months prior to spawning in November and December. Knowledge about the disease status of adults would allow holding negative adults in separate units and minimizing the risk of horizontal transmission.
- QPCR could help determine if vertical transmission is occurring by providing a sensitive, rapid, and efficient technique to test returning adults, and the progeny following egg disinfection.
  QPCR can assist in detecting ISAV in RBT and Brown trout which are known to be carriers with no clinical effects (Nylund et al. 1955a, Mylanund & Jakobsen 1995, Nylund et al. 1997).

In summary, QPCR would provide a highly sensitive and rapid diagnostic tool to effectively detect, monitor and manage the ISA virus. Applying this technology in the area of a newly emerging disease would provide the knowledge and time required to effectively minimize the spread of ISAV and the adverse impacts it could have on Atlantic salmon stocks.

REGION 6

Bozeman Fish Health Center

- Improved detection of Sturgeon Iridovirus. The extreme sensitivity of QPCR would permit non-lethal detection of viruses in sturgeon populations.

- Valuable tool for monitoring *Myxobolus cerebralis* positive stocks in Colorado. Whirling Disease is managed by stocking positive fish into appropriate positive waters based on the number of infection level (number of spores) in the hatchery stock. With QPCR, the sensitivity of detection methods would be greatly increased and infection levels could be more accurately quantified. QPCR also has the advantage of being able to detect the

parasite at any of the developmental life stages. This allows detection of early infective life stages of the parasite, prior to development of spore forms. QPCR would improve sensitivity for all life stages of the parasite, improve stocking strategies for positive waters, and be far more efficient in terms of time and labor required to manage those positive stocks.

- Restoration of grayling and management of captive broodstock is another area that would benefit from QPCR. Currently, captive broodstocks are monitored for *Renibacterium salmoninarum* by Membrane-filtration Fluorescent Antibody Technique (mfFAT). This method is more sensitive than standard FAT but is very labor intensive as it involves filtering ovarian fluid through a fine membrane, and then staining and quantifying the bacteria microscopically. With QPCR, more sensitive and accurate information could be obtained in a single day. With the increase in sensitivity and accuracy in quantifying bacteria, much more information could be gained about BKD disease in graylings and allow more personnel resources to be directed towards understanding the pathology of this disease.

Additional Applications of QPCR in Quality Assurance for the National Fish Health Program

Quality Control / Quality Assurance Program.

- Standardized Assays - QPCR could provide great benefits to the national quality assurance program for fish health centers. Applied Biosystems has expressed interest in creating customized "Assay by Design" systems for testing of significant pathogens such as *Renibacterium salmoninarum* (personal communication, Matt Powell). With the potential for pre-made plates, and reagents (standardized probe concentrations) provided by the manufacturer, the testing for BKD testing will reach new levels of quality control and national standardization of detection methods. Primers currently exist for the following fish pathogens:
    - Infectious Hematopoietic Necrosis Virus (IHNV)
    - *Myxobolus cerebralis*
    - *Myxobolus neurobius*
    - *Nanophyetus salmonis*
    - *Ceratomyxa shasta*
    - *Nucleospora salmonis*

- Quantitative Data for the NWFHS - QPCR would provide quantitative data reporting to the National Wild Fish Health Survey Database for many significant fish pathogens. Quantitative data permits resource managers to accurately evaluate the infection status of a particular group of fish and/or in a particular geographical and draw direct comparisons from distinct regions of the country. The high quality of data generated by QPCR lends scientific credibility and leadership to the Service's fish health program and the NWFHS.

- Development of New Assays - Primer development software included with the QPCR equipment allows fish health centers to develop additional assays to address areas of special studies or pathogens of geographical important to their region.

- Advancement of our understanding of fish physiology - Several primers have already been developed and tested to study fish physiology. Many major proteins and hormones can be detected and quantified by QPCR. This assay will assist fish health centers in conducting special studies in fish health and physiology (i.e., temperature studies, quality smolt assessment, etc) and in monitoring species of concern (i.e., captive broodstock). QPCR is a valuable tool that could advance our knowledge of general physiology, growth and smoltification factors, normal and abnormal metabolic functions. To date, Ken Overturf has developed the following primer sets related to fish physiology in rainbow trout (publications are in press):

  Tumor Necrosis Factor (TNF)
  Hepicidin
  Complement (CD-8, CF3)
  IL-8
  MX-1
  Lysozyme
  T-cell like receptor
  Growth Hormone II (GH II)
  Insulin-like Growth Factor II (ILGF-II)
  Myosin
  Pyruvate kinase
  β-Actin Control

Selected Publications that Illustrate Applicability of QPCR to Current Fish Health Issues (See Bibliography Section also)

Quantitative PCR is currently being used by several researchers and fish health specialists throughout the country. The following is a brief synopsis of peer reviewed publications that attest to the validity and appropriateness of this assay for detecting and studying fish diseases.

Overturf, LaPatra, and Powell (2001) utilized QPCR for the rapid detection and quantification of Infectious Hematopoietic Necrosis virus (IHNV). Utilizing primers and fluorescent labeled probes generated for the specific identification of the virus's nucleocapsid and glycoprotein genes, the method rapidly confirms the presence of virus. In addition to viral detection, QPCR also measures the absolute concentration of virus within a sample.

The study demonstrates the speed and specificity of QPCR which make it ideal for the rapid detection of pathogenic organisms from tissue, water or other potential reservoirs of infection.

Ritchie, et al (2001) employed QPCR to detect infectious salmon anemia virus (ISAV) in Atlantic salmon from Nova Scotia, Canada and demonstrated evidence for functional strain differences in the virus isolates obtained from Europe and the United States. ISAV has become the most serious disease of Atlantic salmon aquaculture resulting in over a 30 million dollar loss in Canadian dollars to the industry according to the New Brunswick Department of Fisheries and Aquaculture. Consequently, surveillance programs to monitor and control the spread of virus have been established in both Canada and now the United States since the finding of the virus in Maine. The standard method for detection of the virus is tissue culture on select salmon head kidney (SHK) cell lines and immunological methods to confirm the virus once cultured. Because tissue culture requires 28 days to complete a diagnosis, more accurate and rapid methods utilizing Reverse Transcriptase (RT) PCR were quickly employed in the surveillance program. In this study, tissue culture did not detect the virus, only additional testing with RT-PCR was able to demonstrate the presence of ISAV in the stock. The study also examined strain variations of viral isolates from different geographical areas, specifically from each side of the Atlantic Ocean. This study also documented the first identification of ISAV in Atlantic salmon in Nova Scotia, Canada, and used RT-PCR to demonstrate the genomic differences in strains from Norway, Scotland and Canada.

