FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Development of selective enrichment culture-polymerase chain reaction (SEC-PCR) for the detection of bacterial pathogens in covertly infected farmed salmonid fish

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Tasmanian Aquaculture & Fisheries Institute
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Aquafin CRC

Atlantic Salmon
AQUACULTURE
SUBPROGRAM

1. NON-TECHNICAL SUMMARY

99/201	Development of selective enrichment culture-polymerase chain reaction (SEC-PCR) for the detection of bacterial pathogens in covertly infected farmed salmonid fish

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OBJECTIVES:

- Develop a procedure for extracting bacterial DNA from selective enrichment media that is suitable for PCR and for processing multiple samples.

- Determine optimum conditions for the PCR tests to maximise specificity and sensitivity of the procedure.

- Optimise PCR conditions to incorporate a PCR protection system to prevent false positive reactions arising from DNA contamination.

- Develop a test procedure based on enzyme hybridization assay (EHA) that will verify any positive PCR reactions using a secondary confirmatory gene probe and in a format suitable for testing multiple samples.

- Optimise the culture conditions and PCR detection process to ensure the minimum test time between sample collection and test result.

- Test populations of salmonids with the optimised SEC-PCR-EHA system to verify test performance and obtain baseline data on carrier prevalence.

NON-TECHNICAL SUMMARY:

Bacterial disease is a major cause of stock loss in aquaculture. The severity of infection may range from acute to chronic through to benign. This latter condition, termed covert infection, is insidious, as fish may appear to be outwardly healthy but during periods of stress, these carriers may breakdown leading to spread of infection and development of a disease outbreak.

Several bacterial pathogens, known to exist in Australia and the cause of significant disease episodes in Atlantic salmon and rainbow trout, can cause covert infections including: atypical *Aeromonas salmonicida*, *Lactococcus garvieae*, *Tenacibaculum maritimum* and *Yersinia ruckeri*.

Early detection of covertly infected fish is considered desirable as it provides a means of determining a suitable disease control strategy such as imposing movement restrictions to prevent the spread of disease, changing management practices to avoid stress or determining the spread of disease in a population at risk of infection. The standard method for identifying carriers is to stress a cohort of fish using a combination of heat and

immunosuppression to force covertly infected fish to breakdown with disease. This form of testing is undesirable for animal welfare considerations, is difficult to accomplish and takes over three weeks to generate results.

The research described in this report has led to the development of a replacement test to identify covertly infected fish. Called Selective Enrichment Culture PCR Enzyme Hybridization Assay (SPE), the laboratory based test only requires the use of skin mucus or faecal samples and positive results are obtained within six days.

The primary step in SPE uses selective enrichment culture to amplify the target pathogen. Media for the salmonid pathogens had been developed previously except for *A. salmonicida*. A selective enrichment medium was developed for the atypical salmonid biovar of *A. salmonicida* and was shown to have a good level of selectivity when tested with over 400 strains of normal flora bacteria commonly associated with salmonids.

Following enrichment culture, DNA is extracted and the target pathogen detected using the DNA amplification technique PCR or RT-PCR coupled with a DNA probe hybridization step to verify positive reactions. Within the amplification process, a PCR contamination control step is included as a measure to reduce false positive reactions.

A rapid two step method for the extraction of DNA and RNA from enrichment culture media using glass microfibre filter plates was developed. Formatted for high throughput testing, 96 samples can be processed simultaneously. Optimised PCR and RT-PCR protocols for the four pathogens could detect as little as 4fg of DNA or RNA, equivalent to one bacterial cell. Coupled with the high throughput DNA/RNA extraction method, the detection level by PCR or RT-PCR ranged from 20 to 80 cells ml^{-1} of culture medium.

Products of PCR and RT-PCR were detected through hybridization with a secondary verification DNA probe. This was achieved using a biphasic PCR format followed by *in-situ* probe hybridization undertaken as a two step process in the one reaction well. High throughput processing was retained using 96 well format plates. PCR contamination control was implemented using the amplicon inactivator isopsoralen IP-10. Integrated into PCR process as a post-amplification step, following UV activation, it was possible to inactivate at least 6×10^7 amplicons, sufficient as a routine control measure.

Field validation of SPE established that the test did not generate unexpected false positive reactions and test specificity was determined to be >99%. Test sensitivity was better than expected and case definitions had to be established for test interpretation. For farms with recent history of disease, RT-PCR was used as it provides an indication of covert infection with live pathogen. SPE in the PCR format was restricted to testing for evidence of exposure to pathogens as the test detects live carriage as well as DNA remnants from dead cells remaining from recent but past infections. From field surveys of farmed salmonids, live carriage levels ranged from 0-18% for *A. salmonicida*, 2% for *L. garvieae*, 1% for *T. maritimum* and 4% for *Y. ruckeri*.

OUTCOME ACHIEVED:
A system has been developed for the practical detection of covert bacterial infections in salmonid fish. The technology is designed for high volume testing and is suitable for large scale population screening. The diagnostic technique, using a hybrid culture-gene probe technology, can be used for quarantine, disease surveillance and disease management purposes and will contribute directly to improved stock health of farmed fish.

KEYWORDS: *Aeromonas salmonicida, Lactococcus garvieae, Tenacibaculum maritimum, Yersinia ruckeri,* Atlantic salmon, rainbow trout, covert infection, diagnosis, PCR, RT-PCR amplification, enzyme hybridization, DNA extraction

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3. BACKGROUND

Bacterial infection can range from a covert condition with an absence of clinical signs to a fulminating florid state leading to death. In overtly diseased fish, pathogen detection can be readily achieved by conventional methodologies such as culture, immuno-staining or the use of gene probes. Identification of covertly infected fish however, is problematic as the pathogen is present in low numbers and is not distributed uniformly within or on the host. Determining the presence of covertly infected fish in a population is important, as these carriers are a source of contagion that may infect naïve individuals. Furthermore, during periods of stress covertly infected fish may breakdown leading to fulminating disease and pen based disease outbreaks or in severe cases, involvement of an entire lease site. The ability to detect covertly infected fish is recognised as an essential disease management and control strategy as it identifies populations at risk and provides a basis to determine appropriate husbandry options to minimise the effect of incipient disease (Ford 1994).

The reference method to identify covertly infected fish is based on the combined effects of elevated temperature and corticosteroid immuno-suppression to initiate overt expression of disease (Bullock and Stuckey 1975). Known as the SIF test (stress inducible furunculosis), this method is used extensively prior to stock movement as a means of preventing translocation of disease caused by *Aeromonas salmonicida* ssp. *salmonicida*, the cause of furunculosis in salmonids (Smith 1991; Hiney *et al.* 1997).

Stress testing is problematic in that it is resource intensive, requiring quarantine holding facilities, is a lethal test that has animal welfare and ethics considerations, and is slow, needing over two weeks to conduct the test. There are few if any truly viable alternatives to the stress test.

Direct culture of fish is unrewarding in that the target pathogen is usually present only at low levels. For *A. salmonicida*, cell number in covertly infected fish may range from as little as 10cfu g^{-1} to 10^4 cfu g^{-1} (Nomura *et al.* 1993; Cipriano *et al.* 1992, 1996); the detection limit by culture of *A. salmonicida* is 10^3 cfu g^{-1} (Cipriano *et al.* 1992). To achieve these levels of detection, sample processing is intensive, as is confirmation, and limits the usefulness and applicability of culture, particularly for mass screening in a population of fish.

Detection of pathogens in covertly infected fish using gene probes and DNA amplification by polymerase chain reaction (PCR) has not been successful due to insufficient test sensitivity. PCR, *sensu stricto*, is exquisitely sensitive; able to detect femtogram amounts of DNA equivalent to a single bacterial genome, this level of sensitivity is of limited value when considered in the context of the test as a whole. The lack of apparent sensitivity of direct PCR in fish tissue is due to at least three factors: PCR inhibiting factors in fish tissue; small tissue sample size that can be used in PCR (typically 50 mg of tissue); and sampling error due to the small numbers of bacteria present in covertly infected fish (Carson 1998). Limits of detection of this format are about 10^4 cells g^{-1} tissue (McIntosh *et al.* 1996; Høie *et al.* 1997; Carson 1998; Byers *et al.* 2002a) with a theoretical limit of 10^3 cells g^{-1} of tissue (Carson 1998). In a comparative study to detect *A. salmonicida* carriers, culture was more efficient than PCR (Byers *et al.* 2002b), further evidence that conventional PCR alone is unsuitable as a means of detecting covertly infected fish.

Direct detection of pathogens by PCR, if not carefully used, has the potential to be highly misleading. Major problems arise not only through false negatives (under reporting) and false positives (over reporting), but also through a fundamental error of not determining a case definition (Meade and Bollen 1994). Determining the significance of positive findings and identifying the qualifiers that must be met to accept a positive PCR finding as evidence of meaningful infection is essential. Difficulties may arise where samples are PCR positive but there is no history of disease in the population under surveillance. Such dichotomies have been reported for screening studies for *A. salmonicida* in Atlantic salmon where there is evidence of carriage but no evidence of infection (Mooney et al. 1995). Similar difficulties have been noted in regard to vaccination where false positive reactions occur as a result of detecting target DNA derived from killed vaccines (Høie *et al.* 1996; Vaneechoutte and van Eldere 1997; Vaneechoutte 1999). Clearly, significance of direct PCR findings in the absence of culture imposes limitations on the usefulness of the technique as does the limitation of test sensitivity, particularly where the target pathogen is present at very low levels such as in covertly infected fish.

Pre-test amplification of bacterial fish pathogens by culture has received little attention, although as a technique it is used extensively to detect food-borne pathogens such as *Shigella, Listeria, Salmonella* and *Campylobacter* species (Uyttendaele *et al.* 2001; Ferron and Michard 1993; Hoorfar and Baggesen 1998; Baylis *et al.* 2000). Enrichment is

achieved using liquid media containing agents that suppress competing non-target organisms but permit the growth of the target species. Target numbers in the sample are invariably low so that the density level determines optimal sample size. An advantage of a liquid medium is that the volume of enrichment medium can be scaled for the sample size, so maximising the likelihood of recovering the target pathogen.

Non-selective enrichment media have been used for the amplification of *A. salmonicida* (Hirvelä-Koski *et al.* 1988) but since the media lack any selective capacity, recovery of the pathogen on subculture is compromised by bacterial overgrowth of competing normal flora. Where samples were collected aseptically, enrichment culture increased detection of *A. salmonicida* compared to plate culture alone. Non-selective enrichment has been used as a means of isolating low levels of bacteraemic flora in penaeids and has been proposed as a monitoring tool for the detection of incipient disease (Sano *et al.* 1996). A limited range of selective indicator media have been developed for bacterial fish pathogens (Shotts 1991), but there use is limited, in part an indication of a limited scope of application and their performance. More recently, four selective enrichment media were developed for the isolation of *A. salmonicida* (goldfish and greenback flounder biovars), *L. garvieae*, *T. maritimum* and *Y. ruckeri* (Carson *et al.*, 2001b). Coupled with capture ELISA as means of detecting the target pathogen, the media were developed as an improved method for the detection of covert infections in fish. The fusion technology of selective-enrichment-culture-ELISA (SEC-ELISA) was at least as effective as conventional stress testing, and for some of the pathogens, was more effective. Improved detection through the use of selective enrichment culture has been achieved for *Renibacterium salmoninarum* using SKDM as an enrichment broth coupled with Western blotting to detect expression of the signature antigen p57 (Griffiths *et al.* 1996).

Although SEC-ELISA was seen as an improvement, the ELISA technology imposed some limitations on the system as a whole. The ELISA test is relatively insensitive and to generate a positive signal it required at least 10^5 cells ml^{-1} of the target pathogen to be present in the enrichment culture medium. While the enrichment efficiency was good for some of the selective media, it was marginal in others and the final concentration of target pathogens could, in some cases, be below the detection threshold of the ELISA. As an immuno-assay, accuracy of the test is dependent on the specificity of the capture antibody. The inherent uncertainty relating to the use of antibodies where a test is

intended for use with samples which may have unknown antigenic diversity is not desirable. Other detection techniques which can detect fewer bacteria and are less susceptible to strain variation in the target pathogen would improve the usefulness of the selective enrichment media.

Detection of food pathogens by selective enrichment has been further refined by coupling the culture process with PCR so that a further level of sensitivity as well as specificity is achieved (Swaminathan & Feng 1994). The technique has been used for the detection of *Vibrio cholerae* (Koch *et al.* 1993) and *Salmonella* species (Bej *et al.* 1994) in oysters. Using selective culture enrichment and PCR as little as one bacterium per 10 g of oyster tissue can be detected consistently and reliably. Use of selective enrichment confers other benefits apart from amplification of the target bacterium through culture. PCR inhibiting factors that may be present in tissue samples are diluted in the culture medium (Cohen *et al.* 1996) which enables a more productive PCR process to occur. Other advantages of selective enrichment culture and PCR is the rapid detection of target pathogen which can be achieved (Thisted Lambertz *et al.* 1996) and based on viable cells rather than non-viable cells containing intact DNA. Enrichment culture, as a pre-PCR strategy to improve detection of fish pathogens, has received little attention despite evident improvements gained in detection efficiency of *A. salmonicida* (Gustafson *et al.* 1992), infectious haematopoietic necrosis virus (Yoshinaka *et al.* 1999) and nodavirus (Iwamoto *et al.* 2001).

Development of highly specific PCR primers for *A. salmonicida*, *L. garvieae*, *T. maritimum* and *Y. ruckeri* (Carson 1998) and corresponding selective enrichment media (Carson *et al.*, 2001b), provides the basis to develop a highly sensitive, rapid and effective system for the detection of bacterial pathogens in covertly infected fish. Importantly, although the technique uses a DNA proxy test to determine presence or absence of a target pathogen, the culture step in the process ensures that test results have biological significance and can be used as a basis for rational interpretation.

4. NEED

Active surveillance of animal populations is considered an important approach in animal health monitoring and disease management (Stärk 1996) and should, in its own right, form part of any national programme that seeks to maintain high health animal stock. Internationally, there is a growing emphasis on defining national disease status for trade purposes regionally within countries and between countries. To that end active consideration is being given by the Office International des Epizooties to the basis for defining disease-free status of farmed aquatic animals (Anon 2002).

A major problem of testing farmed and wild fish is the absence of simple diagnostic tests for the detection of covertly infected fish. Where tests are available, they are resource intensive and time consuming such as the stress inducible furunculosis test for salmonids (Smith 1991). Yet despite the logistical impost, the test is used as an essential disease control measure in eastern Canada and has been instrumental in limiting spread of furunculosis to sea cage farms (Olivier 1992). Identification of covertly infected fish is considered potentially important in managing disease in farmed fish but the lack of suitable diagnostic tools has meant that this approach to maintaining high health stock has not been used (Morrison 1999).

In south eastern Australia, where salmonids are farmed in both freshwater and marine sites, the major bacterial pathogens known to cause significant disease outbreaks are *Aeromonas salmonicida* biovar *acheron*, *Lactococcus garvieae*, *Tenacibaculum maritimum* and *Yersinia ruckeri*. All of these pathogens may be carried in fish as covert infections, a factor that has direct impact on disease management and control.

Development of a generic technology that could be used for active surveillance, was non-destructive and readily undertaken would contribute greatly to the goal of establishing disease free zones. In addition, the technology would have the potential to be used for disease monitoring to determine the extent of an outbreak and also to establish populations at risk as well as those that pose a threat of infection. The technology will also have application for quarantine purposes as a means of controlling stock movements to limit the spread of disease.

5. OBJECTIVES

5.1. Develop a procedure for extracting bacterial DNA from selective enrichment media that is suitable for PCR and for processing multiple samples.

5.2. Determine optimum conditions for the PCR test to maximise specificity and sensitivity of the procedure.

5.3. Optimise PCR conditions to incorporate a PCR protection system to prevent false positive reactions arising from DNA contamination.

5.4. Develop a test procedure based on enzyme hybridization assay (EHA) that will verify any positive PCR reactions using a secondary confirmatory gene probe and in a format suitable for testing multiple samples.

5.5. Optimise the culture conditions and PCR detection process to ensure the minimum test time between sample collection and test result.

5.6. Test populations of salmonids with the optimised SEC-PCR-EHA system to verify test performance and obtain baseline data on carrier prevalence.

6. METHODS

6.1. Develop a procedure for extracting bacterial DNA from selective enrichment media that is suitable for PCR and for processing multiple samples.

6.1.1. Target organisms

The target organisms for this study were: *Yersinia ruckeri* serotype O1b isolated from Atlantic salmon, DPIWE accession number 90/3988; *Lactococcus garvieae* ATCC 49156[T]; *Aeromonas salmonicida* isolated from greenback flounder, DPIWE accession number 93/0956-2; and *Tenacibaculum maritimum* NCIMB 2154[T].

6.1.2. Large scale extraction of DNA for use in determining PCR sensitivity with purified DNA, and as a positive control.

Reference DNA was extracted from the four bacteria using a method based on Marmur (1961) with some modifications due to the mucoid nature of *T. maritimum* and the lysis resistant nature of the cell wall of *L. garvieae*.

A starter culture of 10 ml Brain Heart Infusion broth (BHI) (Oxoid, UK) or Shieh's marine broth (Song *et al.* 1988; Appendix 3) for *T. maritimum* was prepared and incubated at 25°C until turbid. This starter culture was used to inoculate one litre of broth, which was incubated at 25°C until very turbid. For *L. garvieae* the BHI was supplemented with 2 g l^{-1} DL-threonine (Sigma-Aldrich, Missouri, USA) (Klaenhammer *et al.* 1978) and after 12-18 hours incubation 0.12 g l^{-1} penicillin G (Sigma-Aldrich) (Komatsu 1979) was added to assist with cell lysis. For *T. maritimum* the culture was stirred and aerated to encourage dense and even bacterial growth. Purity of all broth cultures was assessed by subculture on sheep's blood agar (Blood Agar No2 [Oxoid] enriched with 7% defibrinated sheep's blood) (SBA) or marine Shieh's agar (MSA) for *T. maritimum*.

The cells were pelleted by centrifugation at 3900g for 20 minutes at 10°C, and the supernatant removed. For *T. maritimum* only, cell surface muco-polysaccharide was removed by washing the pellet in 10 ml saline-EDTA + 20 ml ice cold ethanol, vortexed, made up to 50 ml with saline-EDTA and centrifuged again. For the other bacteria the

pellet was resuspended in 25 ml total saline-EDTA and centrifuged as above. The supernatant was discarded and the tube weighed to determine the amount of packed cells (0.5-1 g).

The pellet was resuspended in 8 ml saline-EDTA to give an even suspension free from clumps. Lysis was initiated by the addition of 1 ml of 20 mg ml^{-1} lysozyme, the suspension vortexed and incubated for 1 hour at 37°C, or for *L. garvieae* overnight. After lysozyme treatment 1 ml of 10% (w/v) SDS and 20 µl of 50 mg ml^{-1} proteinase K was added and the cells incubated at 50°C for 30 minutes in a water-bath. A further 0.5 ml of 10% (w/v) SDS was added to the cells followed by two, 5 minute, 50°C incubation steps with cooling to 4°C between each incubation. At this stage, the degree of cell lysis was assessed by examining the cell suspension by phase contrast microscopy. If the bacterial cells were largely intact short incubation steps at 55°C followed by rapid cooling was repeated until lysis occurred.

After cell lysis 2.75 ml of 5 M sodium perchlorate was added to the cells and shaken for 10 minutes at room temperature. The cell debris was separated from the nucleic acids by adding one volume of chloroform/iso-amyl alcohol 24:1 (Amresco, Ohio, USA), shaking for 30 minutes at room temperature and centrifuging at 3000g for 15 minutes. The top layer was carefully collected using a wide bore pipette and overlayed with two volumes of cold absolute ethanol and the nucleic acids collected by spooling with a glass rod. Any residual ethanol was allowed to evaporate and the nucleic acids were dissolved in 5 ml 10 mM TRIS buffer, pH 8 (Amresco).

Once the nucleic acid mass had separated from the glass rod, the buffer was warmed to 37°C for 15 minutes to completely dissolve the nucleic acids. RNA was hydrolysed by the addition of 250 µl of 0.2% RNase A (Sigma-Aldrich) and incubating for 45 minutes at 37°C (Keller and Manak 1989). A 5 ml volume of phenol saturated TRIS-EDTA buffer (pH 8) (Amresco) was then added, the solution shaken for 10 minutes at room temperature, followed by centrifugation at 1500g for 10 minutes. The upper aqueous layer was collected using a wide-bore pipette, over-layed with two volumes of cold absolute ethanol and the DNA spooled using a glass rod. Residual ethanol was allowed to evaporate and the DNA dissolved in 10 mM TRIS, pH 8. One volume of chloroform/ isoamyl alcohol 24:1 was added to the DNA, shaken for 15 minutes at room temperature, followed by

centrifugation at 3000g for 15 minutes. The upper aqueous layer was collected and the chloroform/isoamyl alcohol procedure repeated. The upper aqueous layer was collected one last time and over-layed with two volumes of cold absolute ethanol and pure DNA spooled using a glass rod. Residual ethanol was allowed to evaporate the DNA dissolved overnight in 5 ml 10 mM TRIS, pH 8 at 4°C, and the DNA stored at -20°C.

The concentration of purified DNA was determined using a Bio-Rad Versafluor Fluorimeter and a Hoechst H33258 DNA specific dye. Zero calibration values were set using TRIS-EDTA-NaCl and Hoechst dye and 100 ng μl^{-1} point determined using TRIS-EDTA-NaCl, Hoechst dye and a calf thymus DNA standard. The DNA samples were measured by adding 2 μl of sample to 2000 μl of TRIS-EDTA-NaCl plus Hoechst dye.

6.1.3. Evaluation of 96-well DNA vacuum extraction units.

Two 96-well vacuum manifold systems were trialed. A Multiscreen™ FB filter plate (Millipore corp., MA, USA, cat# MAFB NOB) and a vacuum manifold from Millipore and the Whatman Polyfiltronics GF/B Uni-filter plate (Whatman, Rockland, MA, USA, cat# 7700-2803) and UNIVAC Teflon coated vacuum manifold (Whatman). The systems were evaluated with respect to cost, ease of use, quality of the system, quality of the DNA extracted from selective-enrichment media, and other problems or factors as they occurred.

6.1.4. Combined protocol for extracting of genomic DNA and ribosomal RNA using UNIVAC manifold and Whatman GF/B 96-well filter plates.

96-well vacuum extraction

Before extracting bacterial RNA, water and sterile plastic troughs were made RNase free by treating with 0.2% (v/v) DEPC (diethylpyrocarbonate) for 30 minutes followed by autoclaving at 121°C 20 min^{-1}. Solutions containing TRIS buffer were not treated with DEPC since TRIS reacts with DEPC.

For the extraction of *L. garvieae* nucleic acids, pre-treatment was required to achieve lysis. A stock solution of 40 mg ml^{-1} lysozyme was prepared and treated with DEPC to final concentration of 0.2%. The lysozyme was then vortexed vigorously and store frozen at

-20°C until use. To 1ml of selective enrichment culture medium, 100 µl of DEPC treated 40 mg ml^{-1} lysozyme was added and then thoroughly vortexed and incubated at 37°C for 1 hour. For DNA extraction only, 2 µl of 50 mg ml^{-1} proteinase K and 100 µl of 10% (w/v) SDS was added, the sample vortexed and incubated at 50°C for 30 minutes. The sample was cooled rapidly for 5 minutes followed by incubation at 100°C for 15 minutes and cooled once more for at least 3 minutes. The proteinase K step was not used when extracting RNA as this resulted in a significant loss in extraction sensitivity.

For all bacteria, lysis, nucleic acid binding and elution were performed using a Polyfiltronics glass microfibre (type GF/B) 800 µl, 96-well Uni-filter plate (Whatman), and a UniVac vacuum manifold (Whatman). Each well on the filter plate was pre-wet for 1-5 minutes with 100 µl of 10 mM TRIS-HCl pH 6.4 with β-mercaptoethanol to a final concentration of 1% and the wells drained by applying a vacuum of about 20 kPa. Lysis buffers L6 and L2 (Boom *et al.* 1990; Appendix 3) were cooled to 4°C and a volume of 500 µl L6 buffer was added to each well using a multichannel pipette followed immediately by 200 µl of selective-enrichment culture. After 15 minutes the resulting lysate was removed from the filter wells by applying a vacuum of about 13.5 kPa. A low vacuum pressure was essential at this step to prevent foaming of the L6 buffer around the drip directors underneath each well, a condition that could lead to sample cross-contamination. Flow rates between wells were balanced by sealing the plate with a flexible membrane (Polyfiltronics VacAssist, Whatman). The filter plate wells were washed twice with 100 µl of cooled L2 buffer, five times with 200 µl of 70% ethanol prepared with DEPC treated water, and once with 100 µl of acetone at a vacuum pressure of 40 kPa. After the last wash, the vacuum was maintained until there was no visible trace of alcohol in the wells or on the drip directors underneath the wells. A 96-well microtitre plate to receive the eluted DNA/RNA was placed in the vacuum manifold and 30 µl of elution buffer (Appendix), pre-heated to 90°C was then added to each filter well. After 10 minutes, DNA/RNA was eluted from the glass microfibre filter plate. A further 30 µl of elution buffer was added to each well, let stand for 5 minutes followed by elution as described. The DNA/RNA was allowed to re-hydrate/dissolve overnight at 4°C.

PCR Thermocycler

All PCR and RT-PCR reactions were undertaken in an MJ Research PTC-100-96V thermocycler (Waltham, Mass., USA). The thermocycler has a conventional 8x12 well

format and accepts 96 well plates, strips or individual tubes; all wells are v-shaped. The manufacturers specifications for well-to-well temperature variation is ±0.3°C within 30 seconds of arrival at 92°C and ±0.5°C of programmed target temperature.

PCR of extracted DNA

The suitability of filter plate extracted DNA for PCR was assessed using 16S rRNA gene primer sets (Carson 1998). PCR reactions contained 200 µM each of dNTPs, 1.375 mM $MgCl_2$ for *A. salmonicida* and 2 mM $MgCl_2$ for *T. maritimum*, *L. garvieae* and *Y. ruckeri*; 1 X PCR buffer (Invitrogen, Ca, USA), 0.5 units Platinum *Taq* DNA Polymerase (Invitrogen), 2 µM each of the two primers, 1 µl of filter plate extracted DNA pre-warmed to 25°C and enough 18 Mohm water to bring the reaction volume up to 20 µl. Cycling conditions were: an initial 3 minutes denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (30 sec), annealing at 65°C (30 sec), extension at 72°C (2 minutes), with a final extension at 72°C for 3 minutes. A positive control and a negative control were included in each PCR. Amplicons were visualised by electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 µg ml^{-1}, and their size measured using a 100 bp marker (Advanced Biotechnologies, Surrey, UK).

RT-PCR of extracted RNA

Reference RNA for sensitivity determinations and positive controls was extracted using a RNAAqueous™-4PCR (Ambion, Austin, Texas, USA) extraction kit following the manufacturer's instructions.