In this study, QPCR provided a much needed assay for a sensitive and robust diagnostic technique that could take into account strain variation to provide effective monitoring to manage a newly emerging virus.

Harms, et al (2000) utilized a competitive PCR technique to study an immunosuppressive cytokine in white perch in the Chesapeake Bay and to compare this quantitative PCR to the traditional immunological assay measuring macrophage bactericidal activity. In this fish health study, the results indicated both temporal and spatial modulation of white perch immune function and demonstrated the utility of quantitative PCR as a molecular biomarker for field assessment of teleost fish immune status.

This study demonstrates the power of QPCR to measure various physiological parameters, in additional to pathogen detection. Validation of existing techniques is another important application of QPCR.

Summary

Major Benefits of QPCR to the National Fish Health Program and Fish Health Management

Advantages of Quantitative PCR

- Extreme Sensitivity – ability to detect early (sub-clinical) and carrier infections
- Rapid diagnostics – detect specific pathogens in a single day.
- Quantification of pathogens – ability to discern biological significance of infection and assess fish health risks with accuracy.

Immediate Benefits to the Fish Health Program

- Advance the scientific capabilities and leadership of the National Fish Health Program:
  - Increased sensitivity and efficiency in detecting significant fish pathogens
  - Standardization of all FHC laboratories
  - Improved reporting (quantitative data) in the NWFHS Database
  - Capability to expand fish health role to many other aquatic organisms
  - Applied research and special studies – aid in understanding biological risks, disease progression, and transmission in both hatchery and wild populations
  - Advance our knowledge in fish physiology and application of fish culture techniques

- Improved capabilities and efficiency and in managing pathogens and fish disease:
  - Improved detection and control of newly emerging pathogens (ISAV, LMBV, Spring Viremia of Carp)
  - Rapid identification of suspect pathogens in the hatchery monitoring program
  - Improved management of T&E species and implementation of recovery plans
  - Capability to test multiple pathogens in a single sample (multiplexing)
  - Ability to utilize non-lethal samples methods without decreases sensitivity
  - Ability to detect pathogens in water supplies and natural environments
  - Detection and control of vertically transmitted diseases from adults to progeny

- Improved Quality Assurance/Quality Control of National Fish Health Program:
  - Quantitative validation of existing collection methodologies and assays
  - Potential for fully customized assays from manufacturer (standardization)
  - Remove subjective interpretation of reading electrophoretic gels
  - Decrease risk of laboratory contamination or cross-contamination of samples (minimal handling of samples and omission of electrophoretic gels)
  - Data is read and stored electronically in computer program
  - Internal standards are used in QPCR to monitor assay performance (stringent quality control built into system prevents false positive results)

Cost Analysis of Quantitative PCR versus Other Laboratory Assays Used to Detect Fish Pathogens

Group of Fish Pathogen:	Screening Assay / (Confirmation Method):	Assay Supplies*	Assay Labor*	Cost per Lot 60 Fish**	Sample Number	Cost per Sample
BACTERIA:						
Y.ruckeri, A.salmonicida,	**CULTURE**	$282.00	$144.00	$426.00	60	**$7.10**
Flavobacterium sp.	FAT	$4.75	$54.00	$58.75	5	**$11.75**
	PCR	$77.25	$252.00	$329.25	5	**$65.85**
Bacterial Kidney Disease (BKD)						
Renibacterium salmoninarum	**ELISA**	$168.00	$324.00	$492.00	60	**$8.20**
	Rs-Nested PCR	$77.25	$252.00	$329.25	5	**$65.85**
VIRUSES:						
IHNV, IPNV, VHSV, OMV	**PLAQUE ASSAY**	$99.00	$144.00	$243.00	12	**$20.25**
	DOT BLOT	$7.50	$108.00	$115.50	5	**$23.10**
	SERUM NUET.	$8.75	$144.00	$152.75	5	**$30.55**
	RT-PCR or Nested PCR	$74.65	$252.00	$326.65	5	**$65.33**
PARASITOLOGY:						
Myxobolus cerebralis	**DIGEST (PTD)**	$42.00	$216.00	$258.00	12	**$21.50**
	Mc Nested PCR	$77.25	$252.00	$329.25	5	**$65.85**
ALL PATHOGEN GROUPS	**QUANTITATIVE PCR**	**$411.00**	**$126.00**	**$537.00**	60	**$8.95**

Due to the extremely high sensitivity and specificity, QPCR meets the needs of both the screening and confirmation methods.

NOTE:

*** Based on laboratory costs developed for the NWFHS and an average labor cost of $18.00 per hour; Formulas include sample collection, but no travel.**

****Lot equals 60 fish; Individual samples or 12,5-pool samples depending on assay performed; subsets of 5 samples are used to determine confirmation testing costs.**

Summary of Laboratory Assay Costs Compared to QPCR

Group of Fish Pathogen:	Combined Screening & Confirmation Method:	Cost per Sample:	Cost per Lot:	Cost Ranges PER LOT: (low)	(high)
BACTERIA:	CULTURE / FAT	$18.85	$484.75		
	CULTURE / PCR	$72.95	$755.25	**$485.00**	**$755.00**
Bacterial Kidney Disease (BKD)	ELISA / PCR	$74.05	$821.25		**$821.00**
VIRUSES:	PLAQUE / DOTBLOT	$43.35	$358.50		
	PLAQUE / Serum Neut.	$50.80	$395.75		
	PLAQUE / PCR	$85.58	$569.65	**$358.00**	**$569.00**
PARASITOLOGY:	PT DIGEST / PCR	$87.35	$587.25		**$588.00**
ALL PATHOGEN GROUPS	QUANTITATIVE PCR	$8.95	$537.00		**$537.00**

Comparison of Costs for Capitol Equipment from Major Manufacturers of QPCR Systems

Manufacturer:	System:	System Cost:	Reagent Costs:	Warranty:
STRATAGENE La Jolla, CA	MX 4000® Multiplex	$59,950	295.00	1 year
ROCHE DIAGNOSTICS, Inc. Indianapolis, IN	LightCycler ®	$57,500	$220.00	1 year
APPLIED BIOSYSTEMS Foster City, CA	ABI Prism® 7000	$42,761	$380.00	1 year

Bibliography and Internet References

Internet Sites for Vendor Publications

Deborah S. Grove. Quantitative Real-Time Polymerase Chain Reaction for the Core Facility Using TaqMan and the Perkin-Elmer/Applied Biosystems Division 7700 Sequence Detector. http://www.abrf.org/JBT/1999/March99/mar99grove.html.

Stratagene: http://www.stratagene.com/

Roche Molecular Biochemicals: http://www.biochem.roche.com

References for PCR Applications in Fish Health

Altinok, I; Grizzle, JM; Liu, Z. 2001. Detection of *Yersinia ruckeri* in rainbow trout blood by use of the polymerase chain reaction. Dis. Aquat. Org. 44:29-34

Andree, KB; MacConnell, E; Hedrick, RP. 1998. A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Org. 34:145-154

Aoki, T; Park, C-I; Yamashita, H; Hirono, I. 2000. Species-specific polymerase chain reaction primers for *Lactococcus garvieae*. J. Fish Dis. 23:1-6

Argenton, F; De Mas, S; Malocco,C; Dalla Valle, L; Giorgetti, G; Colombo, L. 1996. Use of random DNA amplification to generate specific molecular probes for hybridization tests and PCR-based diagnosis of *Yersinia ruckeri*. Dis Aquat. Org. 24:121-127

Aso, Yuihaku; Wani, Jacob; Antonio, D; Klener, S; Yoshhmizu, Mamoru. 2001. Detection and Identification of Oncorhynchns mason virus (OMV) by Polymerase Chain Reaction (PCR). Bull. Fish. Sci. Hokkaido Univ. 52:111-116

Baldwin, TJ; Myklebust, KA. 2002. Validation of a single round polymerase chain reaction assay for identification of *Myxobolus cerebralis* myxospores. Dis. Aquat. Org. 49:185-190

Baliarda, A; Faure, D; Urdaci, M. 2002. Development and application of a nested PCR to monitor brood stock salmonid ovarian fluid and spleen for detection of the fish pathogen *Flavobacterium psychrophilum*. J. Appl. Microbiol. 92:510-516

Barlic-Maganja, D; Strancar, M; Hostnik, P; Jencic, V; Grom, J. 2002. Comparison of the efficiency and sensitivity of virus isolation and molecular methods for routine diagnosis of infectious haematopoietic necrosis virus and infectious pancreatic necrosis virus. J. Fish Dis. 25:73-80.

Bell, AS; Yokoyama, H; Aoki, T; Takahashi, M; Maruyama, K. 1999. Single and nested polymerase chain reaction assays for the detection of *Microsporidium seriolae* (Microspora), the causative agent of `Beko' disease in yellowtail *Seriola quinqueradiata*. Dis. Aquat. Org. 37:127-134

Bowers, HA; Tengs, T; Glasgow, HB Jr; Burkholder, JM; Rublee, PA; Oldach, DW. 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl. Env. Micro. 11:4641-4648

Brown, LL; Iwana, GK; Evelyn, TPT; Nelson, WS; Levine, RP. 1994. Use of the polymerase chain reaction (PCR) to detect DNA from *Renibacterium salmoninarum* within individual salmonid eggs. Dis Aquat Org 18: 165-171

Brown, LL; Evelyn, TPT; Iwana, GK; Nelson, WS; Levine, RP. 1995. Bacterial species other than *Renibacterium salmoninarum* cross-react with antisera against *R. salmoninarum* but are negative for the p57 gene *R.salmoninarum* as detected by polymerase chain reaction (PCR). Dis Aquat Org 21:227-231

Celius, T; Matthews, JB; Giesy, JP; Zacharewski, TR. 2000. Quantification of rainbow trout (*Oncorhynchus mykiss*) zona radiata and vitellogenin mRNA levels using real-time PCR after in vivo treatment with estradiol-17 beta or alpha –zearalenol. J of Steroid Biochem. and Molec. Bio. 75:109-119

Chase, DM; Pascho, RJ. 1998. Development of a nested polymerase chain reaction for amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method for detection of the bacterium in salmonid kidney. Dis Aquat Org 34:223-229

Cook, M; Lynch, WH. 1999. A sensitive nested reverse transcriptase PCR assay to detect viable cells of the fish pathogen *Renibacterium salmoninarum* in Atlantic salmon (*Salmo salar*). Appl. Environ. Microbiol. Vol. 65:3042-3047

Cunningham, CO. 2002. Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture 206:19-55

Devold, M; Krossoy, B; Aspehaug, V; Nylund, A. 2002. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. Dis Aquat Org 40: 9-18

Dyer, A; Soole, K; Matsumoto, G. 2001. Quantitative TaqMan PCR Without a Real-Time Thermal Cycler: An Assay for Fish Insulin-like Growth Factor I Messenger RNA. Marine Biotechnol. 3:16-21

Elliott, DG. 2000. The role of biotechnology in the detection of *Renibacterium salmoninarum* infections in salmonid fishes - promises and pitfalls. Aquaculture Assoc. of Canada, St. Andrews, NB (Canada). Proceedings of the special session 'Biotechnology: What does it mean to growers?' held in conjunction with Aquaculture Canada 2000, Moncton, NB, Bull. Aquacult Assoc Can 100:6-12 2000.

Eszterbauer, E; Benko, M; Dan, A; Molnar, K. 2001. Identification of fish-parasitic Myxobolus (Myxosporea) species using a combined PCR-RFLP method. Dis Aquat Org 44:35-39

Gray, WL; Mullis, L; LaPatra, SE; Groff, JM; Goodwin, A. 2002. Detection of koi herpesvirus DNA in tissues of infected fish. J Fish Dis 25:171-178

Gray, WL; Williams, RJ; Jordan, RL; Griffin, BR. 1999. Detection of channel catfish virus DNA in latently infected catfish. J Gen Virol 80:1817-1822

Griffiths, S; Melville, K. 2000. Non-lethal detection of ISAV in Atlantic salmon by RT-PCR using serum and mucus samples. Bull Eur Assoc Fish Pathol 20:157-162

Harms, CA; Ottinger, CA; Blazer, VS; Densmore, CL; Pieper, LH; Kennedy-Stoskopf, S. 2000. Quantitative Polymerase Chain Reaction for Transforming Growth Factor- beta Applied to a Field Study of Fish Health in Chesapeake Bay Tributaries. Environ Health Perspect 108:447-452

Hiney, MP; Smith, PR. 1998. Validation of polymerase chain reaction-based techniques for proxy detection of bacterial fish pathogens; framework, problems and possible solutions for environmental applications. Aquaculture 162:41-48

Hoeie, S; Heum, M; Thoresen, OF. 1997. Evaluation of a polymerase chain reaction-based assay for the detection of Aeromonas salmonicida ss salmonicida in Atlantic salmon Salmo salar. Dis Aquat Org 30:27-35