The suitability of filter plate extracted RNA for RT-PCR was assessed using 16S rRNA primer sets (Carson 1998). To achieve RNA free of DNA, DMIX buffer (Appendix 3) containing DNase 1 (Promega, USA) was added to an aliquot of each vacuum extracted nucleic acid elute. The DNase was activated by heating the nucleic acids to 37°C for 30 minutes, inactivated by heating to 75°C for 5 minutes and then cooled to 4°C before use as template for RT-PCR.

RT-PCR reactions contained 200 µM each of dNTPs, 1.375 mM $MgSO_4$ for *A. salmonicida* and 2 mM $MgSO_4$ in all other cases, 1 X RT-PCR buffer (Invitrogen), 2 µM of each primer, 0.2 µl Superscript™ One-Step RT-PCR with Platinum *Taq* (Invitrogen), 1 µl DNase treated RNA and sufficient 18 Mohm water to bring the total reaction volume to 20 µl. Cycling

conditions used were: cDNA from RNA at 50°C for 30 minutes, 3 minutes denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (30 sec), annealing at 65°C (30 sec), extension at 72°C (2 minutes), with a final extension at 72°C for 5 minutes. A positive control, a no-sample and a no-RT enzyme negative control were included in each RT-PCR. Amplicons were visualised by electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 µg ml^{-1}, and their size measured using a 100 bp marker (Advanced Biotechnologies).

Quality of nucleic acids

Once the vacuum extraction protocol was formulated, the quality of the resulting nucleic acids was determined. Quality was assessed firstly by the 260/280nm absorbance ratio using a Genequant DNA spectrophotometer (Pharmacia Biotech, Cambridge, UK) and secondly the production of a PCR amplicon.

In order to relate the concentration of DNA with the amount of bacteria added to the vacuum extraction system, decimal dilutions of the target organisms were prepared in selective-enrichment media: HK3C for *A. salmonicida*, CORT for *L. garvieae*, POSI for *T. maritimum* and POST for *Y. ruckeri* (Carson *et al.* 2001b) and viable counts of the suspensions were determined by the Miles and Misra method (Miles *et al.* 1938) using SBA or MSA as appropriate and incubating the plates for 48 hours at 25°C.

6.2. Determine optimum conditions for the PCR test to maximise specificity and sensitivity of the procedure.

6.2.1. Optimisation of PCR and RT-PCR sensitivity without compromising specificity.

Optimisation of existing PCR protocols to obtain maximum sensitivity without compromising specificity was undertaken with the reference DNA obtained in Method 6.1.1.

PCR optimisation was achieved in three ways, firstly by finding the most sensitive *Taq* polymerase. The different enzymes tested were: HotStar *Taq* polymerase (Qiagen); Red Hot DNA polymerase (Advanced Biotechnologies) and Platinum *Taq* DNA polymerase (Invitrogen). Secondly by varying the concentration of magnesium chloride in the PCR

reaction and thirdly by altering the PCR cycling conditions such as annealing temperatures and cycle lengths to suit each bacterium. To test the effectiveness of these variations decimal dilutions of the reference DNA (or RNA) were prepared in sterile 18 Mohm water and PCR (or RT-PCR) was performed until maximum sensitivity was achieved without compromise to specificity. Specificity was assessed at two levels; firstly, using phenotypically similar bacteria, near related species and bacteria likely to be isolated alongside the target bacterium and secondly, using bacteria identified as having some

Table 6.1   Bacteria used for testing specificity of _Aeromonas salmonicida_ PCR and RT-PCR.

Species	Strain no.	Homology[a]	Species	Strain no.
Genotypically similar and related species			**Other relevant species**	
Aeromonas salmonicida	93/0956-2	100%	Vagococcus salmoninarum	NCFB 2777[T]
A. salmonicida	84/09062-B13[b]	100%	Yersinia ruckeri serotype O1b	90/ 3988
A. sobria	ATCC 43979[T]	100%	Escherichia coli	ATCC 25922
A. eucrenophila	ATCC 23309[T]	94%	Tenacibaculum maritimum	NCIMB 2154[T]
A. jandaei	ATCC 49568[T]	94%	Hafnia alvei	95/6404
A. schubertii	ATCC 43700[T]	94%	Proteus rettgeri	96/5494
A. veronii bv sobria	ATCC 9071[T]	94%	Y. intermedia	92/4041
A. veronii bv veronii	ATCC 35624[T]	94%	Aeromonas sp	NF 1[d]
A. hydrophila	ATCC 7966[T]	85%	Aeromonas sp.	NF 2[d]
A. hydrophila	ATCC 7965	85%	Pseudomonas sp.	NF 3[d]
A. hydrophila	UTS 67[c]	85%	Enterobacter sp.	NF 4[d]
A. bestiarum	ATCC 14715	85%		
A. caviae	ATCC 15468[T]	76%		
A. trota	ATCC 49657[T]	76%		
A. media	ATCC 33907[T]	75%		

[a] % homology is in respect of the primer of closest match.
[b] Atypical _A. salmonicida_ isolated from goldfish in Victoria, N. Gudkovs, Australian Fish Disease Laboratory, Australian Animal Health Laboratory, CSIRO, Australia.
[c] Dam water, J. Oakey, University of Technology, Sydney, Australia.
[d] Fish normal flora isolated from Atlantic salmon.

Table 6.2   Bacteria used for testing specificity of _Lactococcus garvieae_ PCR and RT-PCR.

Species	Strain no.	Homology[a]	Species	Strain no.
Genotypically similar and related species			**Other relevant species**	
Carnobacterium piscicola	ATCC 35586[T]	76%	Vagococcus salmoninarum	NCFB 2777[T]
Flavobacterium columnare	NCIMB 2248[T]	71%	Yersinia ruckeri serotype O1b	90/3988
Phenotypically similar			Aeromonas salmonicida	93/0956-2
Streptococcus sp.	CORT 1[c]		Escherichia coli	ATCC 25922
Streptococcus sp.	CORT 2[c]		A. hydrophila	ATCC 7966[T]
Lactococcus piscium	NCFB 2778[T]			
Streptococcus iniae	95.41693/4A[b]			
Enterococcus faecalis	ATCC 29212			

[a] % homology is in respect of the primer of closest match.
[b] Isolated from barramundi, A. Thomas, Queensland Department of Primary Industries, Australia.
[c] Fish normal flora isolated from rainbow trout.

Table 6.3   Bacteria used for testing specificity of *Yersinia ruckeri* PCR and RT-PCR.

Species	Strain no.	Homology[a]	Species	Strain no.
Genotypically similar and related species			Other relevant species	
Yersinia pseudotuberculosis	96/5417-2	96%	*Proteus rettgeri*	96/5494
Y. enterocolitica	96/5440-1B	87.5%	*Vibrio anguillarum*	85/3475-1
Escherichia coli	ATCC 25922	87.5%	*Carnobacterium piscicola*	ATCC 35586[T]
Aeromonas salmonicida	93/0956-2	87.5%	*Vagococcus salmoninarum*	NCFB 2777[T]
Haemophilus influenzae	ATCC 33391[T]	68%	*Lactococcus garvieae*	ATCC 49156[T]
Y. intermedia	92/4041		*Pseudomonas* sp.	NF 3[b]
Phenotypically similar			*Enterobacter* sp.	NF 4[b]
Citrobacter freundii	90/2624-18	87.5%		
Hafnia alvei	95/6404	87.5%		

[a] % homology is in respect of the primer of closest match.
[b] Fish normal flora isolated from Atlantic salmon.

Table 6.4   Bacteria used for testing specificity of *Tenacibaculum maritimum* PCR and RT-PCR

Species	Strain no.	Homology[a]	Species	Strain no.
Genotypically similar and related species			Other relevant species	
Cytophage marinoflava	ACAM 75	65%	*Flavobacterium columnare*	NCIMB 2248[T]
Tenacib. ovolyticum	NCIMB 13127[T]		*F. johnsoniae*	ATCC 17061[Co-T]
Phenotypically similar			*Vibrio splendidus* biovar I	ATCC 25914[T]
FCLB mucoid	89/2244-9[b]		*V. ordalii*	ATCC 33509[T]
FCLB mucoid	89/2756-1[b]		*V. anguillarum*	85/3475-1
FCLB mucoid	96/5171[b]		*Aeromonas salmonicida*	93/0956-2
FCLB mucoid	CRC-2[b]			

[a] % homology is in respect of the primer of closest match.
[b] FCLB: Unidentified *Flexibacter-Cytophaga*-like normal flora isolated from Atlantic salmon.

genotypic similarity in respect to the specific 16S rRNA primer sequence as identified by Carson (1998). The bacteria tested for each of the four pathogens are listed in Tables 6.1 to 6.4.

6.2.2. Optimisation of the selective-enrichment (SEC) vacuum extraction-PCR protocol.

Selective-enrichment media (Carson *et al.* 2001b) were inoculated, and incubated overnight at 25°C. A faintly turbid suspension (approximately 1×10^5 cells ml^{-1}) was made from an overnight broth culture, and decimal dilutions were prepared using the selective-enrichment medium as diluent. Just prior to DNA extraction, viable counts of the suspensions were determined by the Miles and Misra method (Miles *et al.* 1938) using SBA or MSA as appropriate; plates were incubated for 48 hours at 25°C. Bacterial DNA from each of the decimal dilutions was immediately extracted as described in Method 6.1.3. and the DNA amplified by PCR.

After vacuum extraction of nucleic acids from the selective-enrichment media, sensitivity of the SEC-vacuum extraction–PCR protocol was optimised by determining the optimum template volume. Template volumes from 1 to 9 µl were added to the PCR and optimum volume determined by observing the band intensity produced by electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 µg ml⁻¹. Improved PCR sensitivity by increasing template volume was assessed using elution buffers with and without EDTA and at different TRIS concentrations. Agents known to decrease the effect of PCR inhibitors were also trialed including α-casein (Boom *et al.* 1999) and BSA (Kreader 1996; Yu & Mohn 1999).

To determine the ability of the SEC-PCR protocol to detect small amounts of the target bacterium amongst large amounts of non-target bacteria, a *Providencia* sp. was added to the selective-enrichment media just prior to extraction. A 24 hour culture of *Providencia* sp. on SBA was suspended in selective-enrichment medium to a density of about 1×10^{10} cells ml⁻¹. One hundred microlitres of this suspension was added to 900 µl of each decimal dilution containing the target bacterium, to give a final concentration of 1×10^9 cells ml⁻¹ of the *Providencia* sp. DNA from each of the decimal dilutions was extracted using Method 6.1.3. and the DNA amplified by PCR.

6.3. Optimise PCR conditions to incorporate a PCR protection system to prevent false positive reactions arising from DNA contamination.

6.3.1. Addition of Isopsoralen (IP-10) to inactivate amplicons and prevent false-positive PCR reactions.

The photochemical Isopsoralen compound 10 (IP-10) (Cerus Corporation, Ca, USA) was tested for its ability to prevent re-amplification of carried over PCR products in subsequent PCR reactions (Cimino *et al.* 1991). IP-10 was trialed in the following concentrations: 15, 20, 30, 40, 50, 100 and 200 µg ml⁻¹.

PCR technique
16S rRNA primer sets (Carson 1998) were used for the PCR assays. PCR reactions were performed without IP-10 and with IP-10 at each of the seven concentrations specified. The reaction mix contained 200 µM each of dNTPs, 1.375 mM $MgCl_2$ for *A. salmonicida* and

2 mM MgCl$_2$ in all other cases, 1 X PCR buffer (Invitrogen), 10% (v/v) glycerol, IP-10, 2 μM each of the two primers, 0.5 units Platinum *Taq* DNA Polymerase (Invitrogen), 1 μl template DNA (40 ng μl^{-1} unless specified otherwise) and sufficient 18 Mohm water to bring the total reaction volume to 20 μl. Optimum conditions for PCR cycling were: an initial 3 minutes denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (30 sec), annealing at 62°C (30 sec) for *L. garvieae* and *T. maritimum*; and 60°C for *A. salmonicida* and *Y. ruckeri*, and extension at 72°C (1 minute), with a final extension at 72°C for 3 minutes. A positive control and a negative control were included in each PCR run.

PCR product inactivation

After amplification the samples were cooled to 4°C, placed directly onto a transilluminator (Ultra-Lum, Ca, USA) screen and covered with a flexible ice brick cooled to 4°C. The samples were irradiated with UV at 300nm for 15 minutes. In subsequent studies samples were placed on the cooled ice brick and irradiated in a UV crosslinker (XL-1000 Spectorlinker, Spectroline, New York, USA) with UV at 365nm for 900 seconds at 9.5 mW/cm^2. In order to determine the effectiveness of amplicon inactivation by IP-10, PCR product was immediately re-amplified by PCR. To confirm amplification, the remaining PCR product was assayed by electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 μg ml^{-1}.

Efficiency of inactivation

A 1 μl sample of the IP-10 treated PCR product was used as a template for subsequent PCR performed with no IP-10 in the reaction mix. A second 1 μl sample was diluted 1:100 and used to create three 10-fold dilutions of the PCR product. The resulting dilutions were: neat ($\approx$6 x 10^9 amplicons), 10^{-2} ($\approx$6 x 10^7 amplicons), 10^{-3} ($\approx$6 x 10^6 amplicons), 10^{-4} ($\approx$6 x 10^5 amplicons) and 10^{-5} ($\approx$6 x 10^4 amplicons). The presence or absence of amplified product was determined using electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 μg ml^{-1}.

Effect of IP-10 on PCR sensitivity

Reference DNA from Method 6.1.1. was used to prepare dilutions for the sensitivity assays. The DNA was diluted to 10 ng μl^{-1}, 100 pg μl^{-1}, 500 fg μl^{-1}, and then to 5 fg μl^{-1} in 10-fold dilutions, and used as a template for PCR. The sensitivity end-point was

determined by electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 µg ml^{-1}.

6.4. Develop a test procedure based on enzyme hybridization assay (EHA) that will verify any positive PCR reactions using a secondary confirmatory gene probe and in a format suitable for testing multiple samples.

6.4.1. Development of a high-throughput PCR enzyme hybridization assay (EHA) for sensitive, low cost, DNA detection.

NucleoLink^{TM} tubes (Nalge Nunc International, Naperville, IL, USA) were used to perform PCR and EHA in the one tube. In the NucleoLink system one PCR primer is bound covalently to the tube surface (this is called the solid-phase primer). This enables a biphasic PCR reaction to occur in the tube with the resulting amplicon formed free in the liquid phase and also bound to the tube surface, see Figure 6.1. The proprietary term for this process is the Detection of Immobilised Amplified Product in a One-Phase System (DIAPOPS) (Nalge Nunc International).

16S rRNA primer sets and internal probes

16S rRNA primer sets (Carson 1998) were used for the PCR assays. For each PCR-EHA the reverse primer was chosen as the solid-phase primer. Solid-phase primers were modified for use in NucleoLink tubes by addition to the 5' end of a 10 base thymidine (poly-T) linker with a terminal phosphorylation as recommended by Nalge Nunc International. The internal probes used for hybridization to the solid-phase PCR products were developed by Carson (1998) except the *A. salmonicida* probe that was described by Høie *et al.* (1997). All the probes were labelled with biotin at the time of synthesis by the manufacturer (Invitrogen or Sigma-Aldrich).

Determination of sensitivity

Test sensitivity was determined at two levels, firstly with reference target DNA (Method 6.1.1) and secondly with DNA extracted from selective-enrichment media using the vacuum method (6.1.3). For the reference DNA, serial dilutions were prepared and amplified by PCR. For the DNA from selective-enrichment media, a 0.5 McFarland

suspension of bacteria in log-phase of growth was prepared in sterile water. From this a 1:1000 dilution followed by ten decimal dilutions using the selective-enrichment media, as

NucleoLink PCR-ELPHA tube

Binding of primer

Solid phase primer

PCR Primer ratio usually 1:8 in liquid phase

Add PCR reagents

PCR amplicon attached to well

Biphasic PCR

PCR amplicon in liquid phase

Hybridization

Detection

Internal probe

AP + pNPP
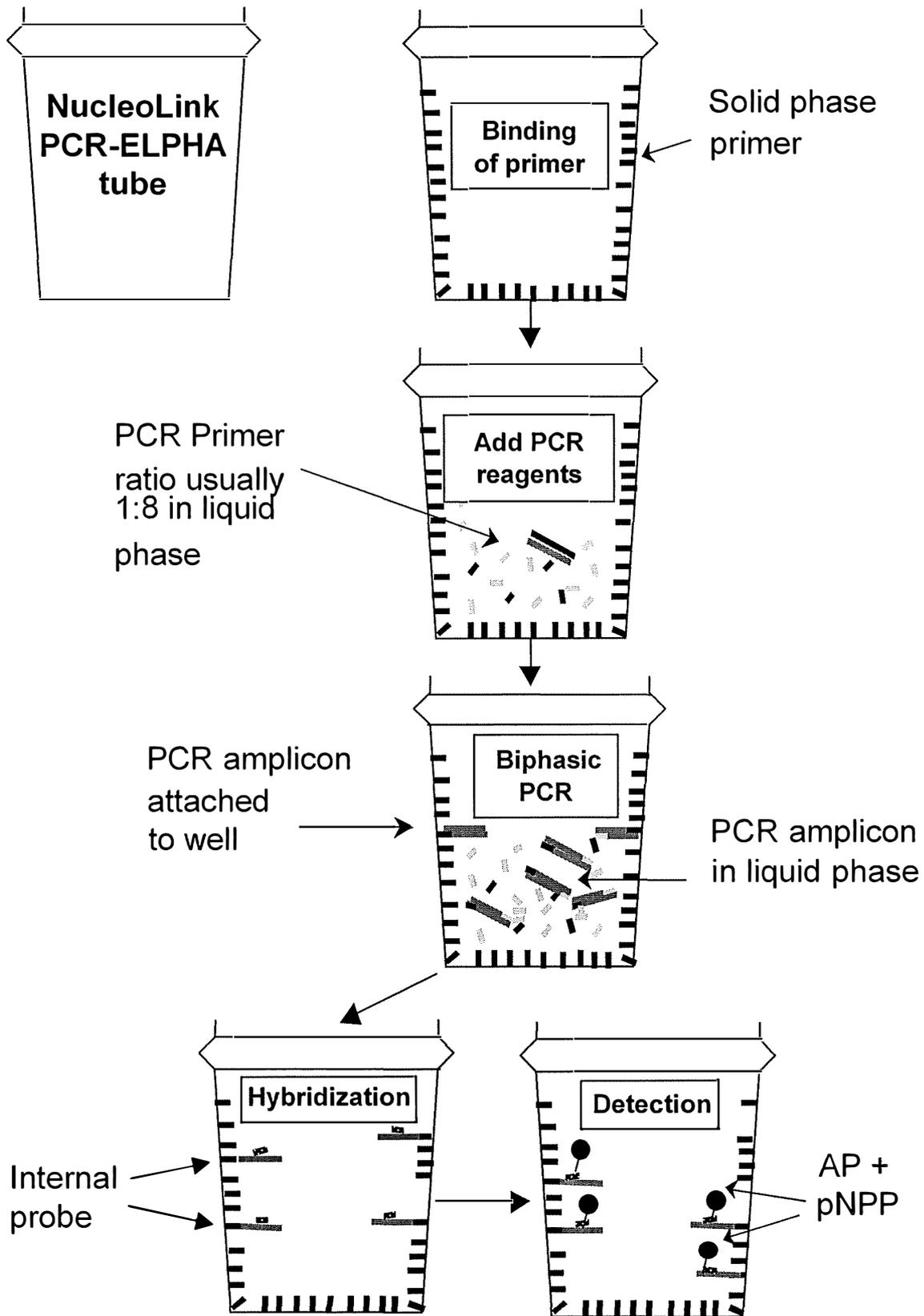
Figure 6.1   Biphasic PCR in NucleLink™ tubes.

diluent was prepared. Just prior to DNA extraction, viable counts of the suspensions were determined by the Miles and Misra method (Miles *et al.* 1938) using SBA or MSA as appropriate; plates were incubated for 48 hours at 25°C. Bacterial DNA from each of the decimal dilutions was immediately extracted and PCR-EHA performed.

Binding of solid-phase primer to micro-wells

The modified reverse primers were covalently bound to the NucleoLink strips by the carbodiimide condensation reaction (CCR) as described for Covalink NH BreakApart™ strips (Rasmussen *et al.* 1994). CCR reagent, sufficient for one strip (8 wells) was prepared by adding 1.63 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to 840 µl of 18 Mohm water, 8.5 µl of 1 M 1-methylimidazole (1-Melm) and 0.85 µl of 1000 ng µl^{-1} of solid-phase primer. NucleoLink strips were placed into a strip frame (Nunc cat. no. 249182) and a 100 µl aliquot of CCR reagent solution was added to each well. The strips were sealed with Tape 8 (Nunc cat. no. 249719) and incubated at 50°C for 5 h. After incubation, non-covalently bound primer was removed by washing three times with 150 µl of pre-warmed 0.4 M NaOH with 0.25% Tween 20. The strips were incubated at 50°C for 15 minutes followed by three more washes. The strips were then washed three times in DIAPOPS buffer (80 mM TRIS-HCl, 20 mM TRIS base, 150 mM NaCl, 0.1% Tween 20, pH 7.5), soaked for 5 minutes and then washed three more times. The wells were thoroughly emptied by tapping the strip sharply several times while upside down on a paper towel. Coated strips could be stored in a clip seal plastic bag at 4°C for up to 2 months.

Asymmetric PCR technique

NucleoLink wells were hydrated and blocked with 200 µl of DIAPOPS buffer with 10 mg/ml Fraction V BSA (prepared in RO water (<2µS) and filter sterilized through a 0.2 µm filter) at room temperature for 1 h. The PCR reaction mix contained 200 µM each of dNTPs, 1.375 mM MgCl$_2$ for *A. salmonicida* and 2 mM MgCl$_2$ in all other cases, 1 X PCR buffer (Invitrogen), 10% (v/v) glycerol, 0.1% Tween 20, 40 µg ml^{-1} IP-10 (Cerus Corporation), 0.5 µM of the forward primer and 0.0625 µM of the reverse primer for *A. salmonicida* and 2 µM of the forward primer and 0.25 µM of the reverse primer for the other three bacteria, 0.5 units Platinum *Taq* DNA Polymerase (Invitrogen), 1 µl template DNA and sufficient 18 Mohm water to bring the total reaction volume to 20 µl. Optimum conditions for PCR cycling were: an initial 3 minutes denaturation at 94°C, followed by 35

cycles consisting of denaturation at 94°C (45 sec), annealing at 62°C (45 sec) for *L. garvieae* and *T. maritimum*; and 60°C for *A. salmonicida* and *Y. ruckeri*, and extension at 72°C (45 sec), with a final extension at 72°C for 3 minutes. A positive control and a negative control were included in each PCR run.

PCR product inactivation

After amplification but before removing the tape from the sealed tubes, the strips were cooled to 4°C and then placed into a strip frame. The samples were irradiated with UV (300 nm) for 15 minutes to render the amplicons resistant to re-amplification in subsequent PCR, see Method 6.3.1. Verification of amplification was undertaken by sampling the liquid phase that contained a proportion of free unbound amplicon. The samples were assayed by electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 μg ml^{-1}.

Denaturation of bound amplicons

Bound amplicons were denatured by washing three times, soaked for 5 minutes and washing three more times in 0.2 M NaOH with 0.1% (v/v) Tween 20 added just prior to use. The wells were washed three times in DIAPOPS buffer, soaked for 5 minutes and then washed a further three times.

Hybridization with biotin labelled probes:

Denatured internal probe and salmon sperm DNA (Invitrogen) were diluted in hybridization buffer (6 x standard saline citrate (SSC), 5 x Denhardt's solution (Amresco), 0.1% Tween 20) to a final concentration of 50 nM and 100 μg ml^{-1} respectively, and 100 μl was added to each well. Hybridization was carried out at 50°C for 1 h. Unbound probe was removed by washing three times in 0.5 x SSC with 0.1% Tween 20, soaking for fifteen minutes at 50°C and then washing three more times. After washing, the wells were thoroughly emptied by sharply tapping the strip several times while upside down on a paper towel.

Colorimetric detection of labelled probes

The conjugate streptavidin-alkaline phosphatase (Promega) was diluted 1:2000 in DIAPOPS buffer and 100 μl was added to each well. The strips were incubated at 37°C for 1-2 h. After incubation the wells were washed three times, soaked for 5 minutes and

washed three more times with DIAPOPS buffer. Then, 100 µl of 10 mg ml⁻¹ p-nitrophenylphosphate (Sigma-Aldrich) diluted in 1 M diethanolamine containing 1 mM MgCl$_2$ (pH 9.8) was added to each well. Colour development was allowed to proceed for 60 minutes in the dark at room temperature and OD readings were taken in an ELISA plate reader at 405 nm. If borderline positive-negative OD readings were obtained (values of about 1.2 times the negative control after 60 minutes), the reaction was allowed to proceed for up to 18 hours. After this time, samples with absorbance readings of at least 1.4 times the negative control corresponded to samples that contained template DNA. If required the EHA reaction was stopped by adding 100 µl of 1 M NaOH to each tube.

6.4.2. Development of a high-throughput RT-PCR enzyme hybridization assay (EHA) for sensitive, low cost, detection of rRNA.

The PCR technique described is based on detection of genomic DNA that is a very stable and enduring molecule. Detection of DNA provides evidence that the target pathogen is present but does not provide *prima facie* evidence that there is live infection even though a precursor culture step is used. Ribosomal RNA is a considerably more labile nucleic acid with a life span of only a few days. Detection of this molecule provides much stronger evidence that the target pathogen at the time of detection is an intact and viable organism. Amplification of RNA uses reverse transcriptase to make copy DNA that is then amplified by conventional PCR. The RT-PCR-EHA method followed that outlined in Method 6.4.1. in every respect, except for the asymmetric PCR, which was modified, and the IP-10 control system was not used. The RT-PCR technique was as follows:

RT-PCR technique

NucleoLink wells were hydrated and blocked with 200 µl of DIAPOPS buffer with 10 mg ml⁻¹ fraction V BSA (prepared in DEPC treated RO water (<2µS) and filter sterilized through a 0.2 µm filter) at room temperature for 1 h. The RT-PCR reaction mix contained 200 µM each of dNTPs, 1.375 mM MgSO$_4$ for *A. salmonicida* and 2 mM MgSO$_4$ in all other cases, 1 X RT-PCR buffer (Invitrogen), 0.1% DEPC treated Tween 20, 3 µM of each primer for *T. maritimum*, 2 µM each primer for *L. garvieae* and 2 µM forward primer and 0.25 µM reverse primer for *A. salmonicida* and *Y. ruckeri*, 0.2 µl Superscript™ One-Step RT-PCR with Platinum *Taq* (Invitrogen), 3 µl DNase treated RNA and sufficient 18 Mohm water to bring the total reaction volume to 20 µl. Optimum conditions for PCR cycling

were: cDNA from RNA at 50°C for 30 minutes followed by a 3 minutes denaturation at 94°C, followed by 35 cycles consisting of denaturation at 94°C (45 sec), annealing at 62°C (45 sec) for *L. garvieae* and *T. maritimum*; annealing at 60°C (45 sec) for *Y. ruckeri* and *A. salmonicida*, extension at 72°C (45 sec), with a final extension at 72°C for 5 minutes. A positive control, a no sample and a no-RT enzyme negative control were included in each RT-PCR run.