Hoeie, S; Dalsgaard, I; Aase, IL; Heum, M; Thornton, JM; Powell, R. 1999. Polymerase Chain Reaction (PCR)-based Typing Analysis of Atypical Isolates of the Fish Pathogen *Aeromonas salmonicida*. Syst Appl Microbiol 22:403-411

Hutchinson, WF; Carvalho, GR; Rogers, SI. 1999. A nondestructive technique for the recovery of DNA from dried fish otoliths for subsequent molecular genetic analysis. Mol Ecol 8:893-894

Kim, YJ; Jung, SJ; Choi, TJ; Kim, HR; Rajendran, KV; Oh, MJ. 2002. PCR amplification and sequence analysis of irido-like virus infecting fish in Korea. J Fish Dis 25:121-124

Kimura, B; Kawasaki, S; Nakano, H; Fujii, T. 2001. Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish. Appl Environ Microbiol 67:206-216

LaPatra SE, Batts WN, Overturf K, Jones GR, Shewmaker WD, Winton JR. 2001. Negligible risk associated with the movement of processed rainbow trout, *Oncorhynchus mykiss* (Walbaum), from an infectious haematopoietic necrosis virus (IHNV) endemic area. J Fish Dis. 4:399-408

Leiro, J; Siso, MIG; Parama, A; Ubeira, FM; Sanmartin, ML. 2000. RFLP analysis of PCR-amplified small subunit ribosomal DNA of three fish microsporidian species. Parasitology 120:113-119

Li, Xinhui; Wu, Shuqin; Li, Kaibin; Bai, Junjie; Pan, Houjun; Luo, Jianren; Jian, Qing. 2001. Polymerase chain reaction (PCR) amplification on diagnosis of Siniperca chuatsi virus disease. J Fish China 25:43-46

Magnussun, HB; Fridjonsson, OH; Andersson OS; Benediktsottir, E; Gudmundsdottir, S; Andresdottir, V. 1994. *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonid fish, detected by nested reverse transcription-PCR of 16S rRNA sequences. Appl Environ Microbiol 60:4580-4583

McBeath, AJA; Burr, KL-A; Cunningham, CO. 2000. Development and use of a DNA probe for confirmation of cDNA from infectious salmon anaemia virus (ISAV) in PCR products. Bull Eur Assoc Fish Pathol 20:130-134

McIntosh, D; Meaden, PG; Austin, B. 1996. A simplified PCR-based method for the detection of *Renibacterium salmoninarum* utilizing preparations of rainbow trout (*Oncorhyncus mykiss*, Walbaum) lymphocytes. Appl Environ Microbiol 62:3929-3932

Mikalsen, AB; Teig, A; Helleman, A-L; Mjaaland, S; Rimstad, E. 2001. Detection of infectious salmon anaemia virus (ISAV) by RT-PCR after cohabitant exposure in Atlantic salmon Salmo salar. Dis Aquat Org 47:175-181

Morris, DJ; Adams, A; Feist, SW; McGeroge, J; Richards, RH. 2000. Immunohistochemical and PCR studies of wild fish for *Tetracapsula bryosalmonae* (PKX), the causative organism of proliferative kidney disease. J Fish Dis 23;129-135

Opitz, HM; Bouchard, D; Anderson, E; Blake, S; Nicholson, B; Keleher, W. 2000. A comparison of methods for the detection of experimentally induced subclinical infectious salmon anaemia in Atlantic salmon. Bull Eur Assoc Fish Pathol 20:12-22

Oreshkova, SF; Shchelkunov, IS; Tikunova, NV; Shchelkunova, TI; Puzyrev, AT; Ilyichev, AA. 1993. Detection of spring viremia of carp virus isolates by hybridization with non-radioactive probes and amplification by polymerase chain reaction. Virus Res 63:3-10

Osorio, CR; Collins, MD; Toranzo, AE; Barja, JL; Romalde, JL. 1991. 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. Appl Environ Microbiol 65:2942-2946

Overturf K, LaPatra S; Powell M. 2001. Real-time PCR for the detection and quantitative analysis of IHNV in salmonids. J Fish Dis 24:325-333

Overturf, K; Hardy, R. 2001. Myosin expression levels in trout muscle: a new method for monitoring specific growth rates for rainbow trout *Oncorhynchus mykiss* (Walbaum) on varied planes of nutrition. Aqua Res Vol 32:315-322

Overturf K, Casten M, Hagemeister C, Hardy R. 2001. Quantification of specific mRNA transcripts in differentiated tissues for assaying growth rates in trout *Oncorhynchus mykiss*. Aquacult 2001: Book of Abstracts pp 504

Pascho, RJ; Elliott, DG; Mallett, RW; Mulcahy, D Comparison of five techniques for the detection of Renibacterium salmoninarum in adult coho salmon. Trans Am Fish Soc 116:882-890

Peng, Xuanxian; Gao, Hua; Wang, Sanying; Zheng, Wenzhu. 2000. 0Universal primer PCR with SSCP and RFLP for identification of fish disease pathogens. J Fish China 24:345-348

Ritchie, RJ; Cook, M; Melville, K; Simard, N; Cusack, R; Griffiths, S. 2001. Identification of infectious salmon anaemia virus in Atlantic salmon from Nova Scotia (Canada): evidence for functional strain differences. Dis Aquat Org 44:171-178

Rodriguez Saint-Jean, S; Borrego, JJ; Perez-Prieto, SI. 2001. Comparative evaluation of five serological methods and RT-PCR assay for the detection of IPNV in fish. J Virol Methods 97:23-31

Sanath Kumar, H; Otta, S; Karunasagar, I*; Karunasagar, I. 2001. Detection of Shiga-toxigenic *Escherichia coli* (STEC) in fresh seafood and meat marketed in Mangalore, India by PCR. Lett Appl Microbiol 33:334-338

Schisler, GJ; Bergersen, EP; Walker, PG; Wood, J; Epp, JK. 2000. Comparison of single-round polymerase chain reaction (PCR) and pepsin-trypsin digest (PTD) methods for detection of *Myxobolus cerebralis*. Dis Aquat Org 45:109-114

Temprano, A; Yugueros, J; Hernanz, C; Sanchez, M; Berzal, B; Luengo, JM; Naharro, G. 2001. 0Rapid identification of *Yersinia ruckeri* by PCR amplification of yrul-yruR quorum sensing. J Fish Dis 24:253-261

Thiery, R; Raymond, JC; Castric. 1999. Natural outbreak of viral encephalopathy and retinopathy in juvenile sea bass, Dicentrarchus labrax: study by nested reverse transcriptase-polymerase chain reaction. J Virus Res 63:11-17