## 6.5.	Optimise the culture conditions and PCR detection process to ensure the minimum test time between sample collection and test result.

6.5.1. Minimum Inhibitory Concentration (MIC) assays towards the development of a selective-enrichment medium for *Aeromonas salmonicida* biovar *acheron*.

A new selective-enrichment medium, HK3C, for *A. salmonicida* biovar *acheron* was developed as the versions for the goldfish and greenback flounder biovars of *A. salmonicida* were found to be inhibitory for this biovar. Information obtained when developing the formulations for goldfish and flounder selective-enrichment media together with new Minimum Inhibitory Concentration (MIC) data for a range of potentially useful antibacterials, see Table 6.5, provided the framework for formulating a

Table 6.5	Antibacterial agents used in the development of HK3C,
a selective medium for *A. salmonicida* biovar *acheron*.

Antibacterial agents	
cis/trans citral	Alexidine
Citronellal	Amoxicillin
Eugenol	Ampicillin
Geraniol	Compound C1
Geranium oil	Compound C2
Lavendulol	Compound C3
Lonone	Colistin
Nerol	Doxycycline
Palmarosa oil	Compound H
β-Pinene	Polymyxin B
R+ -Limonene	Phosphomycin
α-Terpinene	Piromidic acid
α-Terpineole	Compound K
Ofloxacin	Sulphaquinonaline
8-azaguanine	Sulphaquinoxaline
Thymol	Trimethoprim

medium suitable for *A. salmonicida* biovar *acheron*. Bacteria used in the development of the medium were chosen because they had resistance profiles similar to *A. salmonicida*.

These strains had been identified in earlier studies or were isolated during field evaluation of HK3C, Table 6.6.

Table 6.6   Bacteria used for developing HK3C, the selective-enrichment medium for *A. salmonicida* biovar *acheron*.

Tested bacteria	
(B7) *Pseudomonas* sp.	(B11) *Aeromonas* sp.
(B56) *Pseudomonas* sp.	(D32) *Vibrio* sp.
(B91) *Pseudomonas* sp.	(D33) *Vibrio* sp.
(B127) *Pseudomonas* sp.	(B109) *Flavobacterium* sp.
(B128) *Pseudomonas* sp.	(M128) *Flavobacterium* sp.
(M146) *Pseudomonas* sp.	(B9) *Shewanella* sp.
(G49) *Pseudomonas* sp.	(B6) *Enterobacter* sp.
(FT1) *Pseudomonas* sp.	(M147) *Enterobacter* sp.
(W57) *Pseudomonas* sp.	*Proteus* sp.
(W43) *Aeromonas* sp.	*A. hydrophila*

The sensitivity data required for the development of HK3C was obtained using a broth dilution MIC method, based on the National Committee for Clinical Laboratory Standards (NCCLS) method (Sahm and Washington 1991). While concentrations varied depending on the antimicrobial, a typical dilution method is demonstrated using an antimicrobial of 1000 µg ml^{-1} stock concentration to produce a 25 µg ml^{-1} concentration in the first row of a microtitre tray. The antimicrobial was diluted 1:20 in basal medium (modified Brain Heart Infusion Medium) (Appendix) to give a concentration of 50 µg ml^{-1}. Doubling dilutions were prepared as tabled:

Column Number	Initial Concentration (µg ml^{-1})	Final Concentration (µg ml^{-1})
1	50.0	25.0
2	25.0	12.5
3	12.5	6.25
4	6.25	3.13
5	3.13	1.56
6	1.56	0.78
7	0.78	0.39
8	0.39	0.20
9	0.20	0.10
10	0.10	0.05
11	0.05	0.02
12	Control	Control

Young actively growing cultures of the bacterium in the early stage of logarithmic growth were used for the assays (Baker, *et al.* 1983). Suspensions of the test organism were prepared in the basal medium to McFarland standard 0.5 using a Hach Turbidimeter

Model 2100P (Hach, Colarado, USA). This suspension was then diluted 1:100 in basal medium.

Antimicrobial dilutions were made in racked Micronic tubes using 250 µl volumes. Fifty microlitres of each dilution and 50 µl of the test organism suspension were transferred into a microtitre tray using a multi-channel pipette. The microtitre tray was sealed using self-adhesive plastic film sealer (ICN, Ca, USA, cat. no. 77-420-00). The plates were incubated at 25°C until growth (turbidity) could be clearly seen in the control wells. Growth or no-growth was recorded for each organism. The endpoint (MIC) was taken as the lowest concentration showing no growth visible to the naked eye (Waterworth 1978), although for chemotherapy, light haziness in the endpoint well is often acceptable (Turnidge and Stockman 1991). For developing a selective medium however, any growth was considered significant since it yields a large number of colonies if sub-cultured on agar. In addition to identifying MIC, a further parameter was defined as the product limiting concentration (PLC) which was the last well with a turbidity equal to the growth in the control well and signified the concentration of antimicrobial that began to limit growth. The PLC would normally have a concentration of antimicrobial agent less than the MIC. The MIC and PLC were recorded as the concentration of antimicrobial in $\mu g \ ml^{-1}$.

6.5.2. Chequerboard MIC assays towards the development of HK3C a selective-enrichment medium for *Aeromonas salmonicida* biovar *acheron*.

To check for combined antibacterial effects such as antagonism or synergy, chequerboard MIC assays were conducted with those antibacterials that showed potential as ingredients in a selective-enrichment medium. The method is demonstrated with two antibacterial agents H and K.

A 100,000 $\mu g \ ml^{-1}$ stock solution of H was prepared in RO water (<2µS). An aliquot of this stock was diluted 1:16 in modified BHI to give a concentration of 6240 $\mu g \ ml^{-1}$. A 1000 $\mu g \ ml^{-1}$ stock concentration of K was prepared in RO water (<2µS). An aliquot of this stock was diluted 1:80 in modified BHI to give a concentration of 12.5 $\mu g \ ml^{-1}$. From these dilutions, 250 µl of the following doubling dilutions were prepared in Micronic tubes as tabled:

Compound H:

Column Number	Initial Concentration ($\mu g\ ml^{-1}$)	Final Concentration ($\mu g\ ml^{-1}$)
1	6240	1560
2	3120	780.0
3	1560	390.0
4	780.0	195.0
5	390.0	97.50
6	195.0	48.75
7	97.50	24.38
8	48.75	12.19
9	nothing added	nothing added
10 (Row A)	(H)6240	(H)3120
11 (Row A)	(K)12.50	(K)6.250

Compound K:

Row Number	Initial Concentration ($\mu g\ ml^{-1}$)	Final Concentration ($\mu g\ ml^{-1}$)
A	12.50	3.125
B	6.250	1.563
C	3.125	0.781
D	1.563	0.391
E	0.781	0.195
F	0.391	0.098
G	0.195	0.049
H	0.098	0.024

Fifty microlitres of each antimicrobial dilution was transferred to a microtitre tray a shown in Figure 6.2.

Young, actively growing cultures of *A. salmonicida* biovar *acheron,* in the early stage of logarithmic growth, were used for the assays (Baker, *et al.* 1983). Suspensions of the test

Conc. K		Concentrated --------- H ----------→ dilute									H 10	K 11	Control 12
		1	2	3	4	5	6	7	8	9			
↓	A										Conc.	Conc.	
↓	B										↓	↓	
↓	C										↓	↓	
↓	D										↓	↓	
↓	E										↓	↓	
↓	F										↓	↓	
↓	G										↓	↓	
Dilute	H										Dil.	Dil.	

Figure 6.2   Chequerboard microtitre tray layout. Compound H; Compound K

organism were prepared in modified BHI to a density of McFarland 0.5, using a Hach Turbidimeter. This suspension was then diluted 1:100 in modified BHI, and 50 µl was added to each well of the microtitre tray. The microtitre tray was sealed using self-adhesive plastic tape (ICN). The microtitre trays were incubated at 25°C for 3 days and growth or no-growth was recorded for each well. From the growth patterns, the highest concentration of antibacterial in combination that permitted growth of the target pathogen could be determined. Typically two values are obtained, the first with antibacterial A high and B low and in the second antibacterial A low and B high. The optimum concentrations selected was based on using the highest concentrations possible and ensuring there were no adverse interactions arising from interference between the antibacterials.

6.5.3. Most Probable Number (MPN) assays to determine the effect of the selective ingredients on the growth of *A. salmonicida* biovar *acheron*.

To quantify the inhibitory effect of the antibacterial agents a Most Probable Number (MPN) comparison was made between growth in the basal medium and growth in the selective medium HK3C. The MPN method is a means for estimating, without any direct count, the density of organisms in a liquid (Cochran 1950). The MPN was performed in a microtitre tray following the method of Wilson (1996) as summarised below.

A. salmonicida biovar *acheron* medium HK3C and the modified BHI basal medium were each dispensed as 200 µl volumes into half of a microtitre tray using a multistepper pipette. *A. salmonicida* biovar *acheron* was grown in pure culture and from this a McFarland standard 0.5 suspension was prepared using a Hach Turbidimeter and the suspension then diluted by 10^{-5}, to form the stock. A total of six samples were taken from the stock, each sample with a different volume reducing in size by a factor of five for each volume. So that measurable volumes could be used for each sample, the stock suspension was diluted ten fold, twice, by the addition of a volume of uninoculated medium, a system of dilution by addition. The system of inoculation is summarised in Figure 6.3. Five rows of the microtitre tray were used for replicates. Additions, as described, were made for the selective medium and the corresponding basal medium. The plates were sealed using microtitre tray self-adhesive tape.

From stock		From stock diluted 10^{-1}		From stock diluted 10^{-2}		From stock		From stock diluted 10^{-1}		From stock diluted 10^{-2}	
↓		↓		↓		↓		↓		↓	
1	2	3	4	5	6	7	8	9	10	11	12
10μl	5μl	10μl	5μl	10μl	5μl	10μl	5μl	10μl	5μl	10μl	5μl
HK3C						Basal medium					

Figure 6.3   Row A of a microtitre tray depicting volumes used for the MPN dilution series.

The microtitre tray was incubated at 25°C and read at 24 hour intervals for up to seven days. Turbidity in the wells indicated growth and when the same results were read on two consecutive days the final results were recorded.

Statistical Analysis

The Most Probable Number method (McCrady 1915) was used to estimate the density of bacteria in the standard suspension from which could be derived an estimate of the level of inhibition between the basal and corresponding selective medium. The method assumes:

(1) each sample exhibits growth if it contains at least one organism;

(2) the distribution of organisms is random without aggregation of any kind.

The MPN analysis was generated for the number of positive wells obtained for selective and non-selective media using an effective and flexible DOS based computer program by González (1996). Student's t tests were then performed on the data to test for significant difference of estimates (Koopmans 1987).

6.5.4. Evaluation of the capacity of *A. salmonicida* biovar *acheron* to grow in HK3C with competition.

No selective medium is perfect, and some non-target bacteria will always tolerate the antibacterial agents and grow in the medium. Growth characteristics vary enormously between different bacteria and it is possible that a fast growing resistant non-target species could out-compete the target bacterium, thereby limiting the target bacterium's ability to multiply to a level detectable either by culture or by PCR. Growth rates were

assessed by placing together the target bacterium and a known vigorous resistant bacterium in HK3C and monitoring the growth of each bacterium.

Two resistant isolates, *Pseudomonas* sp. (B127) and a mucoid *Pseudomonas* sp. (M146) were used for the study. Both the target organism and the competitor were prepared to a density of McFarland 0.5 and diluted 1:200 to achieve a final concentration of 5×10^{-4} cells ml^{-1} in 10ml of HK3C. At time intervals of 24 hours, 3 days and 5 days, viable counts were made using the Miles and Misra plate count method (Miles *et al.* 1938) to assess the relative proportions of target and competitor. A numerical value for the ability of the target bacterium *A. salmonicida* to grow in competition was calculated using the formula of Rhodes *et al.* (1985) where:

$$EI = \log_{10} EF \quad \text{and} \quad EF = \frac{ER}{IR} \quad \text{and}$$

ER = <u>Viable concentration of target pathogen after enrichment</u>
Viable concentration of competitor after enrichment

IR = <u>Initial viable concentration of target pathogen</u>
Initial viable concentration of competitor

The enrichment index, EI, is a measure of the change in relative concentration of the target organism compared with the competitor over the enrichment period. The larger the EI value the greater the enrichment of the target pathogen compared with the competing bacterium.

6.5.5. Validation of the high-throughput SEC-PCR system.

The 96-well vacuum system and PCR were checked for well-to-well cross-contamination, speed, ease of high-throughput processing, and sample evaporation during PCR. POST selective-enrichment medium seeded with *Y. ruckeri* was added to alternate wells of a glass microfibre filter plate and the DNA extracted (Method 6.1.3). PCR was performed in previously validated thin-walled PCR tubes (Scientific Specialties Inc., Ca, USA) (Method 6.2.1) and in 96-well PCR trays (Scientific Specialties). The results were visualised by electrophoresis in TAE buffer on a 2% (w/v) agarose gel with ethidium bromide at a final concentration of 0.5 µg ml^{-1}.

6.6. Test populations of salmonids with the optimised SEC-PCR-EHA system to verify test performance and obtain baseline data on carrier prevalence.

6.6.1. Trials with fish to test the dynamics of high-throughput SEC-PCR.

The dynamics of the high-throughput system were also evaluated and optimised by performing trials with fish. These fish trials were conducted before development of the SEC-PCR-EHA had been completed. Initial fish trials were conducted from a farm with a history of *Y. ruckeri* infection. A volume of 2 L of POST, selective-enrichment medium for *Y. ruckeri* was prepared and dispensed into 10 ml volumes in sterile glass bottles. One 10 ml volume was inoculated very lightly with the target organism and incubated at 25°C overnight to check for growth in the medium. One hundred and fifty fish were lightly sedated and faecal samples collected by abdominal massage; the fish were revived and returned to their production tank. The collected faeces were placed into individual volumes of POST medium. Thirty of these fish were killed by anaesthetic overdose and spleen samples were placed into a volume of the medium. All samples were then incubated at 25°C, the DNA extracted after 5 days (Method 6.1.3), and PCR performed on the extracted DNA. Positive PCR reactions were checked for cell viability by sub-culture from the selective medium. A second *Y. ruckeri* fish trial was conducted two months later. Faecal samples were collected from 150 fish and enriched in POST. DNA was extracted from the enrichment cultures after 2 and 5 days incubation.

Following a disease outbreak at a NSW rainbow trout farm, 133 fish were tested for *L. garvieae* carriage. For these fish 2800 ml of CORT selective enrichment medium was prepared and dispensed into 10 ml volumes in sterile glass bottles. One volume was inoculated very lightly with the target organism and incubated at 25°C overnight to check for growth in the medium. Spleen and brain samples were collected aseptically, placed in individual volumes of selective medium and incubated at 25°C for 5 days. DNA was then extracted using the vacuum extraction method (Method 6.1.3) and tested by PCR for the presence of *L. garvieae*. All enrichment cultures were subcultured to test for the presence of viable *L. garvieae* and correlated with the PCR findings.

6.6.2. Validation of the selective-enrichment PCR enzyme hybridization assay (SEC-PCR-EHA) and RT- SEC-PCR-EHA systems with field samples.

Validation protocol

Validation of a new technique should follow a recognised protocol. However, practical issues such as context, availability of equipment and animals, time and cost need to be considered. The protocol described in Figure 6.4. is the validation protocol followed here. This protocol is a mixture of the validation protocol described for DNA-based bacterial detection techniques by Hiney and Smith (1998) and suggestions made by Dr Chris Baldock (Ausvet, pers. com.).
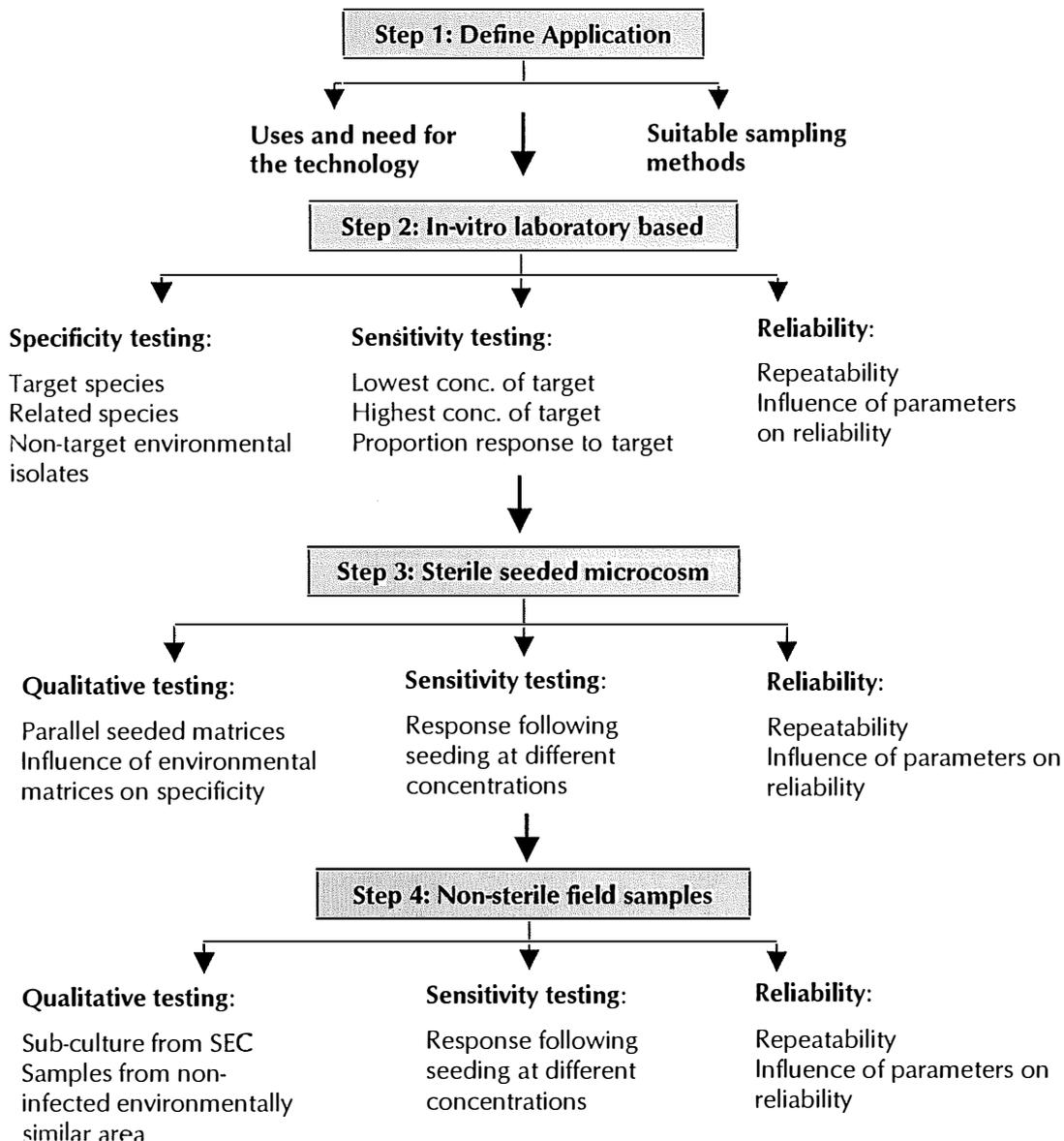
Figure 6.4   Validation protocol for SEC-PCR-EHA. Adapted from Hiney and Smith (1998)

Steps 1-3 of the validation protocol were completed during fulfilment of project Objectives 1 to 5. In order to complete step 4 SEC-PCR-EHA was performed on farmed salmonid fish. Figure 6.5. gives a diagrammatic explanation of SEC-PCR-EHA, and the procedure follows this example: For 100 fish, 750 ml of selective-enrichment medium was
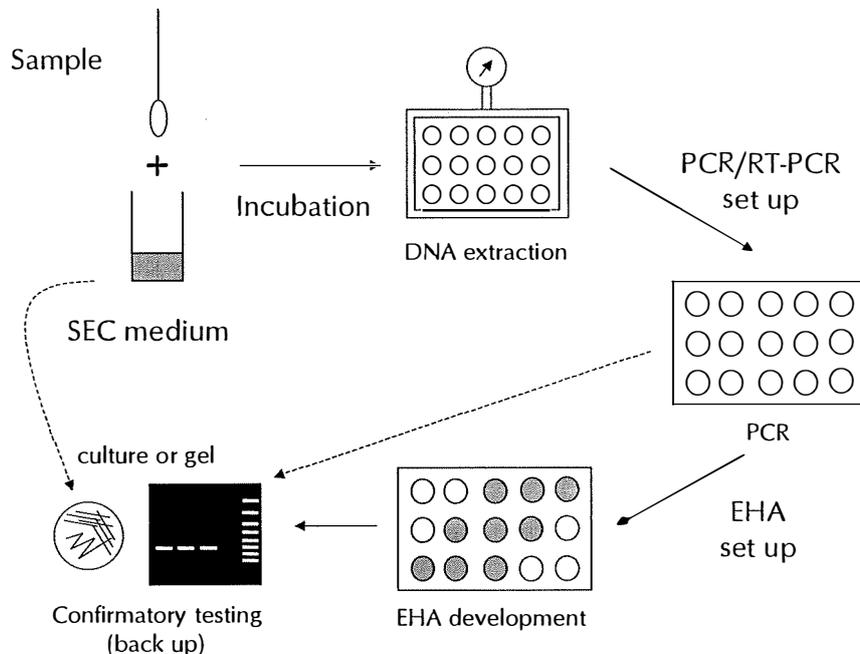
Figure 6.5   Schematic diagram of the SEC-PCR-EHA test system.

prepared and aseptically dispensed into 7 ml volumes in sterile plastic tubes. One 7 ml volume was inoculated very lightly with the target organism and incubated at 25°C overnight to check for growth in the medium. The medium was stored at –20°C until required for a maximum period of three months (unpublished data). When sampling mucus or faeces (*T. maritimum*, *A. salmonicida* and *Y. ruckeri*) the medium was couriered chilled to the farm where sterile swabs were used for non-destructive sampling of lightly sedated fish. For *L. garvieae*, spleen or brain was required, and the fish were sampled in the laboratory. After sampling, the selective-enrichment media were incubated at 25°C for 5 days. After this time, vacuum extraction of nucleic acids (Method 6.1.3), and PCR-EHA (Method 6.4.1) was performed on the samples. In at least one trial for each of the target pathogens, 7 test negative samples were seeded with decimal dilutions of the target bacterium. Bacterial counts by the Miles and Misra method (Miles *et al.* 1938), vacuum extraction and PCR-EHA were then undertaken to check for any inherent inhibitors that may have been present in field derived samples and have caused false negative reactions.

Any DNA PCR positive reactions were tested for live bacterial carriage by RT-PCR-EHA (Methods 6.4.2), and by sub-culture from the selective-enrichment medium.

The number of fish used to validate the system was calculated using the software package Win Episcope 2.0 (de Blas *et al.* 2000). A population size of 10 million fish was assumed and a 95% confidence level was used. Since prevalence data is unavailable for these path-

Table 6.7   Win Episcope 2.0 sample data, maximum number of fish required to obtain a prevalence value for *A. salmonicida*.

Population size	10,000,000
Expected prevalence (%)	50
Sample size	293
Level of Confidence (%)	95
Error (%)	5.73
If the anticipated prevalence is less than the expected value, the true prevalence will be between (44.27-55.73%) given that diagnosis is perfect.	

-ogens and this information is likely to vary greatly depending on environmental factors, where possible an expected prevalence of 50% was assumed for each fish population. As prevalence was unknown for the populations under test, prevalence was assumed to be 50% which ensures a maximum sample size was used and so resulting in better precision (Cameron 2002) of the estimate. The sample data used for the first *A. salmonicida* fish trial serves as an example of the statistical data, see Table 6.7.

Where possible fish trials were conducted at farm sites with recent history of disease, as it would be impossible to effectively validate a system if no positive samples occurred. For the *L. garvieae* SEC-PCR-EHA system full validation was not possible as the pathogen has not been detected in Tasmania since 1991. In order to fulfil the qualitative requirements of step 4, *ie.* validate PCR-EHA positive but culture negative results, disease free fish were sampled at sites with environmental factors close to those found at infected sites. An expected prevalence of 10% with an increased level of confidence (97.5%) was used for these studies.

Sample type

Samples were collected for testing based on procedures used by Carson *et al.* (2001b) which had identified optimal sample types for use with these selective enrichment media.

The details are summarised in Table 6.8. Nearly all the samples required are non-destructive.

Table 6.8   Sample types required for SEC-PCR-EHA testing

Pathogen	Sample	Type
A. salmonicida	skin mucus	non-destructive
L. garvieae	spleen/brain	destructive
T. maritimum	skin mucus	non-destructive
Y. ruckeri	faeces	non-destructive

7. RESULTS

7.1. Develop a procedure for extracting bacterial DNA from selective enrichment media that is suitable for PCR and for processing multiple samples.

7.1.1. Large scale extraction of DNA for use as a reference in determining PCR sensitivity and as a positive control.

Approximately 5 ml of high quality, concentrated DNA was produced for each bacterium. In Figure 7.1A, gel electrophoresis shows a strong clean band of largely un-sheared, high mass DNA extracted from *Y. ruckeri*; in 7.1B, the result of RNase treatment is shown. A similar quality of DNA was achieved for the other three bacteria. The concentration of DNA for each bacterium is given in Table 7.1.

[A]   [B]
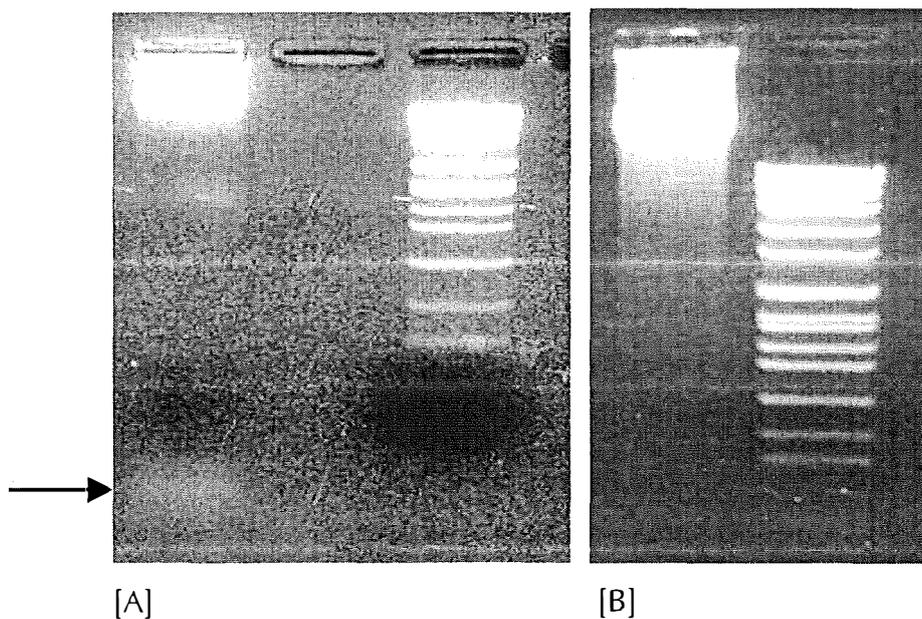
Figure 7.1 **1% agarose gel electrophoresis of *Y. ruckeri* DNA: [A] before RNase treatment, arrow shows RNA [B] after RNase treatment. Marker: SPP-1, 0.36-8.51 kb**

Table 7.1 **Concentration of DNA produced from large-scale DNA extraction.**

Bacterium	DNA concentration
Aeromonas salmonicida	111 ng μl^{-1}
Yersinia ruckeri	146 ng μl^{-1}
Lactococcus garvieae	670 ng μl^{-1}
Tenacibaculum maritimum	685 ng μl^{-1}

7.1.2. Evaluation of 96-well vacuum extraction units.

Two 96-well glass microfibre and vacuum manifold systems were evaluated, see Table 7.2. The Whatman Polyfiltronics filters were better than those from Millipore as they could handle larger sample volumes and were more efficient eluting small quantities of DNA, see Figure 7.2.
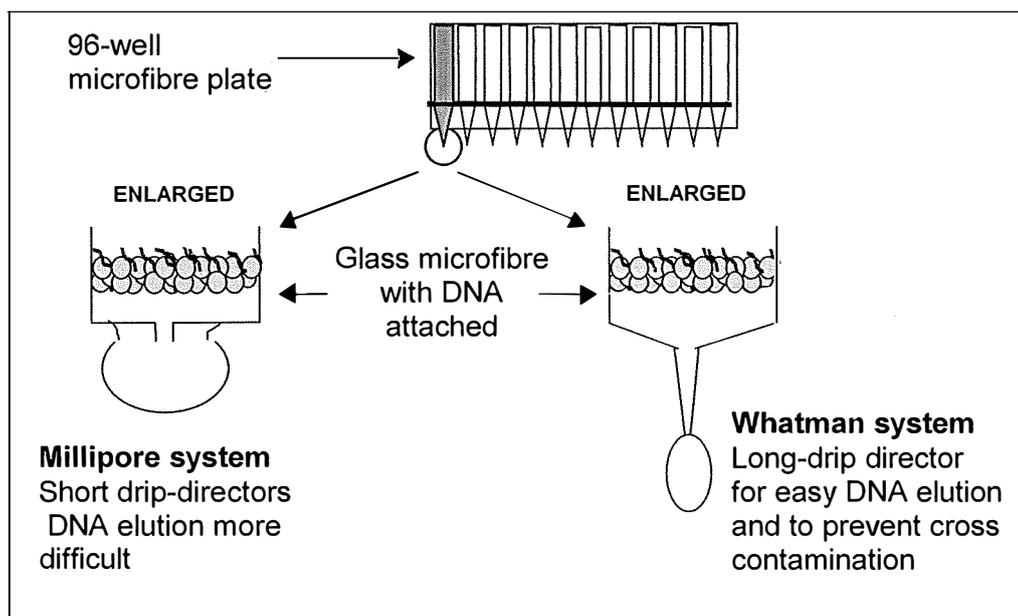
Figure 7.2   Difference in the elution conditions for Millipore and Whatman glass microfibre filter plates.

7.1.3. Extraction of genomic DNA and ribosomal RNA using UNIVAC manifold and Whatman GF/B 96-well filter plates.

Extraction of nucleic acids using the glass microfibre filter plates and the vacuum manifold was very practical. After optimisation of vacuum strength, lysis buffers, volumes and timing, (Method 6.1.4.) the system proved to be an efficient high-throughput nucleic acid extraction system.

Quality of the vacuum extracted nucleic acids was determined by measuring DNA concentration; A_{260}/A_{280} absorbance ratio; ability to act as template DNA or RNA in PCR or RT-PCR and extraction efficiency correlated with bacterial counts measured by the Miles and Misra method, see Table 7.3. Generally the concentration of DNA in the elute decreased as the number of organisms in the media decreased. At dilutions of around 500 cfu ml^{-1}, the DNA concentration became too low to measure spectrophotometrically but

Table 7.2   Comparison of Whatman and Millipore 96-well vacuum filter systems.

Feature	Whatman	Millipore
Cost vacuum manifold	A$1,560	A$995
Cost 96-well filter plates	A$800/ 25 plates (max 2ml volume)	A$600/25 plates (max 200µl volume)
Ease of use	Good	Good
Construction	Very high quality	Good quality
Quality of eluted DNA	High quality	High quality
Problems encountered	Microfibre well blocks with debris	Microfibre well blocks with debris DNA sometimes difficult to elute, see Figure 7.2. Cross-contamination with small elution volumes
Other advantages	Deep-well microfibre plate up-to 2ml available for high sample volumes. Balanced flow rates between wells. Nucleic acids eluted through long drip directors that permit use of small elution volumes and also reduce cross-contamination between wells	Reliable supplier

PCR amplifiable amounts of DNA were still as positive as PCR reactions occurring at higher dilutions. The quality of the extracted nucleic acids was high with most elutes achieving an A_{260}/A_{280} absorbance ratio of between 1.8 and 2.1. The positive PCR and RT-PCR results achievable at very low concentrations are a good indication that high-quality nucleic acids could be obtained from the extraction system.

Table 7.3   Quality of vacuum extracted DNA by decimal dilution.

Bacterium	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
A. salmonicida							
DNA concentration	62	40	58	18	NM	NM	NM
A_{260}/A_{280} ratio	1.735	1.776	1.699	1.808	NM	NM	NM
CFU per 200µl sample	NM	NM	NM	900	90	10	0
PCR	+	+	+	+	-	-	-
RT-PCR	+	+	+	+	-	-	-
L. garvieae							
DNA concentration	62	66	36	56	NM	NM	NM
A_{260}/A_{280} ratio	1.663	1.990	1.791	1.776	NM	NM	NM
CFU per 200µl sample	NM	NM	NM	NM	120	30	4
PCR	+	+	+	+	+	-	-
RT-PCR	+	+	+	+	+	+	-
T. maritimum							
DNA concentration	80	70	26	10	NM	NM	NM
A_{260}/A_{280} ratio	1.823	1.820	1.732	1.796	NM	NM	NM
CFU per 200µl sample	NM	NM	NM	NM	70	3	0
PCR	+	+	+	+	+	-	-
RT-PCR	+	+	+	+	+	-	-
Y. ruckeri							
DNA concentration	52	54	52	NM	NM	NM	NM
A_{260}/A_{280} ratio	2.248	1.715	1.818	NM	NM	NM	NM
CFU per 200µl sample	NM	NM	NM	NM	130	10	0
PCR	+	+	+	+	-	-	-
RT-PCR	+	+	+	+	+	-	-

NM: not measurable

7.2. Determine optimum conditions for the PCR test to maximise specificity and sensitivity of the procedure.

7.2.1. Optimisation of PCR and RT-PCR sensitivity without compromising specificity.

PCR optimisation

Trials of *Taq* polymerase were only conducted with *L. garvieae* and *Y. ruckeri*. Platinum *Taq* DNA polymerase (Invitrogen) was able to amplify the smallest amounts of DNA and considered the most sensitive polymerase. HotStar *Taq* polymerase (Qiagen) was almost
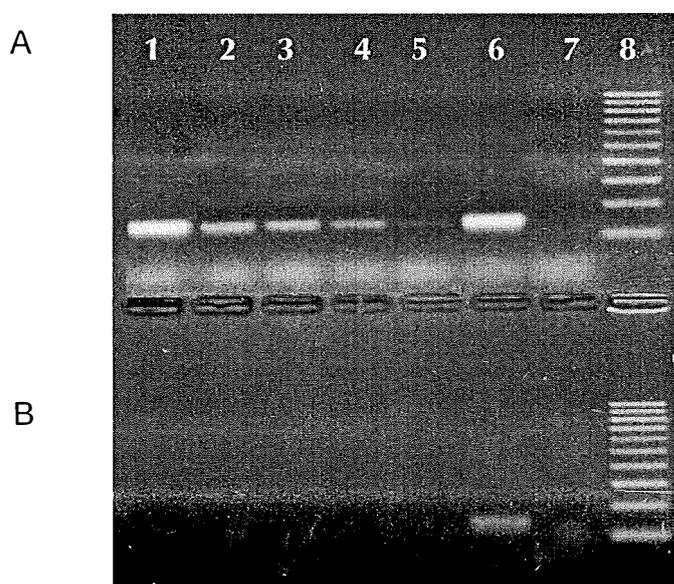
Figure 7.3   Sensitivity of *L. garvieae* PCR with row [A] Platinum *Taq* DNA polymerase and row [B] Red Hot DNA polymerase. Lane 1, 5 pg μl^{-1}; lane 2, 1 pg μl^{-1}; lane 3, 200 fg μl^{-1}; lane 4, 40 fg μl^{-1}; lane 5, 8 fg μl^{-1}; lane 6 10 ng μl^{-1} positive control; lane 7, negative control; lane 8, 100 bp markers.

as sensitive as Platinum *Taq*, however the enzyme was a little more expensive. Red Hot DNA polymerase (Advanced Biotechnologies) was much less sensitive than the other enzymes. See Figure 7.3 for a comparison of Platinum *Taq* DNA polymerase and Red Hot DNA polymerase.

Raising the concentration of magnesium chloride did not increase the sensitivity of the PCR assays significantly. However this increase in concentration did decrease fidelity of the PCR, with spurious bands visible by electrophoresis; this was particularly noticeable when determining *A. salmonicida* specificity, see Figure 7.4.
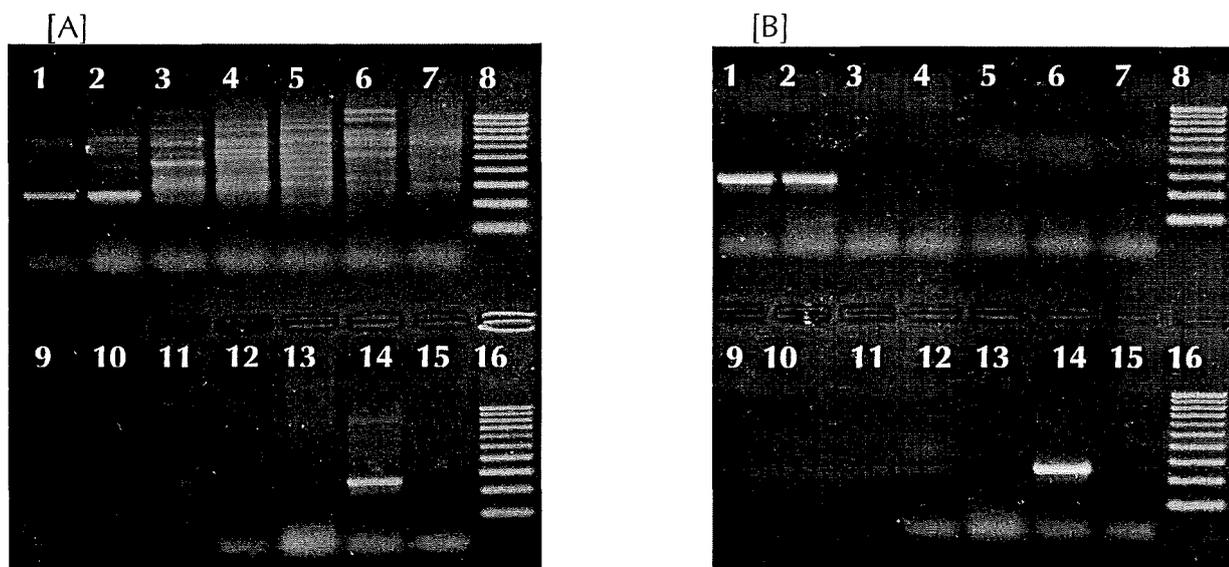
Figure 7.4   Specificity of *A. salmonicida* PCR with [A] 2mM magnesium chloride and [B] 1.375mM magnesium chloride. Lane 1, *A. bestiarum* ATCC 14715; lane 2, *A. hydrophila* ATCC 7965; lane 3, *A. caviae* ATCC 15468[T]; lane 4, *A. eucrenophila* ATCC 23309 [T]; lane 5, *A. sobria* ATCC 43979 [T]; lane 6, *A. media* ATCC 33907 [T]; lane 7, *A. veronii* bv *sobria* ATCC 9071[T]; lane 8, 100 bp markers; lane 9, *A. veronii* bv *veronii* ATCC 35624 [T]; lane 10, *A. schubertii* ATCC 43700 [T]; lane 11, *A. trota* ATCC 49657 [T]; lane 12, *A. jandaei* ATCC 49568 [T]; lane 13, *H. alvei* Accession# 95/6404; lane 14, *Aeromonas salmonicida* 93/0956-2; lane 15, negative control; lane 16, 100 bp markers.

Initial PCR conditions as published by Carson (1998) are given in Table 7.5. Decreasing the annealing temperatures greatly increased PCR sensitivity. However, if annealing temperatures were decreased by too much, some closely related species gave positive PCR reactions. Therefore, the annealing temperatures were decreased as much as specificity restrictions would allow. The annealing temperature for *L. garvieae* and *T. maritimum* PCR was decreased by 3°C, the temperature for *Y. ruckeri* was decreased by 5°C. The annealing temperature for *A. salmonicida* could not be decreased, however, greatly improved sensitivity was achieved by increasing the number of PCR amplification cycles from 30 to 35. After optimisation, the specificity achieved for the 16S primer sets for each bacterium by Carson (1998) was not compromised but sensitivity was greatly improved with detection levels less than 5 bacterial genome equivalents of DNA. The optimised PCR conditions were used for testing the RT-PCR assay and were found to give similarly good sensitivity results.

The final optimised PCR conditions and achieved sensitivities are given in Table 7.4. Figure 7.5. gives an example of a sensitivity increase achieved during cycling variations with *Y. ruckeri* at annealing temperatures of 65°C and 60°C.

Table 7.4   PCR conditions before and after optimisation.

Bacterium	Cycle conditions by Carson 1998	Optimum cycle conditions
A. salmonicida	Red Hot polymerase 1.375 mM MgCl$_2$ 30 cycles 60°C annealing temp 2 min extension time Sensitivity: 10 pg using nested PCR	Platinum *Taq* DNA polymerase 1.375 mM MgCl$_2$ 35 cycles 60°C annealing temp 30 sec extension time Sensitivity PCR: 4 fg Sensitivity RT-PCR: 4 fg
L. garvieae	Red Hot polymerase 2 mM MgCl$_2$ 35 cycles 65°C annealing temp 2 min extension time Sensitivity: 1 ng	Platinum *Taq* DNA polymerase 2 mM MgCl$_2$ 35 cycles 62°C annealing temp 30 sec extension time Sensitivity PCR: 4 fg Sensitivity RT-PCR: 280 ag
T. maritimum	Red Hot polymerase 2 mM MgCl$_2$ 35 cycles 65°C annealing temp 2 min extension time Sensitivity: 1 pg	Platinum *Taq* DNA polymerase 2 mM MgCl$_2$ 35 cycles 62°C annealing temp 30 sec extension time Sensitivity PCR: 4 fg Sensitivity RT-PCR: 40 fg
Y. ruckeri	Red Hot polymerase 2mM MgCl$_2$ 35 cycles 65°C annealing temp 2 min extension time Sensitivity: 10 pg	Platinum *Taq* DNA polymerase 2mM MgCl$_2$ 35 cycles 60°C annealing temp 30 sec extension time Sensitivity PCR: 4fg Sensitivity RT-PCR: 4fg
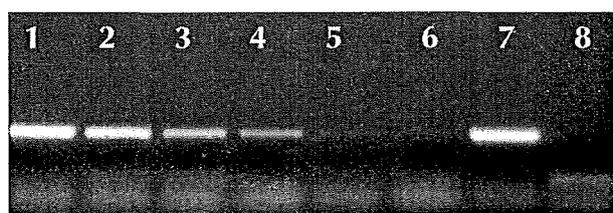
[A]
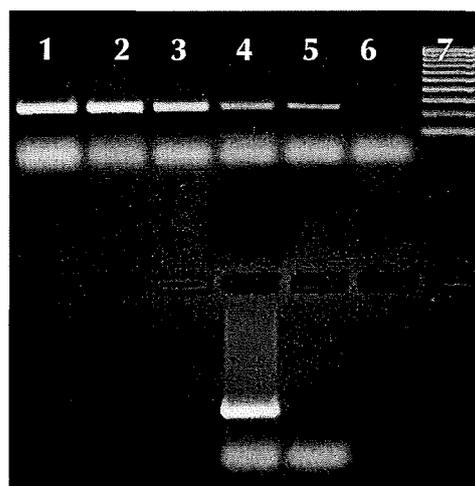
[B]

Figure 7.5   Sensitivity of *Y. ruckeri* PCR [A] with an annealing temperature of 65°C: lane 1, 30 pg µl^{-1}; lane 2, 6 pg µl^{-1}; lane 3, 1.2 pg µl^{-1}; lane 4, 240 fg µl^{-1}; lane 5, 48 fg µl^{-1}; lane 6, 9.6 fg µl^{-1}; lane 7, 10 ng µl^{-1} positive control; lane 8, negative control. [B] with an annealing temperature of 60°C, row 1: lane 1, 10 pg µl^{-1}; lane 2, 1 pg µl^{-1}; lane 3, 200 fg µl^{-1}; lane 4, 40 fg µl^{-1}; lane 5, 8 fg µl^{-1}; lane 6, ~ 1 fg µl^{-1}; lane 7, 100 bp markers. Row 2: lane 4, 10ng µl^{-1} positive control; lane 5, negative control.

7.2.2. Optimisation of the selective enrichment (SEC)-vacuum extraction-PCR protocol.

During the development of the 96-well vacuum extraction technique (Method 6.1.4), PCR detection results compared well with bacterial counts, see Table 7.3. To test the theory that detection sensitivity would increase with increased template volume, a maximum elution volume of 9 µl was tested from each bacterial decimal dilution. All dilutions however gave a negative result indicating PCR failure.

A combination of factors was found to affect the ability to increase template volume. The elution buffer used in the vacuum extraction was critical. TE (TRIS-EDTA) buffer was inhibitory with increased template volumes, but even when 1mM TRIS buffer was used for elution, a larger volume could be used but still not the 9 µl maximum, see Figure 7.6.
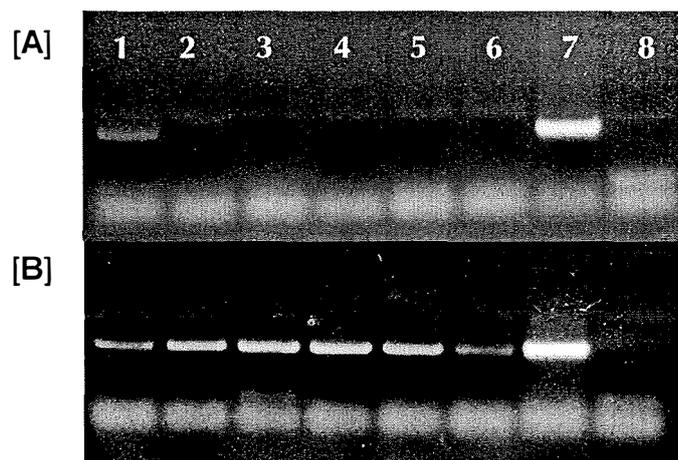
Figure 7.6 **The inhibitory effects of different elution buffers TE (TRIS-EDTA) and 1 mM TRIS, pH 8.0) and template volume on electrophoresis band intensity. Template: *Y. ruckeri* prepared in POST selective medium and extracted using Method 6.1.3, template concentration 150 cfu ml⁻¹. [A] TE buffer and [B] 1 mM TRIS, pH 8.0. Lane 1, 1 µl template volume; lane 2, 3 µl template volume; lane 3, 4 µl template volume; lane 5, 6 µl template volume; lane 6, 7 µl template volume; lane 7, 10 ng µl⁻¹ positive control; lane 8, negative control.**

Adding α-casein to the extraction protocol or to the PCR reaction did not enhance PCR performance. Adding 1.5 µg µl⁻¹ BSA to the PCR reaction mix however, did permit the use of a larger template volume, thereby increasing sensitivity of the PCR from five to 20 fold and increasing the intensity of the resulting electrophoresis bands, see Figure 7.7. With these two modifications, a maximum volume of 6 µl of template DNA per 20 µl PCR reaction volume was achieved. The final sensitivity values for the vacuum extraction from

selective enrichment media followed by PCR (SEC-PCR) and RT-PCR (SEC-RT-PCR) are given in Table 7.5.

Table 7.5 Sensitivity of PCR and RT-PCR systems using nucleic acids extracted using the vacuum system as template.

Bacterium	Sensitivity of PCR cfu 200 μl^{-1} sample	Sensitivity of RT-PCR cfu 200 μl^{-1} sample
A. salmonicida	2	1
L. garvieae	16	9
T. maritimum	1	1
Y. ruckeri	1	1

Performance of the extraction system, assessed as PCR specificity and sensitivity, was determined using a *Providencia* species, known to be enrichment culture resistant. The bacterium was incorporated at a fixed concentration of 1×10^9 cfu ml^{-1} to all dilutions of the target bacterium. In all cases, detection of the target bacterium was not compromised.
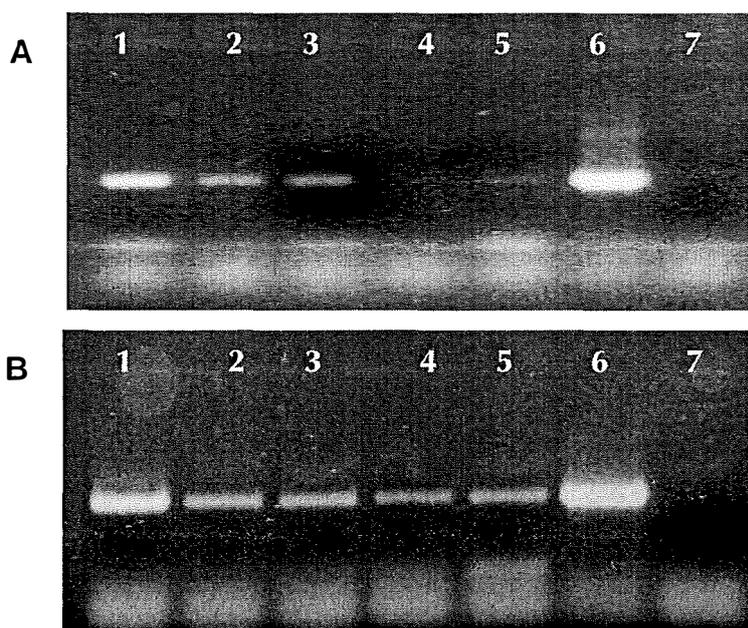
Figure 7.7 The effect of BSA on the ability to increase *Y. ruckeri* PCR template volume of vacuum extracted DNA. PCR with 1500 ng μl^{-1} BSA final concentration, [A] 1 μl template volume and [B] 9 μl template volume. Lane 1, 20000 cfu sample^{-1}; lane 2, 2000 cfu sample^{-1}; lane 3, 200 cfu sample^{-1}; lane 4, 20 cfu sample^{-1}; lane 5, 2 cfu sample^{-1}; lane 6, 10 ng μl^{-1} positive control; lane 7, negative control.

7.3. Optimise PCR conditions to incorporate a PCR protection system to prevent false positive reactions arising from DNA contamination.