Tsamis, V; Mamuris, Z; Panagiotaki, P; Kouretas, D. 2001. Proteins from fish eggs that protect DNA from acid precipitation and inhibit DNA synthesis. Comp Biochem Physiol 129:369-376

Valle, DL ; Zanella, L; Belvedere, P; Colombo. 2002. Use of random amplification to develop a PCR detection method for the causative agent of fish pasteurellosis, *Photobacterium damselae subsp. piscicida* (Vibrionaceae) Aquaculture 207:187-202

Valle, LD; Zanella, L; Patarnello, P; Paolucci, L; Belvedere, P; Colombo, L. 2000. Development of a sensitive diagnostic assay for fish nervous necrosis virus based on RT-PCR plus nested PCR. J of Fish Dis 23:321-327

Villoing, S; Castric, J; Jeffroy, J; Le Ven, A; Thiery, R; Bremont, M. 2000. An RT-PCR-based method for the diagnosis of the sleeping disease virus in experimentally and naturally infected salmonids. Dis Aquat Org 40:19-27

Wiklund, T; Madsen, L; Bruun, MS; Dalsgaard, I. 2000. Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. J Appl Microbiol 88:299-307

Williams, K; Blake, S; Sweeney, A; Singer, JT; Nicholson, BL. 1999. Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. J Clin Microbiol 37:4139-4141

Yokoyama, Hiroshi; Inoue, Daisuke; Sugiyama, Akihiro; Wakabayashi, Hisatsugu. 2000. Polymerase chain reaction and indirect fluorescent antibody technique for the detection of *Kudoa amamiensis* (Multivalvulida: Myxozoa) in yellowtail *Seriola quinqueradiata*. Fish Pathol 35:157-162

Bibliography for Competitive and Quantitative PCR

Araki R, Funimori A, Hamatani K, et al. Nonsense mutation at Tyr-4046 in the DNA-dependent protein kinase catalytic subunit of severe combined immune deficiency. Proc Natl Acad Sci USA 1997;94:2438-2443.

Batt CA. Molecular diagnostics for dairy-borne pathogens. J Dairy Sci 1997;80:220-229.

Brandt ME, Padhye AA, Mayer LW, Holloway BP. Utility of random amplified polymorphic DNA PCR and TaqMan automated detection in molecular identification of Aspergillus fumigatus. J Clin Microbiol 1998;36:2057-2062.

Casteels KM, Mathieu C, Waer M, et al. Prevention of type I diabetes in nonobese diabetic mice by late intervention with nonhypercalcemic analogs of 1,25-dihydroxyvitamin D3 in combination with a short course of cyclosporin A. Endocrinology 1998;139:95-102.

Chen S, Yee A, Griffiths M, et al. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of Salmonella species in food commodities. Int J Food Microbiol 1997;35:239-250.

deKok JB, Hendriks JCM, van Solinge WW, Willems HL, Mensink EJ, Swinkels DW. Use of real-time quantitative PCR to compare DNA isolation methods. Clin Chem 1998;44:2201-2204.

Desjardin LE, Chen Y, Perkins MD, Teixeira L, Cave MD, Eisenach KD. 1998 Comparison of the PE/ABD 7700 system (TaqMan) and competitive PCR for quantification of IS110 DNA in sputum during the treatment of tuberculosis. J Clin Microbiol 1998;36:1964-1968.

Gelmini S, Orlando C, Sestini R, et al. Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to measure c-erB-2 oncogene amplification. Clin Chem 1997;43:752-758.

Gerard CJ, Arboleda MJ, Solar G, Mule JJ, Kerr WG. A rapid and quantitative assay to estimate gene transfer into retrovirally transduced hematopoietic stem/progenitor cells using a 96-well format PCR and fluorescent detection system universal for MMLV-based proviruses. Hum Gene Ther 1996;7:343-354.

Gerard CJ, Olsson K, Ramanathan R, Reading C, Hanania EG. Improved quantitation of minimal residual disease in multiple myeloma using real-time polymerase chain reaction and plasmid-DNA complementarity determining region III standards. Cancer Res 1998;58:3957-3964.

Gibson UEM, Heid CA, Williams PM. A novel method for real-time quantitative RT-PCR. Genome Res 1996;6:995-1001.

Heid CA, Stevens J, Livak KJ, Williams PM. Real-time quantitative PCR. Genome Res 1996; 6:986-994.

Higgins JA, Ezzell J, Hinnebusch BJ, Shipley M, Henchal EA, Ibrahim MS. 5'-Nuclease PCR assay to detect Yersinia pestis. J Clin Microbiol 1998;36:2284-2288.

Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci USA 1991;88:7276-7280.

Kruse N, Pette M, Toyka K, Rieckmann P. Quantification of cytokine mRNA expression by RT PCR in sample of previously frozen blood. J Immunol Methods 1997; 210:195-203.

Livak KJ, Flood SJA, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 1995;4:357-362.

Livak KJ, Goodsaid F. Allelic discrimination using the 5' nuclease assay. Foster City, CA: PE Applied Biosystems Bulletin, 1997.

Lo YMD, Tein MSC, Lau TK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768-775.

Lockey C, Otto E, Long Z. Real-time fluorescence detection of a single DNA molecule. Biotechniques 1998;24:744-746.

Luoh SM, DiMarco F, Levin N, et al. Cloning and characterization of a human leptin receptor using a biologically active leptin immunoadhesin. J Mol Endocrinol 1997;18:77-85.

Marcucci G, Livak KJ, Bi W, Strout MP, Bloomfield CD, Caligiuri MA. Detection of minimal residual disease in patients with AML1/ETO-associated acute myeloid leukemia using a novel quantitative reverse transcription polymerase chain reaction assay. Leukemia 1998;12:1482-1489.

McGoldrick A, Lowings JP, Ibata G, Sands JJ, Belak S, Paton DJ. A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (TaqMan). J Virol Methods 1998;72:125-135.

Mensink E, van de Locht A, Schattenberg A, et al. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. Br J Haematol 1998;102:768-774.

Morris T, Robertson B, Gallagher M. Rapid reverse transcription-PCR detection of hepatitis C virus RNA in serum by using the TaqMan fluorogenic detection system. J Clin Microbiol 1996;34:2933-2936.

Shimokawa T, Kato M, Ezaki O, Hashimoto S. Transcriptional regulation of muscle-specific genes during myoblast differentiation. Biochem Biophys Res Commun 1998;246:287-292.

Suryanarayana K, Wiltrout TA, Vasquez GM, Hirsch VM, Lifson JD. Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. AIDS Res Hum Retroviruses 1998;14:183-189.

Swan DC, Tucker RA, Holloway BP, Icenogle JP. A sensitive, type-specific, fluorogenic probe assay for detection of human papillomavirus DNA. J Clin Microbiol 1997;35:886-891.

Witham PA. PCR based assay for the detection of Escherichia coli Shiga-like toxin genes in ground beef. Appl Environ Microbiol 1996;62:1347-1353.

Absolute Quantitation

Ambs S., S. Dennis, J. Fairman, M. Wright and J. Papkoff. 1999. Inhibition of tumor growth correlates with the expression level of a human angiostatin transgene in transfected B16F10 melanoma cells [In Process Citation]. Cancer Res. *59*:5773-5777, 1999.

Arnold, B.A., Hepler, R.W., and Keller, P.M. One-Step Fluorescent Probe Product-Enhanced Reverse Transcriptase Assay. *BioTechniques* 25(1):98-106, 1998.

Becker K., D. Pan and C.B. Whitley. 1999. Real-time quantitative polymerase chain reaction to assess gene transfer. Hum. Gene Ther. *10*:2559-2566, 1999.

Berg, T., M ller, A.R., Platz, K.P., Hohne, M., Bechstein, W.O., Hopf, U., Wiedenmann, B., Neuhaus, P., and Schreier, E. Dynamics of GB virus C viremia early after orthotopic liver transplantation indicates extrahepatic tissues as the predominant site of GB virus C replication. *Hepatology* 29(1):245-249, 1999.

Chiang, P.W., Wei, W.L., Gibson, K., Bodmer, R., and Kurnit, D.M. A fluorescent quantitative PCR approach to map gene deletions in the Drosophila genome [In Process Citation]. *Genetics* 153(3):1313-1316, 1999.

de Kok, J.B., Hendriks, J.C., van Solinge, W.W., Willems, H.L., Mensink, E.J., and Swinkels, D.W. Use of real-time quantitative PCR to compare DNA isolation methods. *Clin.Chem.* 44(10):2201-2204, 1998.

Fairman, J., Roche, L., Pieslak, I., Lay, M., Corson, S., Fox, E., Luong, C., Koe, G., Lemos, B., Grove, R., Fradkin, L., and Vernachio, J. Quantitative RT-PCR to evaluate in vivo expression of multiple transgenes using a common intron [In Process Citation]. *BioTechniques* 27(3):566-70, 572-4, 1999.

Gerard, C. J., et al. "Improved quantitation of minimal residual disease in multiple myeloma using real-time polymerase chain reaction and plasmid-DNA complementarity determining region III standards." Cancer Res 58 (1998): 3957-3964.

Hartel, C., Bein, G., Kirchner, H., and Kluter, H. A Human Whole-Blood Assay for Analysis of T-Cell Function by Quantification of Cytokine mRNA. *Scand.J.Immunol.* 49(6):649-654, 1999.

Hennig, H., et al. "Lack of evidence for Marek's disease virus genomic sequences in leukocyte DNA from multiple sclerosis patients in Germany [In Process Citation]." Neurosci Lett 250 (1998): 138-140.

Higgins, J. A., et al. "5' nuclease PCR assay to detect Yersinia pestis [In Process Citation]." J Clin Microbiol 36 (1998): 2284-2288.

Houng, H.H., Hritz, D., and Kanesa-thasan, N. Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3'-noncoding sequence. *J.Virol.Methods* 2000.*Apr;86(1):1-11.*

Kafert S., J. Krauter, A. Ganser and M. Eder. Differential quantitation of alternatively spliced messenger RNAs using isoform-specific real-time RT-PCR. Anal. Biochem. *269*:210-213, 1999.

Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T., and Morishima, T. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J.Clin.Microbiol.* 37(1):132-136, 1999.

Klein, D., Janda, P., Steinborn, R., Muller, M., Salmons, B., and Gunzburg, W.H. Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: influence of mismatches on quantification [In Process Citation]. *Electrophoresis* 20(2):291-299, 1999.

Leutenegger, C.M., Klein, D., Hofmann-Lehmann, R., Mislin, C., Hummel, U., Boni, J., Boretti, F., Guenzburg, W.H., and Lutz, H. Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system [In Process Citation]. *J.Virol.Methods* 78(1-2):105-116, 1999

Lo, Y. M., et al. "Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis." Am J Hum Genet 62 (1998): 768-775.

Lo, Y.M., Chan, L.Y., Lo, K.W., Leung, S.F., Zhang, J., Chan, A.T., Lee, J.C., Hjelm, N.M., Johnson, P.J., and Huang, D.P. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res.* 59(6):1188-1191, 1999.

Lo, Y.M., Leung, T.N., Tein, M.S., Sargent, I.L., Zhang, J., Lau, T.K., Haines, C.J., and Redman, C.W. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia [see comments]. *Clin.Chem.* 45(2):184-188, 1999.

Lo Y.M., L.Y. Chan, A.T. Chan, S.F. Leung, K.W. Lo, J. Zhang, J.C. Lee, N.M. Hjelm, P.J. Johnson and D.P. Huang. 1999. Quantitative and temporal correlation between circulating cell-free Epstein-Barr virus DNA and tumor recurrence in nasopharyngeal carcinoma. Cancer Res. *59*:5452-5455.

Lockey, C., E. , Otto, and Z. , Long. "Real-time fluorescence detection of a single DNA molecule." BioTechniques 24 (1998): 744-746.

Luthra, R., et al. "Novel 5' exonuclease-based real-time PCR assay for the detection of t(14;18)(q32;q21) in patients with follicular lymphoma." Am J Pathol 153 (1998): 63-68.

Maudru, T. and K. W., Peden. "Adaptation of the fluorogenic 5'-nuclease chemistry to a PCR-based reverse transcriptase assay." BioTechniques 25 (1998): 972-975.

Nagai, M., Usuku, K., Matsumoto, W., Kodama, D., Takenouchi, N., Moritoyo, T., Hashiguchi, S., Ichinose, M., Bangham, C.R., Izumo, S., and Osame, M. Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP. *J.Neurovirol.* 4(6):586-593, 1998.

Nogva, H.K. and Lillehaug, D. Detection and quantification of Salmonella in pure cultures using 5'-nuclease polymerase chain reaction [In Process Citation]. *Int.J.Food Microbiol.* 51(2-3):191-196, 1999.