7.3.1. Addition of Isopsoralen (IP-10) to inactivate amplicons and prevent false-positive PCR reactions.

PCR amplicons from *A. salmonicida* and *T. maritimum* appeared to be completely inactivated when IP-10 was used at a concentration of 50 µg ml^{-1}. However, when used at this concentration IP-10 decreased the sensitivity of the PCR. Amplicons from *L. garvieae* were not completely inactivated at this concentration or at higher levels; for *Y. ruckeri* and *A. salmonicida*, increasing the concentration of IP-10 to 100 µg ml^{-1} inhibited PCR amplification completely, see Figure 7.8, lanes five and six.
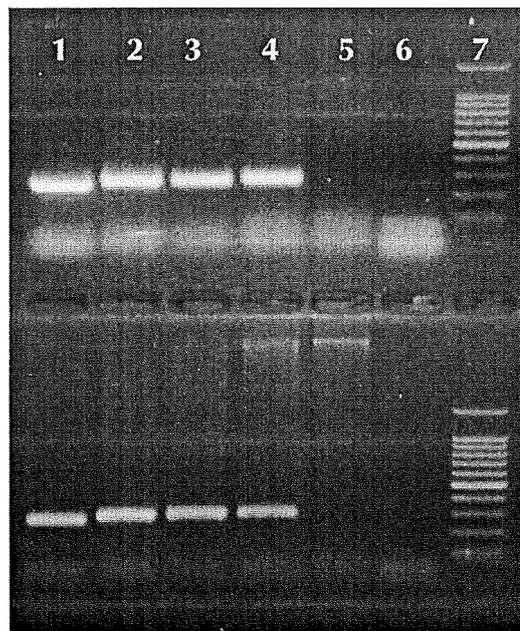
Figure 7.8   **Effect of IP-10 on PCR amplicon mass with increasing concentrations of IP-10. Top row, *Y. ruckeri*: lane 1, amplicons with no IP-10; lane 2, amplicons with 30 µg mL^{-1} IP-10; lane 3, amplicons with 40 µg mL^{-1} IP-10; lane 4, amplicons with 50 µg mL^{-1} IP-10; lane 5, amplicons with 100 µg mL^{-1} IP-10; lane 6, negative control; lane 7, 100 bp markers. Bottom row, *A. salmonicida*: lane descriptions as for *Y. ruckeri*.**

The highest concentration of IP-10 that could be used without adversely affecting specificity or sensitivity was 40 µg ml^{-1}. However, when neat PCR product inactivated with 40 µg ml^{-1} IP-10 was re-amplified, a weak positive band was seen by gel electrophoresis. Assuming that neat PCR product would contain approximately 6×10^9 µl^{-1} copies of the amplicon (HRI Research Inc. 1991), when PCR product was diluted to 6×10^7 amplicons,

IP-10 at 40µg ml^{-1} completely blocked subsequent PCR amplification. This level of inactivation could also be achieved using 30 µg ml^{-1} IP-10 for *T. maritimum* and *Y. ruckeri*. Gel electrophoresis of the re-amplification assay for *Y. ruckeri* at 40 µg ml^{-1} IP-10 is shown in Figure 7.9; the same results were obtained for the other bacteria, figures not shown. To show that this inactivation was not due to UV inactivation alone, *Y. ruckeri* PCR products not containing IP-10 were subjected to UV irradiation and re-amplified by PCR. In Figure 7.10 there is clear evidence of re-amplification demonstrating that UV alone is not capable of inactivating amplicons.
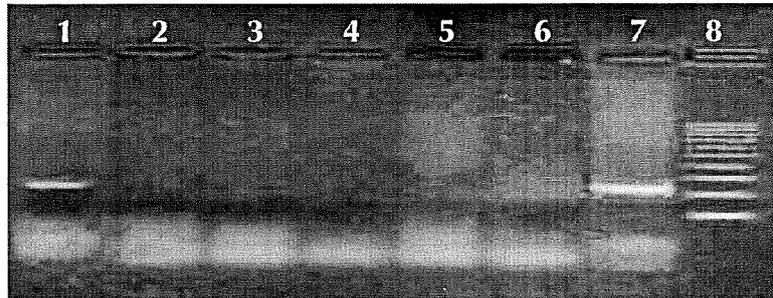
Figure 7.9   **Re-amplification assay using *Yersinia ruckeri* as template, amplicon inactivated with 40 µg mL^{-1} IP-10. Lane 1, approximately 6 x 10^9 amplicons; lanes 2 to 5, decimal dilutions from 6 x 10^7 to 6 x 10^4 amplicons; lane 6, negative control; lane 7, positive control; and lane 8, 100 bp markers.**

Effective inactivation at 40 µg ml^{-1} IP-10 was only successful when the PCR products were cooled during UV activation using a flexible ice brick cooled to 4°C. It was not possible to measure the temperature of the PCR product during UV irradiation but when the ice brick was omitted, IP-10 inactivation was not as efficient (Figure 7.11).
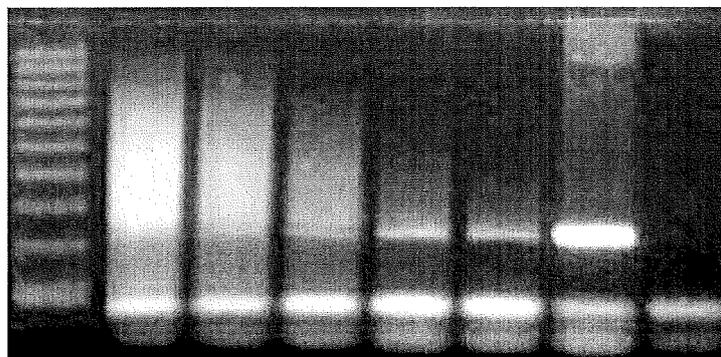
Figure 7.10   **Re-amplification assay using *Yersinia ruckeri* as template, amplicon treated solely with UV. Lane 1, 100 bp markers; lane 2, approximately 6 x 10^9 amplicons; lanes 3 to 6, decimal dilutions from 6 x 10^7 to 6 x 10^4 amplicons; lane 7, positive control; lane 8, negative control.**
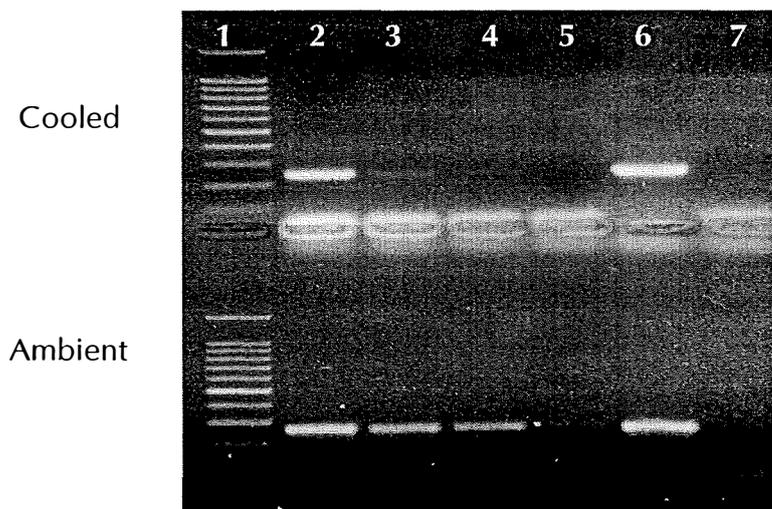
Figure 7.11: Effect of cooling on the efficiency of UV activation of _Y. ruckeri_ PCR amplicons treated with 40 µg mL⁻¹ IP-10. Top row, with cooling: lane 1, 100 bp markers; lane 2, approximately 6×10^9 amplicons; lanes 3 to 5, decimal dilutions of 6×10^7 to 6×10^5 amplicons; lane 6, positive control; lane 7, negative control. Bottom row, without cooling: lane descriptions as for top row.

The binding of IP-10 onto amplicons increased their mass. With increasing concentrations of IP-10, the amplicons migrated more slowly through the gel. The extent of the mass increase is given in detail in Table 7.6.

Table 7.6   Properties of PCR amplicons with and without IP-10.

Amplicon source	A+T ratio[a]	Expected amplicon size	Mass with IP-10[b]	Mass increase[c]
A. salmonicida	45%	261 bp	282 bp	21 bp
L. garvieae	54%	145 bp	160 bp	15 bp
T. maritimum	51%	288 bp	320 bp	32 bp
Y. ruckeri	46%	247 bp	272 bp	25 bp

[a]Ratio of adenine+thymine to guanine+cytosine
[b]Apparent mass of amplicon with 40 µg ml⁻¹ IP-10
[c]Increase in mass due to IP-10

7.4.   Develop a test procedure based on enzyme hybridization assay (EHA) that will verify any positive PCR reactions using a secondary confirmatory gene probe and in a format suitable for testing multiple samples.

7.4.1. Development of a high-throughput PCR enzyme hybridization assay (EHA) for sensitive, low cost, DNA detection.

The lower limit of detection for each bacterium by conventional PCR was 4 fg of reference DNA in thin-walled PCR tubes (Scientific Specialities). Following optimisation in

the NucleoLink tubes, the same level of detection was achieved with the biphasic format of PCR. Three factors were important in achieving this level of detection: cycling times, primer concentration and conjugate (streptavidin-alkaline phosphatase) concentration. Optimum cycling times were determined to be denaturing and annealing of 45 seconds per cycle, as opposed to the standard 30 seconds. The increased times are the result of temperature inertia due to the greater mass of the NucleoLink tubes compared to the thin-walled standard PCR tubes. Optimum primer concentrations varied for each
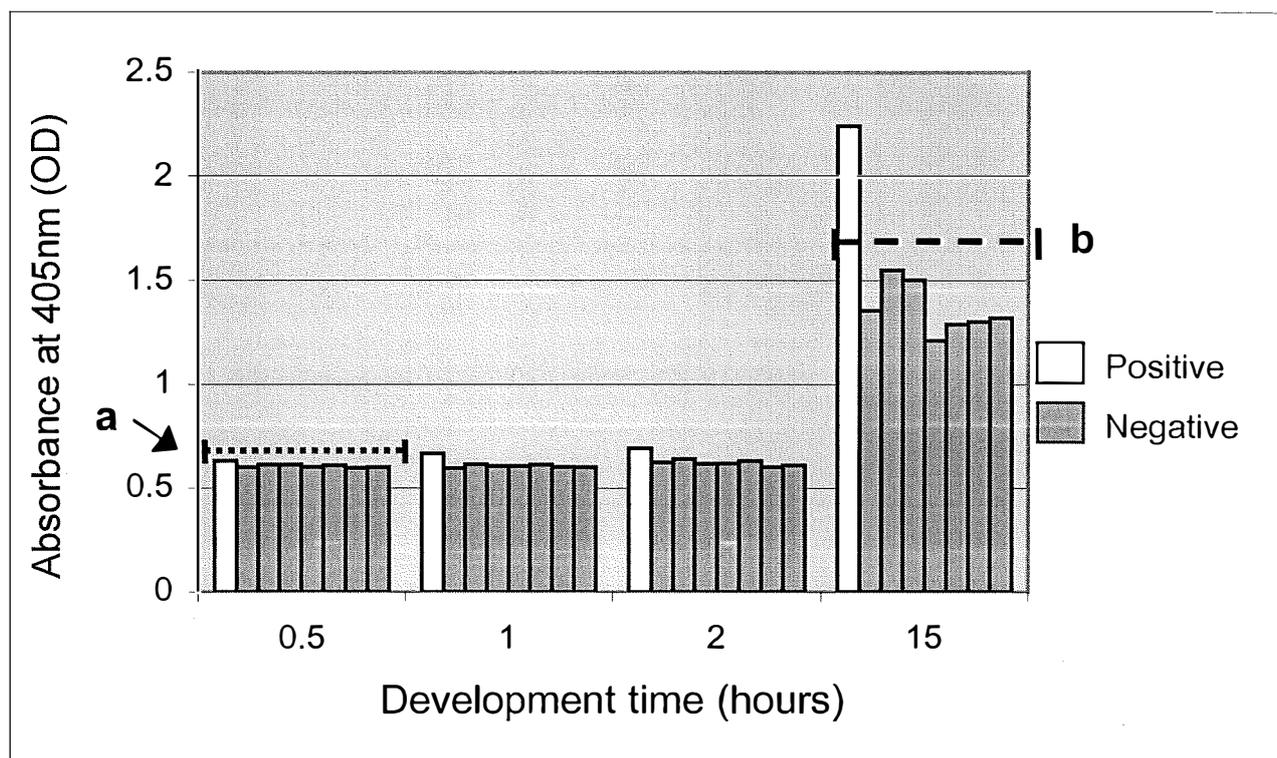
Figure 7.12 **PCR-EHA absorbance values for *Aeromonas salmonicida* showing the difference between weak positive absorbance readings (4 fg of template DNA) and seven negative absorbance readings over time. In this case the weak positive reading (0.632) is less than 1.2 times the highest negative control (1.2*neg= 0.734) after 0.5 hours, shown by line (a). However, the weak positive (2.242) is distinguishable from the negative controls by a factor of at least 1.4 (1.4*neg= 1.697) after 15 hours incubation, shown by line (b).**

bacterium. *A. salmonicida* required 0.5 µM of the forward and 0.0625 µM of the reverse primer, *T. maritimum* required 2 µM of the forward and 0.125 µM of the reverse primer, and *Y. ruckeri* and *L. garvieae* required 2 µM of the forward and 0.25 µM of the reverse primer. A conjugate ratio of 1:2,000 was also critical in achieving this level of detection. A lower ratio increased the background signal so that in some instances it was indistinguishable from weak positives while a higher ratio of conjugate decreased the level of detection.

The ability to distinguish weak positive results from negative background readings was tested by running seven negative PCR reactions alongside a weak positive (4 fg of template DNA). Negative EHA readings were determined to be any value less than 1.2 times the value of the negative control. Occasionally weak positive results were not easily differentiated from the negative controls. In such cases extending the colour development period to 4 to 15 hours was sufficient to separate the weak positive reactions from the negative values by a factor of at least 1.4 (Figure 7.12).

Table 7.7   Detection limits of SEC-PCR-EHA based on spiked samples.

Target bacterium	Detection limit of selective medium	Detection limit of DNA extraction as determined by PCR	Detection limit of SEC-PCR-EHA
A. salmonicida	3*	2	5
Fx. maritimus	4	1	4
L. garvieae	1	16	16
Y. ruckeri	5	1	5

*All values are colony forming units per 200 µl sample.

Once optimised with reference DNA, the PCR-EHA protocol was tested with DNA extracted from selective-enrichment media using the vacuum system. A detection level of 16 cfu per 200 µl sample volume was achieved for L. garvieae, 2 cfu for A. salmonicida and 1 cfu for T. maritimum and Y. ruckeri (see Table 7.7). When the PCR-EHA system is used in conjunction with selective media, the detection limit is compromised as the medium itself has its own limits of detection because of the low level impact of the selective agents on the target bacterium. These inhibitor effects as determined by MPN (Method 6.5.3) are slight, but contribute to the overall achievable level of detection, see Table 7.7.

The high level of specificity achieved during optimisation and development of the conventional PCR (Method 6.2.1) was maintained with the biphasic NucleoLink system. As an example, Figure 7.13 shows the specificity pattern for T. maritimum. A clear, strong signal is seen for the target, but no bands are evident with a range of near related species and all OD values are consistent with the negative control.

Corresponding OD →
EHA value

Corresponding OD →
EHA value
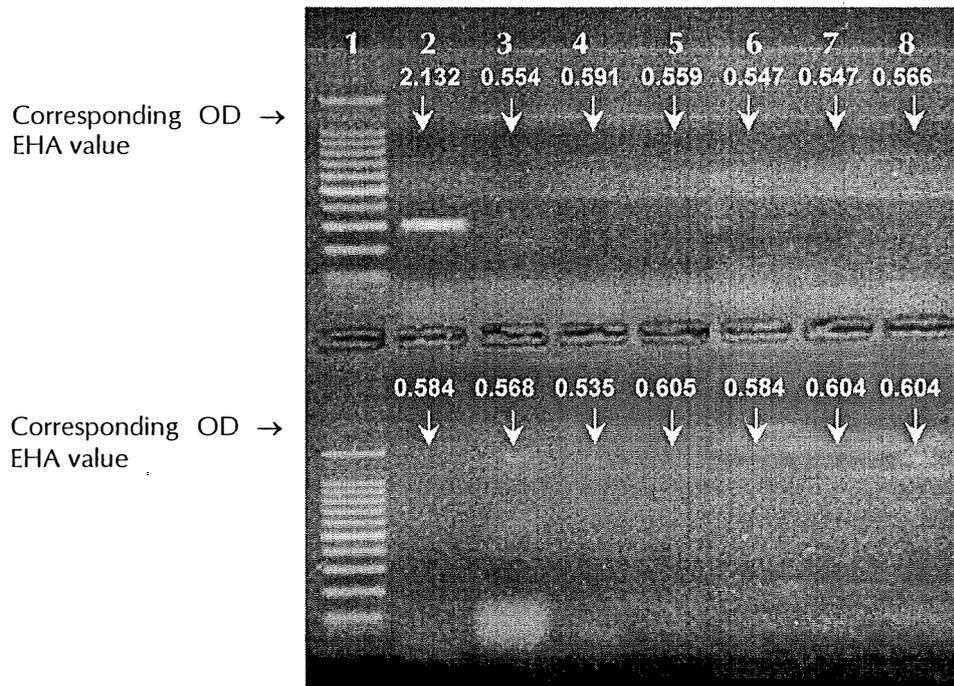
Figure 7.13 **Liquid-phase PCR results and corresponding EHA absorbance readings showing the specificity of the *T. maritimum* PCR-EHA. Top row: Lane 1, 100 bp ladder; lane 2, *T. maritimum* NCIMB 2154^T; lane 3, FCLB mucoid 96/5171; lane 4, FCLB mucoid 89/2244-9; lane 5, FCLB mucoid 89/2756-1; lane 6, FCLB mucoid CRC-2; lane 7, *A. salmonicida* 93/0956-2; lane 8, *Flavobacterium columnare* NCIMB 2248^T. Bottom row: Lane1, 100 bp ladder; lane 2, *Flavobacterium johnsoniae* ATCC 17061^{Co-T}; lane 3, *Vibrio anguillarum* 85/3475-1; lane 4, *V. ordalii*, ATCC 33509^T; lane 5, *V. splendidus* I, ATCC 25914^T; lane 6, *Cytophaga marinoflava* ACAM 75; lane 7, *T. ovolyticum* NCIMB 13127^T; lane 8, negative control.**

7.4.2. Development of a high-throughput RT-PCR enzyme hybridization assay (EHA) for sensitive, low cost, detection of rRNA.

A sensitivity of 4 fg or less of reference RNA was achieved for RT-PCR-EHA for each of the four bacteria. As with the PCR-EHA system, a conjugate ratio of 1:2,000 and optimised primer concentrations was needed for this level of detection. Optimum results were achieved for *A. salmonicida* and *Y. ruckeri* using a primer ratio of 1:8 and primer concentrations of 2 µM for the forward, and 0.25 µM of the reverse primers, see Figure 7.14. This primer ratio proved inefficient for RT-PCR-EHA with other two bacteria. For *T. maritimum* and *L. garvieae* a 1:1 ratio of the two primers at a concentration of 3 µM each for *T. maritimum* and 2 µM each for *L. garvieae* gave the optimal results.
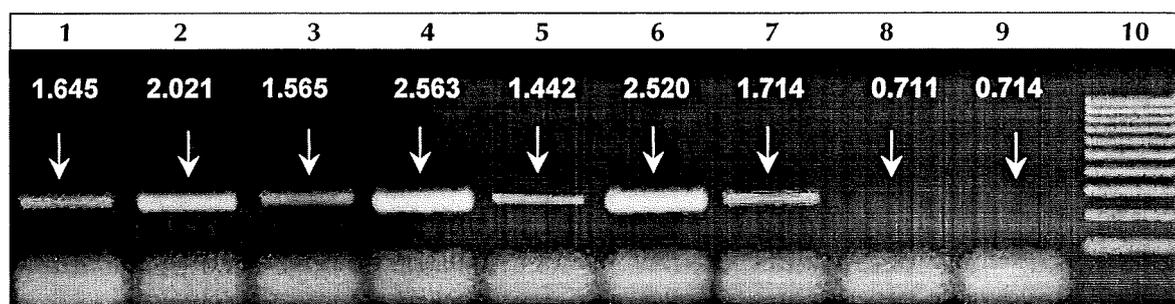
Figure 7.14 Effect of differing primer concentrations on agarose gel electrophoresis and EHA OD values for *Aeromonas salmonicida* reference RNA. Lanes 1 to 7, 1 pg RNA: Lane 1, 2 µM each primer; lane 2, 2 µM forward primer, 1 µM reverse primer; lane 3, 1 µM each primer; lane 4, 1 µM forward primer, 0.5 µM reverse primer; lane 5, 0.5 µM each primer; lane 6, 2 µM forward primer, 0.25 µM reverse primer; lane 7, 2 µM forward primer, 0.5 µM reverse primer; lane 8, negative control with no RT enzyme; lane 9, negative control with no RNA; lane 10, 100 bp markers.

Once optimised with reference RNA, the RT-PCR-EHA was tested on RNA extracted from selective-enrichment media. A sensitivity of 9 cfu per 200 µl sample volume was achieved for *L. garvieae*, 1 cfu for *A. salmonicida* and *T. maritimum* and 3 cfu for *Y. ruckeri*. As for SEC-PCR-EHA, when RT-PCR-EHA is used in conjunction with the selective enrichment media, the overall detection limit is compromised as the medium itself has its own limits of detection. The overall sensitivity of the RT system is slightly better than the SEC-PCR-EHA system with detection limits between 3 and 9 cfu per 200 µl sample, see Table 7.8 for details.

The high level of specificity achieved during the optimisation and development of the RT-PCR (Method 7.2.1) was maintained with the biphasic NucleoLink system.

Table 7.8   Detection limits of SEC-RT-PCR-EHA using spiked samples.

Target bacterium	Detection limit of selective medium	Detection limit of RNA extraction as determined by RT-PCR	Detection limit of SEC-RTPCR-EHA
A. salmonicida	3*	1	3
Fx. maritimus	4	1	4
L. garvieae	1	9	9
Y. ruckeri	5	1	5

*All values are colony forming units per 200 µl sample volume.

7.5. Optimise the culture conditions and PCR detection process to ensure the minimum test time between sample collection and test result.

7.5.1. Test optimisation strategies

Major objectives in development of the nucleic acid extraction system, PCR assay format and verification protocol was to simplify the activities, not only in processing, but also where possible, by integrating the tests so that overall test time was reduced. Streamlining of the system was achieved through the following aspects:

- use of industry standard 96 wells reaction vessels in 8x12 format
- configuration of tests for use with multichannel pipettes in single or multi-stepper modes to speed reagent transfer and dispensing
- nucleic acid extraction technology that permits high throughput processing of 96 samples simultaneously
- integration of PCR with amplicon detection and sequence confirmation in the one reaction vessel
- optimisation of PCR cycling conditions to shorten overall reaction time
- PCR amplicon inactivation technology integrated into PCR process and activated post-amplification in the reaction vessel

The major limiting step of the SEC-PCR-EHA process is the time required for incubation of the enrichment cultures which for all the target pathogens is a period of five days. After incubation however, the PCR-EHA process can be undertaken within 24 hours. In general terms, the times taken for test milestones are as follows:

- nucleic acid extraction – 2 hours
- PCR set up – 1 hour
- Amplification – 2 hours
- Hybridization – 3 hours
- Signal development – 1 hour initial, 18 hours for weak reactions

The times specified are applicable to a range of sample sizes from as few as 8 to 192 (2x96 well plates). The capacity to process large numbers of samples without a major impact on time, stems from the high throughput structure of the assay system.

7.5.2. Minimum Inhibitory Concentration (MIC) assays for the development of HK3C a selective-enrichment medium for *Aeromonas salmonicida* biovar *acheron*.

A. salmonicida biovar *acheron* showed good resistance to the three antibacterials contained in the previously developed *A. salmonicida* selective media, *i.e.* compound H, compound C1 and compound K. Two new potentially useful antibacterial agents were identified during this study, compound C2 and compound C3. Of these, compound C2 inhibited approximately 25% of the normal flora that were resistant to the current version of the selective media, while compound C3 specifically inhibited the growth of *A. hydrophila*, see Table 7.9. The bacteria that show some sensitivity to the antibacterial have their results highlighted.

Table 7.9   MIC results for compound C2 and compound C3 with normal flora resistant to basal selective medium.

Bacterium	C2		C3	
	MIC ($\mu g\ ml^{-1}$)	PLC ($\mu g\ ml^{-1}$)	MIC ($\mu g\ ml^{-1}$)	PLC ($\mu g\ ml^{-1}$)
A. salmonicida biovar *acheron*	2500	625	125	62.5
(B7) *Pseudomonas* sp.	7.8	3.9	125	62.5
(FT1) *Pseudomonas* sp.	500	250	250	125
(W57) *Pseudomonas* sp.	500	250	>500	>500
(B127) *Pseudomonas* sp.	1000	500	>500	>500
(G49) *Pseudomonas* sp.	125	12.5	1.56	0.78
(W43) *Aeromonas* sp.	>500	>500	125	31.25
(B11) *Aeromonas* sp.	>500	>500	>500	>500
(B109) *Flavobacterium* sp.	2000	2000	NT[a]	NT[a]
(B9) *Shewanella* sp.	31.25	15.6	NT[a]	NT[a]
(B6) *Enterobacter* sp.	62.5	31.25	250	125
Proteus sp.[b]	500	125	>500	>500
A. hydrophila [b]	>500	>500	31.25	15.63

[a] Not tested
[b] Bacteria isolated during fish trials with old versions of *A. salmonicida* selective media.

7.5.3. Chequerboard MIC assays in the development of HK3C a selective-enrichment medium for *Aeromonas salmonicida* biovar *acheron*.

Chequerboard MIC assays were performed first between compound H and compound K, two selective agents that are essential components in a selective enrichment medium for *A. salmonicida*. No antagonist or synergistic effects were expected as these have not been seen previously (Carson *et al.*, 2001b). The maximum combined concentration of antibacterials were determined by chequerboard assay as 2000 μg ml^{-1} of compound H and 0.8 μg ml^{-1} of compound K. Chequerboard MIC assays were also used to determine the optimum concentration for compounds C2 and C3 to check for antagonistic or synergistic effects. To perform this assay compounds H and K were added to the base medium at the optimal concentrations identified. No antagonistic or synergistic effects were seen, and four possible combinations of antibiotic concentrations were identified for C2 and C3. These four combinations were tested with those bacteria listed in Table 7.5 and the most effective combinations tested for their inhibitory effect on *A. salmonicida* by the MPN method, Method 6.5.3. The final formulation of medium HK3C is given in Table 7.10

Table 7.10   Selective enrichment medium HK3C for *A. salmonicida* biovar acheron

Component	
Basal medium	CRC for Aquaculture, proprietary formulation
Compound H	200 μg ml^{-1}
Compound K	0.8 μg ml^{-1}
Compound C1	20 μg ml^{-1}
Compound C2	100 μg ml^{-1}
Compound C3	65 μg ml^{-1}

7.5.4. Most Probable Number (MPN) assays to determine the effect of the selective agents on the growth of *A. salmonicida* biovar *acheron*.

The MPN estimates for the growth of *A. salmonicida* biovar *acheron* in the selective (4397 cells ml^{-1}) and non-selective (4307 cells ml^{-1}) media were not significantly different (95% confidence limits); this result was confirmed using Student's t test (Koopmans 1987) on the MPN estimates (P>0.05). It was concluded that the selective components of the medium had no inhibitory effect on *A. salmonicida* biovar *acheron*.

7.5.5. Evaluation of the capacity of *A. salmonicida* biovar *acheron* to grow in the selective medium with competition.

A. salmonicida biovar *acheron* was able to grow in the presence of *Pseudomonas* sp. (B127) and *Pseudomonas* sp. (M146), two vigorous competitors, identified from earlier studies (Carson *et al.*, 2001b). *A. salmonicida* had negative EI values at 24 hours (-0.85), 3 (-0.17) and 5 days (-1.8) when grown in competition with *Pseudomonas* sp. (B127) indicating it was out-competed. Competition is relative, and the cell density of *A. salmonicida* nevertheless increased 1000x after three days incubation. In the presence of the mucoid strain *Pseudomonas* sp. (M146), positive EI values at 24 hours (1.17), 3 (1.29) and 5 days (0.42) showed that *A. salmonicida* could out-compete this strain.

7.5.6. Validation of the high-throughput SEC-PCR system.

During seeding experiments using *Y. ruckeri* as a model, no cross-contamination between either the glass microfibre filter wells or the PCR wells was detected. However, one false negative result was detected when using the 96-well PCR plate (Scientific Specialties). When using rimmed 96-well plates the plastic sealing tape was sometimes cut by the raised rim of the PCR tray, causing the contents to leak by evaporation during PCR thermocycling, a factor known to limit test activity.

7.6. Test populations of salmonids with the optimised SEC-PCR-EHA system to verify test performance and obtain baseline data on carrier prevalence.