Pusterla, N., Huder, J.B., Leutenegger, C.M., Braun, U., Madigan, J.E., and Lutz, H. Quantitative real-time PCR for detection of members of the Ehrlichia phagocytophila genogroup in host animals and Ixodes ricinus ticks. *J.Clin.Microbiol.* 37(5):1329-1331, 1999.

Pusterla, N., Leutenegger, C.M., Chae, J.S., Lutz, H., Kimsey, R.B., Dumler, J.S., and Madigan, J.E. Quantitative Evaluation of Ehrlichial Burden in Horses after Experimental Transmission of Human Granulocytic Ehrlichia Agent by Intravenous Inoculation with Infected Leukocytes and by Infected Ticks. *J.Clin.Microbiol.* 37(12):4042-4044, 1999.

Smith, G.J., et al. "Fast and accurate method for quantitating E. coli host-cell DNA contamination in plasmid DNA preparations." BioTechniques 26 (1999): 518-526.

Wang, S.C., Klein, R.D., Wahl, W.L., Alarcon, W.H., Garg, R.J., Remick, D.G., and Su, G.L. Tissue coexpression of LBP and CD14 mRNA in a mouse model of sepsis. *J.Surg.Res.* 76(1):67-73, 1998.

Wang, T. and Brown, M.J. mRNA Quantification by Real Time TaqMan Polymerase Chain Reaction: Validation and Comparison with RNase Protection [In Process Citation]. *Anal.Biochem.* 269(1):198-201, 1999.

Wang X., X. Li, J.A. Erhardt, F.C. Barone and G.Z. Feuerstein. 2000. Detection of tumor necrosis factor-alpha mRNA induction in ischemic brain tolerance by means of real-time polymerase chain. J. Cereb. Blood Flow Metab. 2000. Jan;20(1):15-20. 20:15-20.

Wang X., X. Li, R.W. Currie, R.N. Willette, F.C. Barone and G.Z. Feuerstein. 2000. Application of real-time polymerase chain reaction to quantitate induced expression of interleukin-1beta mRNA in ischemic brain tolerance. J. Neurosci. Res. 2000. Jan. 15;*59*(2):238-46.

Ying H., T.Z. Zaks, R.F. Wang, K.R. Irvine, U.S. Kammula, F.M. Marincola, W.W. Leitner and N.P. Restifo. Cancer therapy using a self-replicating RNA vaccine. Nat. Med. *5*:823-827, 1999.

Relative Quantitation

Araki, R. and et al Nonsense mutation at Tyr-406 in the DNA-dependent protein kinase catalytic subunit of severe combined immune deficiency mice. PNAS 94:2438-2443, 1997.

Bieche, I., et al. "Novel approach to quantitative polymerase chain reaction using real- time detection: application to the detection of gene amplification in breast cancer." Int J Cancer 78 (1998): 661-666.

Bieche I., P. Onody, I. Laurendeau, M. Olivi, D. Vidaud, R. Lidereau and M. Vidaud. Real-time reverse transcription-PCR assay for future management of ERBB2-based clinical applications. Clin. Chem. 45:1148-1156, 1999.

Casteels, K. M., et al. "Prevention of type I diabetes in nonobese diabetic mice by late intervention with nonhypercalcemic analogs of 1,25-dihydroxyvitamin D3 in combination with a short induction course of cyclosporin A." Endocrinology 139 (1998): 95-102.

Chiang, P.W., Beer, D.G., Wei, W.L., Orringer, M.B., and Kurnit, D.M. Detection of erbB-2 amplifications in tumors and sera from esophageal carcinoma patients. Clin.Cancer Res. 5(6):1381-1386, 1999.

Collins, C., et al. "Positional cloning of ZNF217 and NABC1: genes amplified at 20q13.2 and overexpressed in breast carcinoma." Proc Natl Acad Sci U S A 95 (1998): 8703-8708.

de Kok, J.B., Ruers, T.J., van Muijen, G.N., van Bokhoven, A., Willems, H.L., and Swinkels, D.W. Real-time quantification of human telomerase reverse transcriptase mRNA in tumors and healthy tissues. Clin.Chem.2000.Mar;46(3):313-8.

Dittmer D., C. Stoddart, R. Renne, V. Linquist-Stepps, M.E. Moreno, C. Bare, J.M. McCune and D. Ganem. 1999. Experimental Transmission of Kaposi's Sarcoma-associated Herpesvirus (KSHV/HHV-8) to SCID-hu Thy/Liv Mice. J. Exp. Med. 190:1857-1868.

Dolken, L., F. , Schuler, and G. , Dolken. "Quantitative detection of t(14;18)-positive cells by real-time quantitative PCR using fluorogenic probes." BioTechniques 25 (1998): 1058-1064.

Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Danenberg, P.V., and Laird, P.W. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. Cancer Res. 59(10):2302-2306, 1999.

Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Blake, C., Shibata, D., Danenberg, P.V., and Laird, P.W. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic.Acids.Res.2000.Apr.15;28(8):E32.

Eder, M., Battmer, K., Kafert, S., Stucki, A., Ganser, A., and Hertenstein, B. Monitoring of BCR-ABL expression using real-time RT-PCR in CML after bone marrow or peripheral blood stem cell transplantation. Leukemia 13(9):1383-1389, 1999.

Fink, L., et al. "Real-time quantitative RT-PCR after laser-assisted cell picking." Nat Med 4 (1998): 1329-1333.

Haugland, R.A., Vesper, S.J., and Wymer, L.J. Quantitative measurement of stachybotrys chartarum conidia using real time detection of PCR products with the TaqMan(TM)fluorogenic probe system. Mol.Cell Probes. 13(5):329-340, 1999.

Hirayama, Y., et al. "Concentrations of thrombopoietin in bone marrow in normal subjects and in patients with idiopathic thrombocytopenic purpura, aplastic anemia, and essential thrombocythemia correlate with its mRNA expression of bone marrow stromal cells." Blood 92 (1998): 46-52.

Johnson, M.R., Wang, K., Smith, J.B., Heslin, M.J., and Diasio, R.B. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. Anal.Biochem.2000.Feb.15;278(2):175-84.

Krauter J., M.P. Wattjes, S. Nagel, O. Heidenreich, U. Krug, S. Kafert, D. Bunjes, L. Bergmann, A. Ganser and G. Heil. 1999. Real-time RT-PCR for the detection and quantification of AML1/MTG8 fusion transcripts in t(8;21)-positive AML patients. Br. J. Haematol. 107:80-85.

Kruse, N., Pette, M., Toyka, K., and Rieckmann, P. Quantification of cytokine mRNA expression by RT PCR in samples of previously frozen blood. J.Immunol.Methods 210(2):195-203, 1997.