7.6.1. Fish trials to test the dynamics of the high-throughput SEC-PCR system.

During the first *Y. ruckeri* fish trial 14% of the 150 faecal samples and 10% of the 30 spleen samples were positive by SEC-PCR. Verification of the positive samples by sub-culture was only successful with 20% of the positive faecal samples; all of the non-confirmable samples had resistant normal flora that may have masked the presence of *Y. ruckeri* in subculture. These resistant flora were checked for cross-reaction with *Y. ruckeri* by PCR; all were negative. The SEC-PCR positive results varied greatly in the intensity shown by electrophoresis, see Figure 7.15. Some bands were so weak that they were labelled 'ghosts'; *Y. ruckeri* was not isolated by culture from any of the samples that

produced 'ghost' bands suggesting that such samples contained trace quantities of amplifiable *Y. ruckeri* DNA but not viable cells that could be recovered in culture.
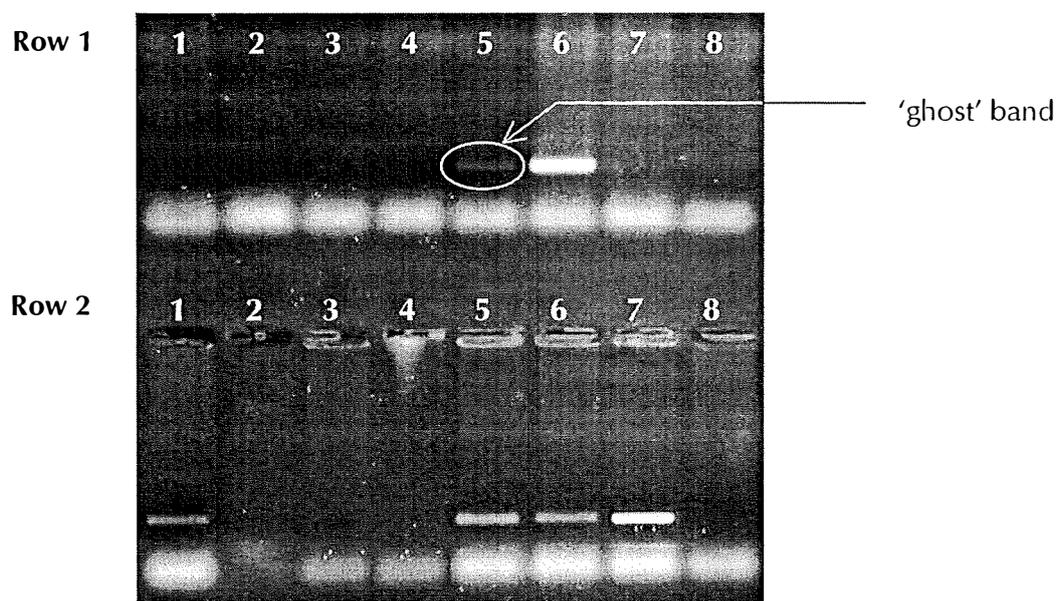
Figure 7.15 **Variation in intensity of electrophoresis bands between samples from a *Y. ruckeri* fish trial. Row 1: lanes 1 to 8 are samples 1 to 8. Row 2: lanes 1 to 6 are samples 88 to 93, sample 5 band intensity is so weak that it is described as a 'ghost'; lane 7, 10pg µl⁻¹ positive control; lane 8, negative control.**

Verification of the PCR positive spleen samples was successful, with *Y. ruckeri* isolated from all samples. During the second *Y. ruckeri* fish trial, 20% of the 150 faecal samples were positive by SEC-PCR after 5 days incubation. *Y. ruckeri* was not isolated by culture from any of these positive samples. As seen in the first *Y. ruckeri* trial, some of the SEC-PCR positive results showed very weak 'ghost' bands by electrophoresis. The SEC-PCR results varied during the period of culture incubation. Some samples gave strong bands after 2 and 5 days of incubation; others formed weak bands after 2 days and became stronger at 5 days, but about 30% of the 'ghosts', evident after 2 days incubation, were not visible after 5 days. Resistant normal flora was isolated by subculture but no cross-reactions occurred with these isolates when tested by PCR. Since there were several instances of unconfirmed positive SEC-PCR results, 18 fish were collected from a farm with no history of *Y. ruckeri* infection and tested by SEC-PCR; all samples were clearly SEC-PCR negative indicating that the positive PCR results from the previous trial were the result of amplification of *Y. ruckeri* DNA.

Like the *Y. ruckeri* fish trials, a greater proportion of *L. garvieae* samples were positive by SEC-PCR than by culture. Of 55 fish tested, 12 were positive by PCR and only one of these fish was positive by subculture from the enrichment medium. Based on the sample size tested, the prevalence was 22%. Like the trials with *Y. ruckeri*, 'ghost' bands were seen in the agarose gels indicating weak PCR reactions.

7.6.2. Validation of the SEC-PCR-EHA and SEC-RT-PCR-EHA systems by field trial testing.

Each SEC-PCR-EHA, and where applicable, reverse transcriptase (RT) system, was extensively validated in field trials. The exception was *L. garvieae*. SEC-PCR was tested with fish with a recent history of infection but for SEC-PCR-EHA, the system could only be tested on disease free fish, as there had been no recent outbreaks of streptococcosis at the time of validation. The number of fish tested for each bacterium and the disease status of each farm is given in Table 7.11. The decision to test by RT-PCR was based on farm need and related to the need to determine a history of exposure (DNA-PCR) or evidence of live carriage of the target pathogen (RT-PCR); see Discussion 8.6.2 for more details.

Table 7.11   Number of fish tested for each bacterium and the disease status of the farms tested.

Bacterium	Farm disease history	Number of fish tested by SEC-PCR-EHA	Total number of fish tested with by SEC-PCR-EHA	RT system tested
A. salmonicida	No disease	163	478	No
	Disease one year previous	130		
	Current disease	185		
L. garvieae	No history of disease in 12 years	48	181	No
	History of disease	133*		
T. maritimum	History of occasional disease	96	242	Yes
	History of occasional disease	96		
	No disease	50		
Y. ruckeri	History of disease	96	258	Yes
	History of disease	96		
	No disease	48		
	No disease	18		

*SEC-PCR only

Aeromonas salmonicida

The first *A. salmonicida* SEC-PCR-EHA field trial was conducted in February 2001. For this trial 163 skin mucus samples were taken from Atlantic salmon from two farm sites with no

history of diseases. The sampled fish were from three age groups: 4 months post smolt; 10 months post smolt and harvest ready fish. SEC-PCR-EHA was performed on all samples. No positive SEC-PCR-EHA reactions occurred from the sampled fish using the negative cut-off value of 1.2 times the negative control proposed from laboratory experiments. Retrospective testing was undertaken by seeding several samples with *A. salmonicida* to check for the presence of inhibiting agents; all such samples gave positive SEC-PCR-EHA reactions with an inoculum level of 7 cfu ml^{-1} (the lowest dilution tested).

A second *A. salmonicida* trial was conducted in late March 2001. In this trial, SEC-PCR-EHA was used to test 130 Atlantic salmon from a cage of fish, which had experienced a disease outbreak with *A. salmonicida* 12 months previously in January 2000. This second trial produced very different results to the earlier trial. Initial SEC-PCR-EHA results suggested a 30% carriage of *A. salmonicida* amongst the fish tested. However, no *A. salmonicida* was recovered by sub-culture from the enrichment cultures although, in all cases, a resistant flora comprising mixed aeromonads was present. Because of the known cross-reaction of some *A. hydrophila* strains with the 16S rRNA gene primer set for *A. salmonicida*, confirmatory testing by PCR was performed using the *vap*A and PAAS primer sets (Byers *et al.* 2002a). All tests with PAAS were negative but about 19% of 16S rRNA positive samples were positive with the *vap*A primers. Screening of the resistant mixed aeromonads isolated from the selective enrichment cultures established that a strain of *A. hydrophila* was responsible for the positive *vap*A reactions. From this data, it was established that there was no evidence that the group of fish tested were carriers of *A. salmonicida*.

A third *A. salmonicida* trial was conducted in January 2002 due to the coincidental occurrence of a disease outbreak in Atlantic salmon. In this trial, 184 fish were tested by SEC-PCR-EHA. Samples were collected from live fish from 5 separate cages considered high risk, as well as from some moribund or dead fish. A smaller sample set was collected from apparently healthy fish from a nearby farm with no history of disease, see Table 7.12. for details. The fish sampled from the farm with no history of disease gave negative SEC-PCR-EHA results. By contrast, fish sampled from cages 1 and 2, which were dead or moribund, were almost all positive. The remaining 155 fish tested showed no clinical signs of disease and were randomly selected from several cages considered at-risk but with no elevated levels of mortality or signs of disease. Positive identification of *A. salmonicida*

was achieved by sub-culture from the selective media from 18% of the SEC-PCR-EHA positive samples.

Table 7.12 Prevalence of *Aeromonas salmonicida* according to SEC-PCR-EHA, results by cage number and disease status.

Farm	Sample status	Number of samples	% SEC-PCR-EHA positive	Apparent prevalence
No history of disease	No clinical signs	3	0	0%
History of disease	Dead or moribund fish (cage 1 & 2, selected samples)	19	17	NA*
	Dead fish (cage not specified)	7	1	14%
	No clinical signs (cage 3)	30	1	3%
	No clinical signs (cage 4)	32	2	6%
	No clinical signs (cage 5)	30	1	3%
	No clinical signs (cage 6)	33	6	18%
	No clinical signs (cage 7)	30	0	0%

*NA: not applicable

Tenacibaculum maritimum

The first *T. maritimum* fish trial was conducted in August 2001 at a farm with no recent evidence of disease but with a previous history of infection. Mucus samples from near the pectoral fin, an initiation site for infection, were collected from 96 Atlantic salmon and the SEC-PCR-EHA and reverse transcriptase (RT) systems used to determine prevalence. The SEC-PCR-EHA was used to determine a history of disease and the RT system as evidence of live carriage of the organism. SEC-PCR-EHA established a *T. maritimum* carriage rate prevalence of 25 percent. Of these, 16% of samples were strong positives (above 1.4 times the negative control) and the remaining 9% were weak positives with OD values between 1.2 and 1.4 times the negative control. No *T. maritimum* was isolated by sub-culture from the positive samples, however a large variety of *Flavobacterium*-like normal flora were recovered as resistant flora from the selective enrichment medium. No PCR positive reactions were detected with any of the flora, indicating that the positive reactions detected by PCR-EHA were due to the presence of *T. maritimum* and not a cross reaction with the resistant flora. Given the relatively high amount of resistant flora in the enrichment cultures, it is possible that *T. maritimum* may not have been detected by subculture. Presence of *T. maritimum* was tested by use of follow-up RT-PCR-EHA as a means of detecting the presence of live target pathogen using RT-PCR. By this test only one sample was positive, equivalent to a 1% live carriage of *T. maritimum*. During this fish trial, the RT-PCR-EHA system did not run as smoothly as was anticipated. The results of the

EHA were not visible until after 6 hours incubation. The assay was repeated and the same unexpectedly long incubation time was again required. Further experimentation determined, that on storage, the activity of the streptavidin-alkaline phosphatase conjugate declines. To compensate for the loss in activity, the incubation time for the biotin-streptavidin binding step was extended from one to two hours.

A second *T. maritimum* trial was conducted in December 2001 at the same farm as originally tested. Mucus samples were taken from 96 Atlantic salmon and the SEC-PCR-EHA and reverse transcriptase (RT) systems used to determine carriage prevalence. SEC-PCR-EHA detected 7% carriage of the organism, however, no *T. maritimum* was isolated by culture from the selective medium. PCR was performed on any contaminating normal flora and no cross-reaction was found. Follow up by SEC-RT-PCR-EHA showed the live carriage rate was only 1%.

Due to the number of positive SEC-PCR-EHA results that were not confirmed by culture, a small-scale fish trial was conducted from a sea farm with no history of disease with *T. maritimum*. Figure 7.16. shows the distribution of the absorbance values resulting from the SEC-PCR-EHA. No samples were close to the 1.2 times negative control cut-off value, confirming that the positive reactions seen in the previous two trials were due to the detection of *T. maritimum* and not the result of non-specific cross-reactions with resistant flora.

Yersinia ruckeri

The first *Y. ruckeri* fish trial was conducted in June 2001 from a farm with a long history of yersiniosis. Faecal samples were collected from 96 Atlantic salmon and the SEC-PCR-EHA and reverse transcriptase (RT) systems used to determine carriage prevalence. SEC-PCR-EHA detected a *Y. ruckeri* prevalence of 23% using a negative cut-of value of 1.4 times the negative control after overnight development of the EHA. As seen in Figure 7.15. the strength of the band seen by gel electrophoresis varied greatly, with some bands weak enough to be considered 'ghosts'. During this trial however, *Y. ruckeri* was not isolated
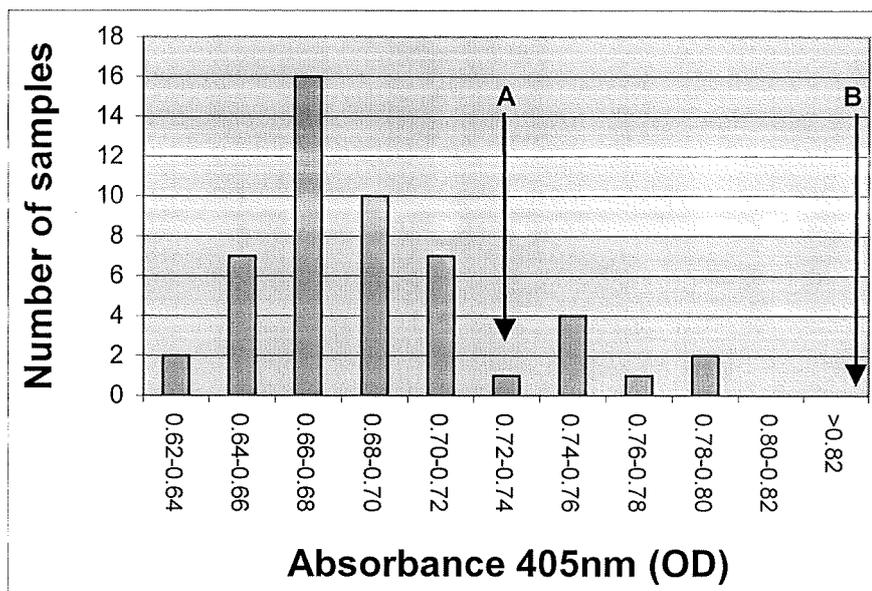
Figure 7.16 **SEC-PCR-EHA absorbance values for _T. maritimum_ with 100 fg of template DNA and replicate negative controls. [A] represents the value of the negative control after 0.5 hours development time, [B] is the negative cut-off value at 1.2 times the negative control (8.7).**

from any of the POST, the selective enrichment cultures. No cross-reactions were detected by _Y. ruckeri_ PCR when the resistant flora was tested. Follow-up RT-PCR-EHA on the positive samples showed that there was a 4% live carriage of the pathogen.

The second _Y. ruckeri_ trial was conducted in January 2002 from the same farm as originally tested. Faecal samples were taken from 48 fish that had been vaccinated against _Y. ruckeri_ and from 48 fish of the same age that had not been vaccinated. The _Y. ruckeri_ SEC-PCR-EHA and reverse transcriptase (RT) systems were used to determine a history of infection and evidence of active carriage of the bacterium. Using SEC-PCR-EHA and follow-up SEC-RT-PCR-EHA, 4% of the vaccinated fish showed a history of infection but with no active carriage of _Y. ruckeri_ as determined by RT-PCR. In contrast, 8% of the unvaccinated fish showed a history of infection of which 4% was active carriage as determined by RT-PCR. For all PCR positive samples however, _Y. ruckeri_ was not recovered from the enrichment cultures.

As for the _T. maritimum_ trials, a large number of _Y. ruckeri_ positive SEC-PCR-EHA results could not be confirmed by culture. In order to test for non-specific cross-reactions, a small-scale trial was conducted with rainbow trout at a farm with only low grade,

infrequent episodes of yersiniosis. Rainbow trout were used because of their inherent resistance to infection with *Y. ruckeri* serotype O1b, the strain extant in Australia. Figure 7.17. shows the distribution of the absorbance values resulting from the SEC-PCR-EHA. It can be seen that all but one of the EHA absorbance readings was well below the negative cut-off of 1.2 times the negative control OD value. The positive sample was cultured and yielded a culture of *Y. ruckeri*, thereby giving confidence in the capacity of the system to detect *Y. ruckeri*. Decimal dilutions of a suspension of *Y. ruckeri* were added to the selective-enrichment cultures to check for false-negative reactions possibly arising from PCR inhibitors that may have been co-extracted and eluted during DNA extraction. Positive SEC-PCR-EHA reactions were achieved where there was less than 1 cfu 200 μl^{-1} sample, indicating the sensitivity of the system and freedom from PCR inhibitors.
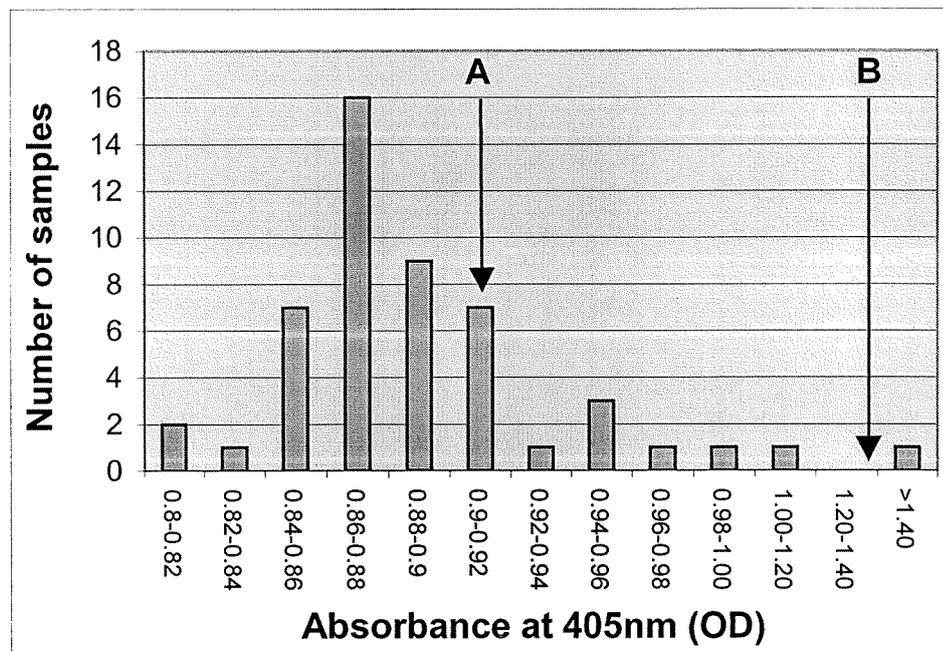
Figure 7.17 SEC-PCR-EHA absorbance values for *Y. ruckeri* with 24 fg of template DNA and replicate negative controls. [A] represents the value of the negative control after 0.5 hours development time, [B] is he negative cut-off value of 1.2 times the negative control.

Lactococcus garvieae

No fish farms in Tasmania have had any reported or tested episodes of infection with *L. garvieae* since 1990. Consequently, no large-scale fish trials were undertaken. A smaller trial was undertaken to establish specificity of the system with field samples. Spleen samples were taken from 48 rainbow trout and tested by SEC-PCR-EHA. After a three hour development time, the SEC-PCR-EHA results were all negative using a negative cut-

off of 1.2 times the OD value of the negative control. One sample was close to the negative cut-off, however, upon repeat testing this sample was clearly negative. Decimal dilutions of *L. garvieae* were added to the selective-enrichment cultures to check for false-negative reactions due to PCR inhibitors. The level of detection by SEC-PCR-EHA was 8 cfu 200 μl^{-1} sample, indicating the sensitivity of the system and freedom from PCR inhibitors.

8. DISCUSSION

8.1. Develop a procedure for extracting bacterial DNA from selective enrichment media that is suitable for PCR and for processing multiple samples.

8.1.1. Large scale extraction of DNA for use in determining PCR sensitivity with purified DNA and as a positive control.

The large scale DNA extraction method resulted in highly concentrated, PCR quality reference DNA, which did not noticeably decline in quality after three years stored at – 20°C. The variations to the procedure for *T. maritimum* and *L. garvieae* were essential in achieving efficient DNA extraction. Ethanol treatment of *T. maritimum* cells to remove the outer mucopolysaccharide layer facilitated action of the lytic reagents on the cell wall, while threonine and penicillin treatments of *L. garvieae* weakened the cell wall and enhanced lysozyme activity. The reference DNA was used for all aspects of test development and was used as an internal standard to monitor PCR efficiency during field trials to determine carriage levels in fish. Monitoring was seen as essential since the heterogeneity of fish tissue samples meant there was always a possibility of co-elution of PCR inhibitors which could lead to a reduction in the number of positive samples.

8.1.2. Evaluation of 96-well vacuum extraction units.

The Millipore and Whatman 96-well systems produced high-quality DNA from the selective-enrichment media. The Whatman filter system was selected because of the large sample volume capacity and the design of the 'drip-directors' underneath the filter plates, which gave consistent and efficient performance when processing small DNA elution volumes. Whatman filterplates are available in volume capacities to a maximum of 2 ml. In the high capacity format a 1 ml sample could be processed thereby increasing test sensitivity further if required. With the Millipore system, a maximum sample volume of only 100 µl could be used and because of the design of the filterplates, a larger volume of DNA elution buffer was required which had the effect of diluting the extracted DNA and thereby reducing test sensitivity. When an elution volume of less than 50 µl was used with the Millipore plates, the elution process was difficult to perform and sometimes resulted in cross-contamination of samples.

8.1.3. Extraction of genomic DNA and ribosomal RNA using UNIVAC manifold and Whatman GF/B 96-well filter plates.

PCR quality DNA and RT-PCR quality RNA could be simultaneously extracted from the selective-enrichment media using the Whatman GF/B 96-well filter plates and UNIVAC manifold. For the Gram-negative bacteria lysis and extraction could be undertaken entirely in the filterplate and, with multichannel pipettes, processing of 96 samples simultaneously was quick, efficient and well suited to screening large numbers of fish. The guanidinium buffer was effective in lysing bacterial cell walls and binding the released nucleic acids to the glass microfibres in the filter. Extracting 96 samples using this system took about one hour to perform. Unfortunately, but not unexpectedly, lysis of *L. garvieae* required pre-treatment with lysozyme and proteinase K which had to be undertaken as a separate step in a 96 well microwell tray. The extra step increased sample processing and nucleic acid extraction took 2.5 hours to perform, more than double the time for the Gram-negative bacteria. The cost of nucleic acid extraction by the system described was 55^c per sample compared with A\$3-4 per sample for equivalent commercially available systems.

While performing vacuum extraction, particular stages of the procedure were critical and deviation from the method given in Method 6.1.4 could reduce extraction efficiency significantly. Critical steps were identified as follows:

- The lysis buffers must be cooled to 4°C before use
- β-mercaptoethanol must be added to the buffers just before use for efficient RNA extraction.
- Vacuum strength for removal of the L6 lysis buffer is of particular importance as the buffer contains the detergent Triton X-100, which will cause foaming underneath the filter plate if the vacuum pressure is too great. Foaming has the potential to cause cross-contamination between samples during extraction.
- When using less than half of the 96 wells in the filter plate during an extraction, the VacAssist™ membrane was essential for creating an even vacuum pressure across the filter plate, and a balanced flow of buffer through the filter plate wells.
- Heating of the elution buffer was essential to release all of the nucleic acids from the glass microfibre filter.
- After elution, complete rehydration of nucleic acids overnight at 4°C was essential to avoid false-negative PCR results (Koller *et al.* 2000), and was particularly necessary

when the concentration of eluted DNA was low. The effect of inadequate rehydration was particularly evident with *L. garvieae* in the presence of co-extracted non-target DNA.

When the protocol described in the methods was followed closely, extraction efficiency using the Whatman vacuum system was reproducible and there was no evidence of sample cross-contamination. The system allowed for rapid, high-throughput and inexpensive nucleic acid extraction.

8.2. Determine optimum conditions for the PCR test to maximise specificity and sensitivity of the procedure.

8.2.1. Optimisation of PCR and RT-PCR sensitivity without compromising specificity.

As detection of carrier status in fish was the ultimate aim of the project, optimisation of PCR and RT-PCR protocols with reference nucleic acids was crucial in achieving optimum sensitivity of the complete system. It was equally important to retain the integrity of test specificity and this limited the range of PCR optimisation possibilities. In the case of *A. salmonicida*, magnesium chloride concentration was critical but for the other pathogens, annealing temperature was the crucial factor. The ability to detect very small amounts of nucleic acid by PCR or RT-PCR was only possible with the combination of improved *Taq* polymerase, and PCR cycling conditions. Detection levels using reference nucleic acids were used as measures of performance when extracting nucleic acids from the selective-enrichment media with and without fish tissue inocula. Establishing performance of the assays under these conditions enabled future PCR performance to be monitored to determine if test performance had been compromised through inefficient extraction of nucleic acids or by the presence of inhibitors arising from unusual fish samples.

PCR and RT-PCR specificity were not compromised beyond the inherent limitations of the PCR primers themselves and their corresponding internal confirmation probes. Specificity was 100% for the *T. maritimum, L. garvieae* and *Y. ruckeri* PCR and RT-PCR protocols using the range of sequence similar and phenotypically relevant organisms. Similarly, specificity was good for *A. salmonicida* with only the expected cross-reaction of some

strains of *A. hydrophila* and *A. bestiarum* (Carson 1998). Due to this cross-reaction, positive *A. salmonicida* reactions should always be confirmed by sequencing the PCR amplicon or by recovering the organism in culture and verifying its identity by phenotyping (Carson *et al.* 2001a).

8.2.2. Optimisation of the selective-enrichment (SEC) vacuum extraction-PCR protocol.

An obvious strategy to improve detection sensitivity was to increase the sample volume of eluted nucleic acid. When template was added to the PCR reaction-mix at a ratio of 1:10, the eluted DNA obtained from the filter plate was suitable for direct PCR. However, when a greater volume of eluted DNA was used, false negative PCR results occurred. High volumes of TRIS-EDTA inhibited the PCR amplification by chelating magnesium ions in the PCR reaction-mix. Other PCR inhibitors, possibly the carry-over of salts during extraction (Boom *et al.* 1999) were neutralised by the addition of BSA to the PCR reaction-mix. BSA levels of 400 ng μl^{-1} or less (Romanowski *et al.* 1993; Kreader, 1996; Yu and Mohn 1999) gave inconsistent results. Complete relief from inhibitors was achieved when BSA concentration was raised to 1.5 μg μl^{-1} and it was possible to add DNA template to the reaction-mix in a ratio of 2:5. High concentrations of BSA do not appear to have a negative impact on PCR and concentrations as high as 6μg μl^{-1} have been used to facilitate PCR in the presence of inhibitors (Al-Soud and Rådström 2000).

The detection levels (see Table 7.5) for the four target pathogens range from 1-16 cfu 200μl^{-1} and compare very favourably with other methods of extracting DNA from enrichment media. Giesendorf *et al.* (1992) were able to detect 100 cfu *Campylobacter* spp. in 200 μl of medium, while Lindqvist (1999) could detect 1-2.0 x 10^4 cfu *Shigella* spp. in 200 μl of enrichment broth.

Using the reference DNA, the detection level for the four target pathogens was approximately 4 fg or one bacterial genome equivalent, to one bacterial cell (Table 7.5). These values represent an absolute for the assay as configured and it is apparent that the detection values achieved from the enrichment media and the filter plates is not as high (Table 7.6). The difference is likely to be due to lysis inefficiency, nucleic acid capture, elution efficiency or co-elution of inhibitors. Increasing sensitivity by increasing template volume was only partially successful. The PCR used in this study is in a micro format,

which has the advantage of reagent economy, and faster thermodynamics which improve fidelity of the reaction as well as shortening cycle times and hence test running time. Larger volumes of template could have been accommodated but this would have necessitated increasing reagent volume and consequently the cost of the test. As formatted, about a quarter of the 35 μl of eluted DNA is used. It was felt that doubling the template volume to 16 μl as a means of further increasing sensitivity was not justified given the already very high sensitivity of the assay and the fact that the test as a whole has a culture enrichment step to amplify low numbers of bacteria.