Lee, L.Y., Patel, S.R., Hackett, N.R., Mack, C.A., Polce, D.R., El-Sawy, T., Hachamovitch, R., Zanzonico, P., Sanborn, T.A., Parikh, M., Isom, O.W., Crystal, R.G., and Rosengart, T.K. Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for vascular endothelial growth factor 121. Ann.Thorac.Surg.2000.Jan;69(1):14-23;.discussion.23-4.

Leutenegger C.M., C.N. Mislin, B. Sigrist, M.U. Ehrengruber, R. Hofmann-Lehmann and H. Lutz. 1999. Quantitative real-time PCR for the measurement of feline cytokine mRNA. Vet. Immunol. Immunopathol. 71:291-305.

Maeda, I., Takano, T., Matsuzuka, F., Maruyama, T., Higashiyama, T., Liu, G., Kuma, K., and Amino, N. Rapid screening of specific changes in mRNA in thyroid carcinomas by sequence specific-differential display: decreased expression of acid ceramidase mRNA in malignant and benign thyroid tumors [In Process Citation]. Int.J.Cancer 81(5):700-704, 1999.

Mandigers, C.M., Meijerink, J.P., Raemaekers, J.M., Schattenberg, A.V., and Mensink, E.J. Graft-versus-lymphoma effect of donor leucocyte infusion shown by real- time quantitative PCR analysis of t(14;18) [letter]. Lancet 352(9139):1522-1523, 1998.

Marcucci, G., et al. "Detection of minimal residual disease in patients with AML1/ETO-associated acute myeloid leukemia using a novel quantitative reverse transcription polymerase chain reaction assay." Leukemia 12 (1998): 1482-1489.

Mensink, E., et al. "Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR." Br J Haematol 102 (1998): 768-774.

Moody, A., Sellers, S., and Bumstead, N. Measuring infectious bursal disease virus RNA in blood by multiplex real-time quantitative RT-PCR. Virol.Methods 2000.Mar;85(1-2):55-64.

Patterson, B. K., et al. "Repertoire of chemokine receptor expression in the female genital tract: implications for human immunodeficiency virus transmission." Am J Pathol 153 (1998): 481-490.

Pongers-Willemse, M.J., Verhagen, O.J., Tibbe, G.J., Wijkhuijs, A.J., de Haas, V., Roovers, E., van der Schoot, C.E., and van Dongen, J.J. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. Leukemia 12(12):2006-2014, 1998.

Pugazhenthi, S., Nesterova, A., Sable, C., Heidenreich, K.A., Boxer, L.M., Heasley, L.E., and Reusch, J.E. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J.Biol.Chem.2000.Apr.14;275(15):10761-6.

Raggi, C.C., Bagnoni, M.L., Tonini, G.P., Maggi, M., Vona, G., Pinzani, P., Mazzocco, K., De Bernardi, B., Pazzagli, M., and Orlando, C. Real-time quantitative PCR for the measurement of MYCN amplification in human neuroblastoma with the TaqMan detection system. Clin.Chem. 45(11):1918-1924, 1999.

Ringel, M.D., Balducci-Silano, P.L., Anderson, J.S., Spencer, C.A., Silverman, J., Sparling, Y.H., Francis, G.L., Burman, K.D., Wartofsky, L., Ladenson, P.W., Levine, M.A., and Tuttle, R.M. Quantitative reverse transcription-polymerase chain reaction of circulating thyroglobulin messenger ribonucleic acid for monitoring patients with thyroid carcinoma. J.Clin.Endocrinol.Metab. 84(11):4037-4042, 1999.

Sangoram, A. M., et al. "Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription." Neuron 21 (1998): 1101-1113.

Sgroi D.C., S. Teng, G. Robinson, R. LeVangie, J.R. Hudson, Jr. and A.G. Elkahloun. 1999. In vivo gene expression profile analysis of human breast cancer progression. Cancer Res. 59:5656-5661.

Shayesteh, L., Lu, Y., Kuo, W.L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G.B., and Gray, J.W. PIK3CA is implicated as an oncogene in ovarian cancer [see comments]. Nat.Genet. 21(1):99-102, 1999.

Shimokawa, T., et al. "Transcriptional regulation of muscle-specific genes during myoblast differentiation." Biochem Biophys Res Commun 246 (1998): 287-292.

Taoufik, Y., Froger, D., Benoliel, S., Wallon, C., Dussaix, E., Delfraissy, J.F., and Lantz, O. Quantitative ELISA-polymerase chain reaction at saturation using homologous internal DNA standards and chemiluminescence revelation. Eur.Cytokine.Netw. 9(2):197-204, 1998.

Virtanen M., H. Torma and A. Vahlquist. 2000. Keratin 4 upregulation by retinoic acid In vivo: A sensitive marker for retinoid bioactivity in human epidermis. J. Invest. Dermatol. 2000. Mar;114(3):487-93.

Wingo, S.T., Ringel, M.D., Anderson, J.S., Patel, A.D., Lukes, Y.D., Djuh, Y.Y., Solomon, B., Nicholson, D., Balducci-Silano, P.L., Levine, M.A., Francis, G.L., and Tuttle, R.M. Quantitative reverse transcription-PCR measurement of thyroglobulin mRNA in peripheral blood of healthy subjects. Clin.Chem. 45(6 Pt 1):785-789, 1999.

Yajima, T., et al. "Quantitative reverse transcription-PCR assay of the RNA component of human telomerase using the TaqMan fluorogenic detection system." Clinical Chemistry 44 (1998): 2441-2445.

Yamada Y., N. Itano, H. Narimatsu, T. Kudo, S. Hirohashi, A. Ochiai, A. Niimi, M. Ueda and K. Kimata. 1999. Receptor for hyaluronan-mediated motility and CD44 expressions in colon cancer assessed by quantitative analysis using real-time reverse transcriptase-polymerase chain reaction. Jpn. J. Cancer Res. 90:987-992.

Yoneyama H., A. Harada, T. Imai, M. Baba, O. Yoshie, Y. Zhang, H. Higashi, M. Murai, H. Asakura and K. Matsushima. 1998. Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. J. Clin. Invest. 102:1933-1941.

Zhang, A.W., Hartman, G.L., Curio-Penny, B., Pedersen, W.L., and Becker, K.B. Molecular Detection of Diaporthe phaseolorum and Phomopsis longicolla from Soybean Seeds. Phytopathology 89(9):796-804, 1999.

Manufacturer's Product Information

ABI Prism 7000 System®

Roche LightCycler System®

Stratagene MX4000®