When a selective-enrichment medium is used to target a particular pathogen, the chance that a competing organism may out-grow the target is very real. The DNA from a competitor will be co-extracted with the target pathogen and has the potential to decrease PCR sensitivity (Giesendorf *et al.* 1992; Swaminathan and Feng 1994; Weaver and Rowe 1997). In extraction trials using a *Providencia* sp. as the competitor, no reduction in detection sensitivity was demonstrated at any of the ratios tested so that as little as 1 cfu 200μl^{-1} selective-enrichment medium of the target could be detected at a competitor concentration of 2×10^8 cfu 200μl^{-1}. This ratio represents the worst case likely to be encountered and equates to the target failing to grow and competitors growing in the medium without constraint. The capacity of the system to detect very small numbers of the target pathogen, under adverse conditions, indicates the robustness of the assay system.

In summary, the SEC-PCR protocol was optimised to detect, by PCR or RT-PCR, between 1 and 16 bacteria 200 μl^{-1} of culture in a convenient, rapid and high-throughput format suitable for testing large numbers of samples simultaneously.

8.3. Optimise PCR conditions to incorporate a PCR protection system to prevent false positive reactions arising from DNA contamination.

8.3.1. PCR contamination control strategies

While PCR provides a fast and sensitive method for detecting the presence of pathogens, its inherent sensitivity means the technique is prone to false-positive results arising from amplicon carry-over (Fahle, *et al.* 1999; Vaneechoutte and Van Eldere 1997). The

frequency of these erroneous results can be greatly reduced by following strict containment procedures such as separate rooms for sample preparation and amplification, dedicated equipment, barrier pipette tips, etc. (Kwok and Higuchi 1989). Containment procedures however, are not always sufficient to prevent carry-over contamination, the result of aerosol formation or pipetting PCR amplicons (Cimino, *et al.* 1990; Sarksena, *et al.* 1991).

Several PCR sterilization strategies have been developed with varying degrees of success. UV irradiation of reactants before PCR commences can be used to reduce the chance of false-positive PCR reactions (Ou *et al.* 1991; Padua *et al.* 1999). While this method can help to eliminate low-level template contamination, UV irradiation can compromise the integrity of primers and thermostable polymerases, which can cause a reduction in assay sensitivity which can in turn be the cause of false-negative reactions. Uracil N-glycosylase (UNG) inactivation is reported to greatly reduce the chance of false-positive results due to amplicon carry-over (Burkardt 2000; Pang *et al.* 1992). However, the UNG method has some disadvantages in that efficiency is partially determined by the amount of thymine in the amplicon sequence and length (Isaacs *et al.* 1991). UNG is expensive and care must be taken to prevent residual UNG activity from degrading amplified product prior to analysis (Rys and Persing 1993; Thornton *et al.* 1992).

Both UV irradiation and UNG treatment are pre-PCR contamination control methods, which have no downstream capacity to render amplicons 'sterile' after amplification. Post-PCR amplicon inactivation, a preferred and more conservative control strategy, is possible using psoralens. On UV activation, psoralens form stable cross linkages in double stranded DNA, which prevents polymerase activity in any subsequent PCR. The versatility of the system and its capacity to inactivate amplicon *in situ* makes this a useful and practical control measure.

8.3.2. Addition of isopsoralen (IP-10) to inactivate amplicons and prevent false-positive PCR reactions.

Psoralens are a type of furocoumarin, a class of planar, tricyclic compounds that intercalate between base pairs of nucleic acids and may be linear in form or occur as an angular isomer, isopsoralen. Psoralens and isopsoralens inactivate in a similar way and are

equally effective (Cimino *et al.* 1991). During UV activation psoralens form monoadducts or can bifunctionally bind to both strands of DNA to form interstrand derivatives. The effect of such modification prevents *Taq* polymerase from processing along the amplicon and thereby stopping PCR amplification (Persing and Cimino 1993), see Figure 8.1. The photochemical IP-10, a proprietary amino derivative of isopsoralen (Cerus Corporation), forms monoadducts as cyclobutane rings at adjacent thymine pyrimidine nucleotides when activated by UV light at 300-400nm. As isopsoralen does not form interstrand adducts, the DNA molecule can be analysed post-PCR using hybridization techniques that require single stranded DNA (Cimino *et al.* 1991).

UV

IP-10 is incorporated into the PCR reaction mix prior to amplification

A   C   T.T   A   C.C   A   A
T   G   A   A   T   C   G   T   T

After amplification, UV irradiation causes IP-10 to form monoadducts, principally between adjacent thymine and sometimes cytosine pyrimidines, that block polymerase processing in subsequent PCR. As no interstrand adducts are formed, subsequent hybridization reactions are not affected.

IP-10 monoadduct

Figure 8.1   Effect of UV activation of the photochemical IP-10 on double stranded DNA

Complete amplicon inactivation requires IP-10 to create at least one adduct per strand of DNA. In practice, to ensure an adequate level of binding, Poisson statistics require a minimum average of 20 effective adducts per strand. Since isopsoralens can create one adduct per 11 to 100 nucleotides (Persing and Cimino 1993), depending on A+T ratio and UV dose, complete inactivation requires amplicons to be at least 300-350 bp in length or have a strong bias towards pyrimidine nucleotides (Espy, et al. 1993; Persing and Cimino 1993). Amplicon length of the four target pathogens are typically short and range from 145 to 288 bp and have A+T percentages which are as low as 45%. Given these inherent structural limitations of the amplicons, total inactivation could not be achieved. While the definition of effective inactivation generally depends on the requirements of individual laboratories, inactivation of 10^5 amplicons is considered a minimum requirement (HRI Research Inc. 1991). De la Viuda *et al.* (1996) and Espy *et al.* (1993)

successfully inactivated 10^5 amplicons of *Borrelia burgdorferi* (156-bp) and human papillomavirus type 16 (180-bp) using 100 µg ml^{-1} IP-10. Using the definition of 10^5 amplicon inactivation we were able, using the protocol reported in this study, to inactivate at least 6×10^7 amplicons, a hundred fold increase in efficiency using an IP-10 concentration of 40 µg ml^{-1}. In real terms, inactivation of 10^5 amplicons would decrease the chance of contamination due to aerosols (Cimino *et al.* 1990) and the increased level of efficiency reported here improves the level of PCR protection against contamination events.

Amplicons treated with IP-10 appeared to have an increased mass compared with amplicons not containing IP-10. This increase in mass occurs as IP-10 binds to thymine nucleotides, increasing the apparent size of the amplicon. Therefore, an increase in the mass of the amplicon is evidence of the formation of adducts and is an important factor in determining the effectiveness of IP-10 inactivation (Cimino *et al.* 1991). The larger amplicons of *T. maritimum*, *A. salmonicida* and *Y. ruckeri* had a greater increase in mass than the smaller *L. garvieae* amplicons as expected, see Table 7.6. Increase in mass however, is not just a function of amplicon size but is also dependent on the proportion of pyrimidines which can form adducts with IP-10. Although the amplicon of *Y. ruckeri* is smaller than that of *A. salmonicida*, the increase in mass was greater in *Y. ruckeri* as it has a higher A+T ratio than *A. salmonicida* and hence has more IP-10 binding sites. For the four species investigated, an increase in size of the amplicons appeared to match the efficiency of IP-10 inactivation.

Incorporating IP-10 into the PCR protocol effectively prevented false-positive results without affecting the specificity or sensitivity of the PCR. Existing protocols were readily adapted to include IP-10 and adequate inactivation was achieved using a standard UV transilluminator or UV crosslinker and the use of a freezer brick to cool the sample during irradiation. While this protocol renders a large proportion of the amplified product unable to re-amplify, it should be used to complement strict containment procedures to prevent false-positive PCR reactions by laboratories that frequently perform PCR, especially those involved in disease diagnosis.

8.4. Develop a test procedure based on enzyme hybridization assay (EHA) that will verify any positive PCR reactions using a secondary confirmatory gene probe and in a format suitable for testing multiple samples.

8.4.1. Development of a high-throughput PCR enzyme hybridization assay (EHA) for sensitive, low cost, DNA detection.

When biphasic PCR was performed in NucleoLink tubes using the same cycle conditions as the regular PCR, the sensitivity decreased from 4 fg to about 40 fg for each bacterium. This reduction in amplicon yield was attributed to the thicker walls of the NucleoLink tubes. Increased mass slows the thermodynamics of the system, which can have a profound effect on PCR reaction kinetics. The sensitivity of the test was restored to the low femtogram range by increasing reaction time parameters of the PCR, a strategy that ensured that sufficient time was programmed for the different reaction stages of the cycle. Further optimisation was achieved by varying primer concentration and conjugate (streptavidin-alkaline phosphatase) concentration. The sensitivity values achieved compare favourably with the 100-300 fg of *Campylobacter jejuni* and *C. coli* DNA detected using NucleoLink tubes by Grennan *et al.* (2001). The sensitivity achieved was dependent on the primer concentration used. Only *A. salmonicida* gave optimum results using the primer levels previously reported by Oroskar *et al.* (1996) as generally suitable for use in NucleoLink tubes (0.5 µM of the forward and 0.0625 µM of the reverse primer). For the three other target pathogens lower primer concentrations, typically half those of *A. salmonicida*, gave the best detection levels. Optimum primer concentrations were defined as those concentrations that gave maximum sensitivity, and gave complimentary results with both agarose gel electrophoresis and EHA OD values. Reactions that gave a strong gel and weak EHA readings or reactions that gave strong EHA and weak gel readings were considered sub-optimal. Optimisation of the colour development time resulted in two cut-off values, 1.2 times the negative control after 0.5 to 2 hours and 1.4 times the negative control to a maximum of about 16 hours. Due to the occasional occurrence of weak positive readings not easily distinguishable from negative values after 0.5 hours, every PCR-EHA assay was allowed to develop for 16 hours. This incubation period could not be extended beyond about 18 hours, as after this time some false-positive results occurred.

When performing PCR-EHA using DNA extracted from selective-enrichment media as a template, the sensitivity achieved for *L. garvieae* was less than that achieved for the other bacteria. This was probably due to inefficient lysis during the DNA extraction procedure. Increasing the concentration of lysozyme or lengthening the incubation time did not improve sensitivity. The sensitivity for the other three bacteria was similarly reduced compared with pure DNA, probably the result of inefficiencies with the DNA extraction procedure. The main limitation to sensitivity in the procedure is the ability to add only one quarter of the eluted DNA to a single PCR reaction. During extraction, the bacterial DNA in a 200 μl sample of enrichment-culture medium is concentrated into about 35 μl of DNA elute. To add this quantity to a single reaction, the total volume of the PCR mix would need to be significantly increased. While this is possible, the cost per test would be substantially increased. If the total volume of eluted DNA were used in one PCR reaction, the sensitivity of the PCR-EHA system for 200 μl of enrichment broth would be 4 cfu for *L. garvieae* and 1 cfu for the three other bacteria.

The specificity of the PCR-EHA system was identical to that achieved for conventional PCR discussed in section 8.2.1. Clear, unambiguous reactions were evident, even with low concentrations of template DNA, and there was complete agreement between the presence of amplicon in the liquid-phase of the PCR as detected by gel electrophoresis and the solid-phase amplicon detected by EHA.

In conclusion, a highly sensitive and specific system that detects DNA from the four target pathogens was developed. The system is optimised at every step to provide streamlined high-throughput sampling. The one-tube system decreases processing time and the risk of cross-contamination by reducing the handling of PCR amplicons. NucleoLink tubes are available at a low cost of A$12 per 96-wells for a combined PCR/hybridization test, compared with the main alternative, streptavidin-coated plates which cost from A$45 to A$50 per 96-wells for hybridization only.

8.4.2. Development of a high-throughput RT-PCR enzyme hybridization assay (EHA) for sensitive, low cost, detection of rRNA.

The sensitivity achieved for RT-PCR-EHA was equal to or greater than that achieved for PCR-EHA. As with the PCR-EHA the sensitivity achieved for *L. garvieae* was less than that

achieved for the other bacteria, this was due to the limitations of lysis as discussed in section 8.4.1. Like the PCR-EHA, the sensitivity achieved using RNA extracted from the selective-enrichment medium was slightly less than that achieved with reference RNA, due to inefficiencies in lysis, binding and elution. If the total volume of eluted RNA could be used in one RT-PCR reaction, the sensitivity of the RT-PCR-EHA system for 200 µl of enrichment broth would be 1 cfu for each bacterium. The theoretical detection limit compares favourably with the four target pathogens which ranged between 9cfu for *L. garvieae* and 1cfu for *A. salmonicida* and was considered to be sufficiently sensitive in conjunction with the selective enrichment media.

The specificity of the RT-PCR-EHA system was identical to that achieved for the conventional RT-PCR protocol discussed in section 8.2.1. Clear, unambiguous reactions were evident, even with low concentrations of template RNA, and there was good agreement between the presence of amplicon in the liquid-phase of the RT-PCR detected by gel electrophoresis and solid-phase amplicon detected by EHA. With the RT-PCR-EHA system, positive EHA results were sometimes seen where gel electrophoresis was negative. This only occurred when the sample was know to contain RNA, therefore in some cases the EHA was more sensitive than the electrophoresis in detecting the presence of RNA.

IP-10 for contamination control was attempted with RT-PCR but caused a significant decrease in test sensitivity. Steps to minimise this effect included decreasing the temperature for the cDNA step, adding extra magnesium and increasing the number of amplification cycles. While decreasing the cDNA temperature improved sensitivity, the level of inhibition was still unsatisfactory. To ensure a useful threshold of detection the use of IP-10 was abandoned with RT-PCR.

8.5. Optimise the culture conditions and PCR detection process to ensure the minimum test time between sample collection and test result.

Emphasis on streamlining high-throughput processing was applied at all stages of the project as a means of fulfilling the requirements of Objective 5; specific aspects are described in 8.5.1. The usefulness of the system was improved by the development of HK3C, a selective enrichment medium specific for *A. salmonicida* biovar *acheron*, a new

atypical strain, the cause of significant disease in marine farmed Atlantic salmon. Details relating to the development and testing of the medium are discussed in this section.

8.5.1. Test optimisation strategies

A major emphasis of the project was to develop a detection system that could be used for screening large numbers of fish with a practical, convenient and inexpensive technology. The capacity to test large numbers of samples was considered essential as detecting prevalence in populations requires the testing of large numbers of individuals, particularly if prevalence is low. Assuming a test has very high level of specificity and a good level of sensitivity, it requires 150 fish from a population of 100,000 to be tested to detect a 2% prevalence (des Clers, 1994). Given sample sizes of this magnitude, processing must be streamlined if prevalence surveys are to be undertaken with any likelihood that the process can be achieved on a realistic and achievable basis.

The primary sample in the selective-enrichment medium has to be undertaken in tube format to ensure an adequate sample size, a prerequisite for the test as a whole. After incubation, the remainder of the test steps is conducted in a high-throughput format using multichannel pipetting devices and 96-well format reaction vessels at all subsequent steps. A flow diagram of the process is given in Figure 8.2. The system devised represents an

Step	Format	High throughput	Function
Enrichment culture	Racked culture tubes	No	Primary amplification of target pathogen
↓			
Nucleic acid extraction	96 well	Yes	Simultaneous processing of up 96 samples: cell lysis, DNA purification, concentration and extraction. Complete process in the one action.
↓			
PCR-EHA RT-PCR-EHA	96 well	Yes	Biphasic PCR followed by probe hybridization to confirm result and indicate positive finding. Two step process in the one reaction well.

Figure 8.2 **Flow diagram showing major process steps of SEC-PCR-EHA and stages with high throughput formats**

economic and practical methodology for testing populations of fish for the presence of covert infections. The SEC-PCR-EHA test is comprised of three components, of which two are formatted as high throughput processes, each of which are run in the one reaction vessel. The advantage of this arrangement is the reduced number of operations and need to handle test products, thereby reducing the risk of process error or contamination with the attendant dangers of generating false positive reactions as a consequence.

Although not part of test performance, an estimate of cost of materials for undertaking both SEC-PCR-EHA and SEC-RT-PCR-EHA was determined as a guide. For the PCR form of the test, the cost per sample is A$2.50 while for RT-PCR the cost is A$3.30 per sample. The major cost items in both cases are summarised in Table 8.1. The single most expensive item is *Taq* polymerase required for PCR, while for RT-PCR, the cost of the reverse transcriptase and combined *Taq* polymerase is the most expensive component; these costs do not include labour. While the individual cost of each test is low, when

Table 8.1   Major cost (A$) items for SEC-PCR-EHA test, per 150 sample

Material	DNA-PCR	RT-PCR
Selective medium	$40	$40
Lysis buffers with guanidinium salt	$30	$3
96 well filter plates	$50	$50
Pipette tips	$45	$47
DNA *Taq* polymerase	$60	$0
dNTPs	$10	$0
RT-*Taq* polymerase	$0	$180
Primers	$8.50	$8.50
ELISA developing substrate	$30	$30

undertaking large scale survey programmes involving many populations of fish, the cost of surveillance will be substantial. Costs could be lowered if samples are pooled, but the rationale for doing so would be dependent on the nature of the survey and the data required.

8.5.2. Minimum Inhibitory Concentration (MIC) assays for the development of HK3C a selective-enrichment medium for *Aeromonas salmonicida* biovar *acheron*.

The new atypical form of *A. salmonicida* biovar *acheron* grew poorly in the selective media that had been developed previously for the greenback flounder and goldfish ulcer

biovars of *A. salmonicida*. Given that *A. salmonicida* biovar *acheron* was the cause of a significant disease event in farmed Atlantic salmon, there was a real need to develop an efficient medium that could be coupled with the PCR-EHA process. *A. salmonicida* biovar *acheron* showed good resistance to three core ingredients of the selective medium developed for *A. salmonicida* (Carson *et al.*, 2001b). The antibacterials, compounds H, K and C1, have been shown previously to inhibit 93% of a library of 400 normal fish flora of salmonids. Following selective screening of additional compounds, C2 showed good potential for inhibiting some resistant *Pseudomonas* spp. and Enterobacteriaceae and raised the overall inhibitory level in respect of the library of normal flora to 94%. During the *A. salmonicida* fish trial conducted in March 2001, a particularly resistant strain of *A. hydrophila* was isolated from the prototype selective medium. Since some strains of *A. hydrophila* are known to cross react with the 16S rRNA primers for *A. salmonicida* (Carson 1998) further screening of antibiotics was undertaken with a view to improving selectivity further. Using MIC assays, compound C3 at a concentration range of 31.25 - 62.5 $\mu g\ ml^{-1}$ was found to inhibit the growth of this resistant strain of *A. hydrophila* without inhibiting *A. salmonicida* biovar *acheron* which was resistant at these concentrations.

8.5.3. Chequerboard MIC assays in the development of HK3C a selective-enrichment medium for *Aeromonas salmonicida* biovar *acheron*.

As no antagonistic or synergistic effects were found between any of the selective agents for the proposed enrichment medium HK3C, the concentrations of antimicrobial used in the final formulation were determined by the highest concentrations that had no suppressing effect on the growth of *A. salmonicida* biovar *acheron*. Chequerboard titrations can identify several possible optimal concentrations of the selective agents as the assay is usually asymmetric in regard to the assayed components with $c_1\uparrow$ and $c_2\downarrow$ or $c_2\uparrow$ and $c_1\downarrow$, see Figure 8.3.

Sequential testing of these antibacterial combinations identified the optimal concentrations in respect of *A. salmonicida* biovar *acheron*. Final assessment of the formulated medium was undertaken using the library of 400 normal fish flora and was shown to be able to suppress 97% of the flora. Selective enrichment media are unlikely to suppress all non-target floras and it is expected that some competitors will be encountered with normal samples collected from target sites. There are few reports that

measure directly the level of suppression of potential competitors, the performance of most enrichment media are assessed in comparative studies to determine relative efficiency in the recovery of the target organism. In a recent study (Baylis *et al.* 2000) however, the rate of false positives based on resistant non-target species is given for several selective enrichment media for *Campylobacter* species. From the false positive rate it is possible to infer the proportion of the flora in the sample population that were inhibited and ranged from 77% to 88% depending on the type of selective enrichment broth. The inhibition capacity of 97% for HK3C compares favourably with the performance of these *Campylobacter* selective enrichment media.

		Concentrated ———— H ————— → dilute									H	K	Control
Conc. K		1	2	3	4	5	6	7	8	9	10	11	12
↓	A												
↓	B												
↓	C												
↓	D												
↓	E												
↓	F				B								
↓	G			A									
Dilute	H												
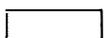
▨ Growth   ▨ PLC   ☐ No growth

$A = c_1\uparrow, c_2\downarrow$ $B = c_1\downarrow, c_2\uparrow$ $c_1 =$ compound K, $c_2 =$ compound H   PLC = product limiting concentration

Figure 8.3   **Typical chequerboard titration with asymmetric assay results for two antibacterials, H and K.**

8.5.4. Most Probable Number (MPN) assays to determine the effect of the selective agents on the growth of *A. salmonicida* biovar *acheron*.

If the choice of selective agents and the concentration they are used at are not well calibrated, the selective medium may be too stringent and the recovery of target pathogen can be compromised. Medium composition may affect both sensitivity and specificity in that different strains of the target pathogen may perform differently in the presence of the selective agents. Performance of HK3C in this regard was assessed by MPN assay by comparing the success of initiating growth with varying inoculum size using HK3C and the non-selective basal medium. Using MPN, it is possible to use inoculum sizes containing as little as three cfu. Even at very low inoculum levels, it was established that there was no significant difference between the selective and non-selective form of HK3C. Theoretically therefore, if three viable cells of *A. salmonicida* biovar *acheron* are inoculated into the selective medium they would have as much

chance of multiplying to a detectable level as if they had been placed in the non-selective medium. It should be borne in mind that this is a comparative assay and assumes that the inoculum contains cells that are capable of multiplication. Similarly, caution should be applied in extrapolating the results to field samples which themselves may compromise performance with the introduction of fish tissue and resistant flora that may act as direct competitors. Nevertheless, the MPN assay does indicate that the medium has been well optimised and inherently does not appear to compromise growth of *A. salmonicida* biovar *acheron*.

8.5.5. Evaluation of the capacity of *A. salmonicida* biovar *acheron* to grow in HK3C with competition.

Performance of selective enrichment media needs to be assessed in relation to likely competitors, that is, the flora resistant to the selective components of the medium. Resistance is a relative term in that a competitor may be weakly or strongly inhibited. A weakly inhibited competitor may grow rapidly and have a pronounced negative impact on the target species while a competitor that is strongly inhibited may only grow slowly and have little effect on the target. Performance of HK3C was measured by determining EI values with a strong and weak competitor, two types of pseudomonad known to tolerate the inhibitory agents of the medium. The EI values for *Pseudomonas* sp. B127 and *Pseudomonas* sp. M146 at 5 days were −1.8 and 0.42 respectively indicating that strain B127 clearly out-competes *A. salmonicida* biovar *acheron* but not strain M146. The level of competition and the effect on the target organism has to be assessed in regard to the purpose of the medium. EI values of +2 or less indicate a medium with weak selective enrichment potential (Rhodes *et al.* 1985). This criterion is based on recovering the target organism by subculture, which requires a high ratio of target to competitor for visual detection of colonies on culture plates. Where PCR is the detection method and not subculture, enrichment efficiency does not need to be as good because the test format (nucleic acid extraction + PCR) provides a high level of sensitivity and may compensate for a lower enrichment efficiency. Even where *Pseudomonas* strain B127 clearly out-competed *A. salmonicida* biovar *acheron* there was a 1000 fold increase in cell density, a level more than sufficient to be detected by PCR. For *A. salmonicida* biovar *acheron* the lower detection limit, even in the presence of competitor DNA, was 2cfu 200 μl^{-1} of medium, equivalent to 10cfu ml^{-1} (see Table 7.8)

8.5.6. Validation of the high-throughput SEC-PCR system.

Physical performance of the system and practicality of use was assessed as summarised in 8.5.1. Of major concern was the risk of contamination during nucleic acid extraction arising from cross-well contamination and during set up of large scale PCR across a 96 well PCR plate. Similarly, uniformity of PCR dynamics across 96 wells was also considered a potential problem with a possibility of different reaction kinetics at different wells. As indicated in the results, there was no evidence of well-to-well cross contamination during extraction. Great care is required to control vacuum pressure to the values specified and to ensure that the flow regulator plate is used to balance well-to-well flow. This was particularly important when 96 samples were being processed, as vacuum pressure was greater when all wells were used. Plate sealing for PCR was found to be critical as any loss of vapour from a reaction well may lead to a false negative reaction. Sealing was a problem when raised rim plates were used and care is required to ensure that the plate and seal types are fully compatible. NucleoLink strips and their proprietary tape sealing technology provided trouble free sealing.

Well-to-well variation of PCR reaction, due to variability in thermodynamics across the PCR thermocycler, is largely a function of instrument design. All the work in this study was undertaken using an MJ Research PTC-100-96V thermocycler and with this instrument, there was no evidence that well-to-well thermodynamic variation was sufficient to cause false negative reactions in any of the 96 wells.

8.6. Test populations of salmonids with the optimised SEC-PCR-EHA system to verify test performance and obtain baseline data on carrier prevalence.

8.6.1. Performance of SEC-PCR-EHA and SEC-RT-PCR-EHA with fish survey field samples.

High throughput performance of the integrated system
An overriding consideration during all stages of development of the system was to ensure that large numbers of samples could be processed in a practical and economic fashion. Testing of the components of the assay, from nucleic acid extraction through to amplicon detection and verification, was always undertaken with a view to high throughput processing. Given this emphasis, testing of the integrated system with field samples

proved to be straightforward and there were few unexpected problems either logistically or from test chemistries.

Like all such high volume testing, preparation of materials prior to testing was an important aspect of the process. Many of the materials and reagents required for the assay have good shelf lives, including reagents specifically prepared in-house. During test development and trials it became particularly evident that some key reagents have a limited life and laboratories contemplating using this technology must have in place good systems to monitor reagent quality. Components known to have a limited period of activity include:

- dNTPs (12 months or as advised by manufacturer)
- streptavidin-alkaline phosphatase (6 months or as advised by the manufacturer)
- lysis buffers L6 (4 weeks, DNA; 2 weeks, RNA) and L2 (3 weeks)
- primer coated NucleoLink tubes for biphasic PCR (2 months)
- IP-10 titration with new batches to verify optimum concentration

Following the incubation period required for the selective enrichment phase of the test, processing of the culture media for PCR and verification can be undertaken in less than 9 hours. This time frame is adequate where strong positive reactions occur, but to develop any weak reactions a further 12-18 hours incubation is required. If testing is not urgent, the assay can be halted at several points without jeopardising the results. Break points occur following nucleic acid extraction where the sample can be frozen and held for several months; once PCR commences then the test should be completed through to the conclusion of hybridization and colour development. Given that the colour development period may require up to 18 hours incubation, this is best undertaken overnight. A practical work flow for testing is given in Table 8.2 and was found useful where testing is

Table 8.2   Work flow plan for SEC-PCR-EHA

Task	Day	Timing
Enrichment culture	1-5	Culture incubation
Nucleic acid extraction	5	Extract and rehydrate overnight
PCR-EHA	6	PCR and primary colour development, continue overnight
EHA development	7	Final reading of weak reactions

not urgent. Given that much survey work is undertaken for health monitoring, the work flow plan is well suited for this purpose.

Care was also given to the use of positive controls when running the test. High template concentrations in the region of 1 pg DNA can be used as a gross indicator of test performance but using a control at this level neither reveals how well the system is optimised nor its capacity to detect low template concentrations. In all assays conducted using field samples, it essential that a low template concentration of 100 fg DNA is used. While this concentration is well above the detection threshold of the test, it is sufficiently close to the threshold to be a good marker of test performance.

PCR and RT-PCR as detection strategies

During preliminary field trials, anomalous results were obtained when comparing PCR-EHA results with subculture from the selective enrichment medium to recover the target pathogen. It was anticipated that there would be agreement between PCR and culture result, however in many cases there appeared to be a high rate of false positive reactions with PCR, assuming culture to be the gold standard reference. In many instances, non-target resistant flora that can tolerate the culture medium were recovered. In such cases, the resistant flora was checked for PCR specificity to ensure that there was not cross-reaction. In all such cases the results were negative, providing further evidence of PCR fidelity. In other instances, weak PCR-EHA reactions were detected, but on subculture, the medium appeared to be sterile as no bacteria were recovered. In both situations culture and PCR result could not be reconciled.

A potential limitation of enrichment culture as a means of amplifying the target pathogen is a problem associated with recovering viable cells that may be injured or in a viable but non-culturable condition (Busch and Donnelly 1992; Wai *et al.* 2000). In addition, the tissue sample inoculum may contain remnants of the target pathogen as cells or DNA fragments which, depending on test sensitivity, may be detected by PCR (Høie *et al.* 1996; Miriam *et al.* 1997; Waage *et al.* 1999). PCR coupled with enrichment culture has been shown to be more sensitive than culture alone (Chiu and Ou 1996; Thisted Lambertz *et al.* 1996; Feder *et al.* 2001) and it is likely that the PCR positive, culture negative reactions are due to non-viable cells or remnant DNA. A cornerstone of the SEC-PCR-EHA technology has in large part relied on growth of the target organism in the

enrichment medium as a means firstly of amplifying the numbers of target organism, and secondly, by adding a measure of confidence to positive PCR findings on the basis that these have been derived from live cells in the culture medium. The observation that PCR positive/culture negative (P+/C-) reactions may occur required some adjustment to the basis for interpreting results, the type of PCR test that should be used, and its application.

Of primary concern was establishing the reliability and significance of these anomalous results. Given the sensitivity of the PCR assay that has been developed and that the assay may detect remnant DNA, non-viable and viable cells, it is considered that P+/C- results are of meaning, and proof that the sample contains direct evidence that the fish tissue was exposed to the target pathogen. Acceptance of this view is supported by observations arising from testing fish with known history of disease.

In all cases of P+/C- samples, the band in agarose gels was always faint, a so-called 'ghost' band. In time course studies of P+/C- samples, a band was visible two days after sample inoculation and remained at the same level of intensity when the culture was tested five days later. In 30% of these cases the 'ghost' bands had disappeared, suggesting degeneration of target DNA during this period of incubation. In contrast, band intensity of P+/C+ samples was clearly evident at day two and by day five band intensity had increased, as expected, following further growth of the target pathogen in the selective enrichment medium.

Further evidence was obtained to support the contention that P+/C- samples were the result of detection of non-viable target pathogen and not the result of cross-reactions with non-target resistant flora. Rainbow trout, clinically normal and intrinsically resistant to infection with *Y. ruckeri* serotype O1b, were tested by SEC-PCR-EHA. These fish were from a site that also hold Atlantic salmon which had had several low grade disease outbreaks with *Y. ruckeri* preceding the time of sampling. Of the 18 fish tested, all were PCR negative and culture negative except for one fish, which was P+/C+ for *Y. ruckeri*. Given the evidence, it is concluded that PCR positive findings are the result of detecting target pathogen DNA.

From the field testing undertaken, it is apparent that using SEC-PCR may be misleading in that it suggests that all positive findings are the result of viable target pathogens. In one of

the first *Y. ruckeri* trials of fish with a history of sporadic episodes of yersiniosis, the carriage rate was determined to be 23% but in follow up confirmatory testing, the true carriage rate was only 4%. The evidence here indicates that positive findings may arise from viable and non-viable cells and the test does not provide *prima facie* evidence of live carriage by fish of the target pathogen. It does however indicate clearly that there has been a *history* of carriage, which may still be active. To resolve the dichotomy of interpretation all PCR positive samples could be tested by subculture to recover the target pathogen and so confirm the status of the PCR reaction. Depending on the source of the sample, detection of the target may not be possible where there is heavy overgrowth by resistant competing flora, which masks the presence of the target pathogen. Reliance on culture alone as a means of confirming the presence of viable cells is problematic and some other proxy measure is required.

RNA is a labile species of nucleic acid that occurs in the cell as three distinct forms: messenger, ribosomal and transfer. Of these types, mRNA is the most labile and is considered a good indicator of cell viability (Hellyer *et al.* 1999) as it has a half-life of only a few minutes (Kushner 1996). Ribosomal RNA is more stable than mRNA but is nevertheless closely associated with cell viability (Rosenthal and Iandolo 1970). RNA can be detected using RT-PCR. In this form of PCR, reverse transcriptase makes a DNA copy of the target RNA sequence and this DNA copy is then amplified by conventional PCR. The primer sets developed for the four target pathogens are all based on the gene that codes for rRNA and the primers could be used for RT-PCR without modification. Although primers for mRNA may provide a better indication of cell viability than rRNA which is more stable (McKillip *et al.* 1998; Yaron and Matthews 2002), it would require a major change in primer design from a directed to a random target strategy and would require extensive testing to establish specificity (Carson 1998). Given that rRNA is a good proxy measure of cell viability and is less stable than DNA, use of 16S rRNA primers is justified.

Implementation of a RT-PCR detection system provides the basis for a differential test system that can be used for distinguishing live and dead target pathogens. The ability to determine the viable status of target pathogens has a major impact on the basis on which testing is undertaken and the type of information sought regarding the disease history of the population of fish under surveillance (see Table 8.3). Failure to differentiate between PCR, a proxy test and cell viability has the potential to misrepresent the true biological

status of covertly infected fish. Microcosm studies with *A. salmonicida* ssp. *salmonicida* have shown that only P+/C+ samples can infect fish while P+/C- samples failed to cause infection (Stanley *et al.* 2002). Where the purpose of testing however, is intended to demonstrate evidence of any infection, either past or present, then PCR would be appropriate, as the assay uses as template, DNA, known to be stable and enduring (Dupray *et al.* 1997). In such instances any evidence of infection would be of interest.

Table 8.3   Application of PCR and RT-PCR for detecting covert infections in fish

Investigation	Requirement	Target	Test	Outcome
Quarantine	Evidence of infection	DNA	SEC-PCR-EHA	Live or dead bacteria; remnants of cells – evidence of exposure
Stock movement	Evidence of infection	DNA	SEC-PCR- EHA	Live or dead bacteria; remnants of cells – evidence of exposure
Surveillance	Evidence of active carriage	rRNA	SEC-RTPCR- EHA	Live bacteria only
Risk assessment	Evidence of active carriage	rRNA	SEC-RTPCR- EHA	Live bacteria only

Where it is important to establish carriage rates in populations with a history of disease it is important to differentiate between carriage *per se*, as indicated by PCR, and active infection involving viable organisms. The capacity to distinguish between types of carriage may provide a basis of determining if fish are in a period of convalescence following an outbreak based on changing ratios of dead/viable cells over a period of time. If the population is convalescing, then it is anticipated that the ratio of dead/viable cells will decrease, as might the carriage rate decline with time. Further testing of populations would be required to verify this response and establish its usefulness as a health management tool.

8.6.2. Validation of the SEC-PCR-EHA and SEC-RT-PCR-EHA systems by field trial testing.

If, during the initial field trials, every positive SEC-PCR result could be validated by a corresponding positive culture, then validation of the SEC-PCR-EHA technique would be straightforward. As discussed, it was not possible to confirm PCR results by culture in all cases. Consequently testing was undertaken using populations of fish with no history of disease as well as those with recent disease outbreaks as recommended by Hiney and Smith (1998) as a means of validating PCR based tests.

Aeromonas salmonicida

The *A. salmonicida* SEC-PCR-EHA system was successfully validated with 315 fish sampled from farms with some history of disease and 163 fish sampled from farms with no history of disease. The fish from the non-diseased farm came from the same location as the diseased farms. All the samples from the fish with no evidence of disease were negative by SEC-PCR-EHA (the more stringent assay) while positive reactions were obtained from fish at the site with a recent disease episode. The absence of positive reactions in the negative population provided good evidence of the specificity of the test and gave confidence that subsequent positive SEC-PCR-EHA reactions were not the result of cross reaction with non-target organisms.

The second trial tested the system with fish from a farm with no recent history of disease, although the fish had experienced a minor outbreak 12 months previously. Testing of this group of fish required extensive follow up confirmation arising from cross reactions with strains of *A. hydrophila* and provided much additional and valuable information regarding the specificity of PCR primer sets, other than 16S rRNA, used for the identification of *A. salmonicida*.

The initial apparent prevalence for the fish was determined to be 30%, as tested by SEC-PCR-EHA using 16S rRNA primers. Verification of these positive findings was undertaken through sub-culture from the enrichment media. *A. salmonicida* was not recovered from any of the enrichment cultures although, in all cases, a resistant flora comprising mixed aeromonads was present. Given the fastidious nature of *A. salmonicida* it was possible that the pathogen had been overgrown by the more robust aeromonads. Based on previous testing (Carson 1998) the 16S rRNA primers are known to have a high level of specificity for *A. salmonicida sensu lato,* but have a known cross reactivity with some strains of *A. hydrophila* and *A. bestiarum.* By subculture it was established that two strains of *A. hydrophila* formed the predominant component of the resistant flora recovered. In subsequent testing, it was confirmed that these strains cross reacted with the *A. salmonicida* 16S rRNA primers so accounting for the positive SEC-PCR-EHA reactions.

In addition to using the 16S rRNA primer set, all the positive selective enrichment cultures were tested using the *vap*A and PAAS primer sets for *A. salmonicida;* these latter primers are reported to have a combined specificity of >99% (Byers *et al.* 2002a). All tests with

PAAS were negative but 19% of those tested were positive by *vap*A. Follow up testing showed that the *vap*A positive reactions were due to cross reaction with one of the *A. hydrophila* strains resistant to the enrichment culture medium. Hitherto, the *vap*A primer set has been considered to be specific for *A. salmonicida* and not to be found in *A. hydrophila*. Given the significance of the finding, the amplicon was sequenced to confirm that it had been derived from the *vap*A gene and was not the result of a non-specific reaction. Sequencing confirmed that the amplicon had a sequence expected for the *vap*A gene, and by further analysis, it was shown that this strain of *A. hydrophila* has the complete *vap*A gene (N. Gudkovs, Australian Fish Disease Laboratory, CSIRO). The cross reaction of the *vap*A primer set with a strain of *A. hydrophila* is unexpected but given the close genotype of *A. hydrophila* and *A. salmonicida* this finding is perhaps not unexpected. It does also mean that this primer set can now no longer be considered specific for *A. salmonicida* and has important implications for the identification and detection of *A. salmonicida*.

When used for population screening, cross reactivity of the 16S rRNA primer set will generate false positive reactions and, when used alone, will over estimate the true level of *A. salmonicida*. Based on the design of the primer set, the 16S rRNA primer will detect all instances of *A. salmonicida*, biovars and subspecies. Use of other primer sets specific for *A. salmonicida* also presents problems in that both PAAS and *vap*A do not detect all strains of *A. salmonicida*. If the primers are used in tandem then the specificity is >99%, but if used independently, specificity for PAAS and *vap*A is 93% and 93.3% respectively (Byers *et al.* 2002a). Although the *vap*A primer set is considered species specific, it would appear that confidence in the primers is not justified as they cross react with *A. hydrophila*, a species closely related genotypically to *A. salmonicida*. Only the PAAS primer set appears to be specific for *A. salmonicida*, albeit with a level of false negative reactions. Given the known limitations of the available primers it is recommended that populations are screened using the 16S rRNA primer set and any positives are confirmed using the PAAS primers and *A. salmonicida* recovered from the enrichment culture. The basis for screening is summarised in Figure 8.4 and is a framework for testing and confirmation for *A. salmonicida*.

```
                          ┌──────────────┐
                          │  16S rRNA    │
                          │  Positive    │
                          └──────────────┘
                                 │
                                 ▼
    PAAS                 ┌──────────────────┐              PAAS
    Negative             │ Verify with PAAS │              Positive
                  ┌──────┤                  ├──────┐
                  │      └──────────────────┘      │
                  ▼                                 ▼
        ┌──────────────────┐              ┌──────────────────┐
        │  Negative for    │              │  Positive for    │
        │  A. salmonicida  │              │  A. salmonicida  │
        └──────────────────┘              └──────────────────┘
                  │                         ┌──────┴──────┐
                  ▼                         ▼             ▼
        ┌──────────────────┐   ┌──────────────────┐ ┌──────────────────┐
        │  Confirm by      │   │  Confirm by      │ │ Test for A.      │
        │  culture if      │   │  culture if      │ │ salmonicida      │
        │  critical        │   │  critical        │ │ ssp. salmonicida │
        └──────────────────┘   └──────────────────┘ │ with Miyata      │
                                                     │ primers          │
                                                     └──────────────────┘
```
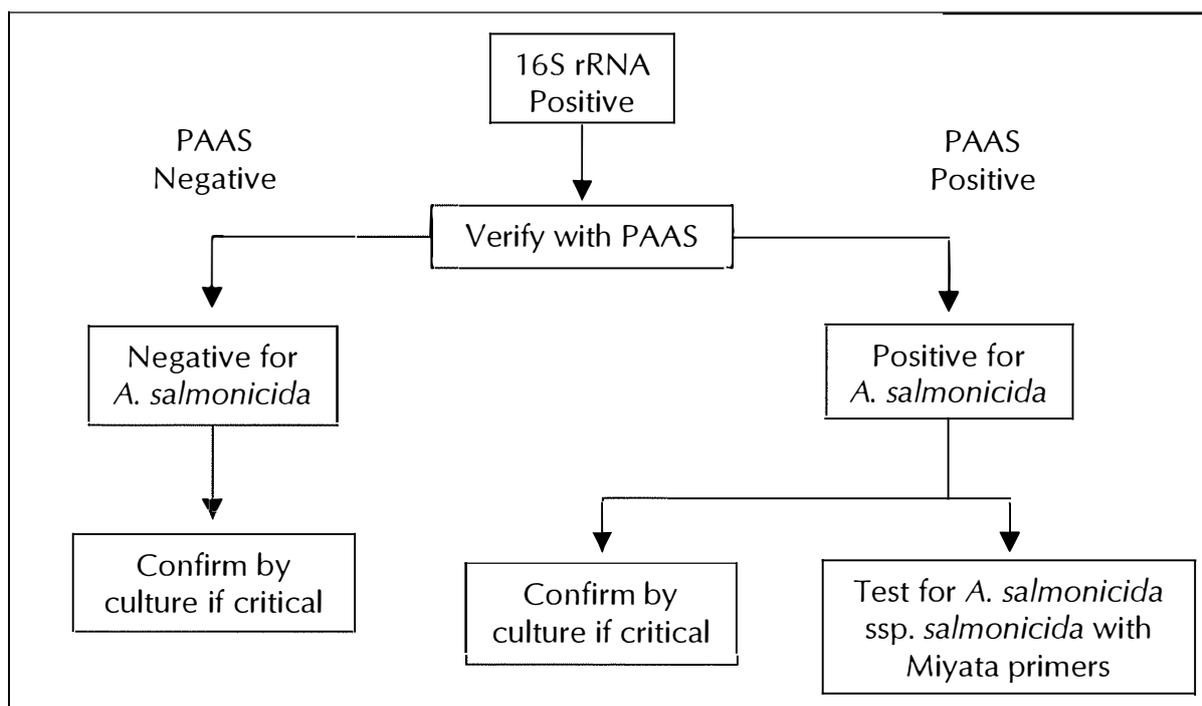
Figure 8.4   PCR screening and confirmation options for the detection of *A. salmonicida*

The recovery of *A. hydrophila* from the selective enrichment medium and the confounding results that this caused, highlighted the need to improve specificity of the medium. The medium was re-formulated to inhibit the growth of these two strains of *A. hydrophila* to arrive at medium HK3C as detailed in section 8.5.2. In the third field trial, the HK3C formulation of the medium was used and this time no strains of *A. hydrophila* were isolated. This trial involved testing fish with a recent history of disease. Samples were collected from cages with different histories ranging from active disease, at-risk and not at risk. None of the tests gave ambiguous results and it was possible to establish the overall prevalence for the lease site as well as cage-to-cage prevalence, which ranged from 0 to 18% carriage of *A. salmonicida* (see section 7.6.2).

Tenacibaculum maritimum

The specificity of the test for *T. maritimum* was very high with no evidence of positive reactions when populations of fish with no evidence of disease were tested. The absence of confounding positive reactions provided a measure of assurance that all positive reactions by SEC-PCR-EHA arose from the presence of *T. maritimum* in the sample. Fish were screened initially using PCR, and all positives were retested by RT-PCR-EHA to determine the status of the reaction: resolved infection or active infection respectively. The SEC-RT-PCR-EHA system proved to be useful for approximating live carriage at farms

with a known history of disease. Carriage rates using PCR ranged from 7% to 25% but using RT-PCR active carriage was typically 1%. All PCR positive enrichment cultures were independently subcultured and recovery of *T. maritimum* matched the RT-PCR results providing further evidence that ribosomal RNA correlates well with cell viability. Specificity for the test was considered better than 99%.

Yersinia ruckeri

It was difficult to determine specificity of the assay by testing *Y. ruckeri* free populations of the target species, Atlantic salmon as infection is widespread in hatcheries. A compromise was reached by testing rainbow trout, a host known to be resistant to infection with *Y. ruckeri* serotype O1b, enzootic in Australia. The population of fish tested was from a hatchery that raises both Atlantic salmon and rainbow trout; the hatchery had a known history of yersiniosis in Atlantic salmon. The specificity of the test was good with no positive reactions detected except for one fish, confirmed by recovering *Y. ruckeri* from the enrichment culture. Based on this testing, the specificity for the test was considered better than 99%.

Testing of SEC-PCR-EHA with Atlantic salmon demonstrated that the prevalence level obtained by PCR could not be substantiated by confirmatory sub-culture. Using subculture as the arbiter, PCR in all cases, overestimated carriage. In the first *Y. ruckeri* trial, PCR-EHA gave a carriage level of 23% but a RT-PCR-EHA of only 4%. In the second trial similar figures were obtained with a carriage level of 8% by PCR-EHA and 4% active carriage by RT-PCR-EHA. In both cases, *Y. ruckeri* could not be recovered in culture. A possible explanation may lie with using rRNA as a proxy measure of viability. Although viability is associated with intact rRNA, it is, compared to mRNA, a stable molecule that may be quite persistent and not always correlate well with culture (Hellyer *et al.* 1999). Based on these results with *Y. ruckeri*, rRNA, while a useful measure of viability, may still overestimate the true level of live carriage.

In the second trial, SEC-PCR-EHA and SEC-RT-PCR-EHA were used as a means of evaluating carriage levels of *Y. ruckeri* in fish vaccinated against yersiniosis. From the limited trial, the live carriage rate in vaccinated and unvaccinated fish was 0% and 4% respectively and the carriage level based on history of exposure was 4% and 8% respectively. The significance of this finding from a vaccination and fish health perspective

is unclear as testing was limited in scale (n=48 per group). It does illustrate however, the potential of this technique as a novel tool for disease investigation and health assessment.

Lactococcus garvieae

Specificity testing of the SEC-PCR-EHA was undertaken using rainbow trout with a known history of freedom from disease caused by *L. garvieae*. All fish tested were negative by the assay and indicated a test specificity of better than 99%. Testing of the complete system was not undertaken with fish known to have a recent history, as there had been no recent disease episodes at the time of testing. Earlier, a farm in New South Wales had an outbreak of streptococcosis and the SEC-PCR system, which, at that point, had not been integrated with the EHA process, was evaluated. Like the assays for the other pathogens where some samples were P+/C-, the test worked well and at least one sample was P+/C+ demonstrating that the system was amenable to confirmatory testing where samples contained viable organism. Although positive SEC-PCR-EHA samples from the field were not available to add to the validation data, there is sufficient evidence based on seeded samples to indicate the utility of the system.

Specificity and validation

A protocol for the validation of PCR based tests has been proposed (Hiney and Smith 1998) based on a rolling system of tests as summarised in Figure 6.4. The process has been followed for all the target pathogens using the SEC-PCR-EHA test. In Step 1 the use and need for the technology must be identified. Application of the test is summarised in Table 8.3 and is intended as a means of detecting covert infections in fish using, in nearly all cases, non-destructive sampling except for *L. garvieae* which requires spleen for testing. Specificity and sensitivity testing through *in vitro* modelling was undertaken as required in Step 2. All the tests were highly specific for the target species and there was no evidence of cross reaction with near related or non-target species, except for *A. salmonicida*. Strategies for safeguarding and confirming positive results for *A. salmonicida* are discussed, see section 8.6.2. In Step 3 the assays were tested for performance when fish tissues were added to the medium to determine the impact of fish tissue on test specificity and sensitivity. For all assays, these additions had no negative effect on test performance. Lastly, in Step 4 testing of the assays were undertaken using field samples to determine specificity through the use of specific pathogen free fish. Confirmatory testing, by the use of low dose retrospective challenge, was undertaken as a means of confirming

test sensitivity. In all cases, SEC-PCR-EHA performed as expected. It is considered that the conditions outlined in the protocol of Hiney and Smith (1998) to validate the PCR based tests have been met.

For epizootiology, test specificity and sensitivity are important test parameters as they have a profound effect on calculating sample size when determining prevalence for establishing freedom from disease. Test characteristics can be inferred through comparative studies where an existing test is already in use. In the absence of a comparable technique, establishing specificity and sensitivity parameters of the test at a population level is problematic. Of the two parameters, specificity has the most profound effect on sample size. Small reductions in test specificity will require a large number of samples, while reduced test sensitivity has much less impact on sample requirements. The number of samples required to determine freedom from disease was calculated using the software program FreeCalc version 2 (Cameron, 1998, 2002) and are given in Table 8.4.

Table 8.4   Relative effects of test specificity and sensitivity on sample size for determining freedom from disease.

Specificity %	Sensitivity %	Sample size*
100	100	56
100	90	66
100	80	74
99	100	128
99	90	166
99	80	183
98	100	188
98	90	223
98	80	263

*Assumed population size 100,000, minimum expected prevalence 5%

Given the importance of establishing test specificity, all the target pathogens were tested with populations of fish known to be specific pathogen free. In the trials conducted, the test for all four pathogens had a specificity of >99%. Test sensitivity is not truly known although based on laboratory testing, it would appear to be high. As given in Table 8.4, true sensitivity is less critical in determining sample size.

9. BENEFITS

The major beneficiaries of the SEC-PCR-EHA technology are identified as:

- Fish farmers
- Health care providers
- Regulators
- Fish health diagnostic laboratories
- Fish health researchers

Development of a system for the identification of covert infections in fish provides farms, for the first time, with a practical basis with which to assess the level of infection either prior to, or immediately after, a disease outbreak. The data generated provides the farmer, health care provider and regulator with a basis to judge the severity of a disease and make a forecast regarding the ultimate impact of the outbreak.

The test format has been developed to ensure that it is efficient and able to be used for high-throughput testing, an essential pre-requisite for population assessment of disease status. The technology, while optimised for the known major bacterial pathogens of salmonids, is a generic platform that can be applied to a wide range of other bacterial pathogens associated with finfish, crustacea and shellfish. The unit systems developed can be applied to a range of other purposes, specifically the DNA extraction protocol and the one tube biphasic PCR and internal probe verification system. These processes are all well suited for a variety of high-throughput screening activities.

The major beneficiaries are the Tasmanian salmonid industry but the techniques have application in southern states of Australia where salmonids are farmed. Of particular application is the test capability for *Lactococcus garvieae* in rainbow trout and *Yersinia ruckeri* in Atlantic salmon as disease caused by these two agents are enzootic in New South Wales and Victoria respectively. While the system was optimised for *A. salmonicida* biovar *acheron* in Atlantic salmon, it can be readily optimised for use with goldfish and would be a powerful tool for monitoring stocks for goldfish ulcer disease for disease management or stock movement controls.

10. FURTHER DEVELOPMENT

The technology as described can be considered 'laboratory ready', but consideration could be given to further developments as means of adding additional value to SEC-PCR-EHA.

Test sensitivity

Considerable effort was made to maximise test sensitivity using large template volumes, optimising PCR assay conditions and the use of agents to relieve the effects of PCR inhibitors. The thresholds of detection consequently were very low with detection in the range of 5 – 80 cfu ml^{-1} of medium. While this level of detection was seen as desirable at the time of test development, when used in early field trials, a significant proportion of fish were enrichment culture negative but weak PCR positive. Since these fish had a history of infection, it was concluded that the positive PCR reactions were the result of remnant DNA from earlier infection episodes. It is possible that the PCR-EHA test as formatted is too sensitive and consideration could be given to reducing test performance to eliminate weak positive reactions. To determine an optimal level of sensitivity to achieve consistency of PCR positive and culture positive, extensive field testing would be required to validate the desired level of sensitivity.

PCR as a measure of pathogen viability

While DNA, and to a lesser extent ribosomal RNA, are good signature molecules for a target pathogen, their usefulness as evidence of cell lability is limited. DNA is a stable, durable molecule and is unsuitable as a marker of cell viability while ribosomal RNA, although less durable is considered relatively stable and its detection can not be reliably correlated with viability. The durability of rRNA in a complex matrix of fish tissues and enrichment culture medium is unknown and should be assessed to determine the reliability of this marker as an indicator of cell viability. Based on published information it may not be particularly useful, although trials with fish tissues suggest it may be an adequate proxy measure. Evaluation of rRNA stability is worth considering, given that the PCR primers target this molecule and their specificity has been extensively evaluated and found reliable. Alternatively, primers could be designed that target messenger RNA, a molecule with a half-life of only a few minutes. While mRNA is recognised as a good indicator of cell viability, it would require entirely new RT-PCR primers to be designed that would be specific for the four target pathogens. The new primers would need to target a

gene other than the 16S rRNA gene and would need to be both intra-species specific as well as inter-species specific. Design and evaluation of new primers is time consuming where no convenient markers have been previously identified. Primers have been designed for *Renibacterium salmoninarum* using the p57 gene as target sequence and successfully used with RT-PCR (Cook and Lynch 1999). Primer sets, not based on 16S rRNA, have been developed for *A. salmonicida* (Hiney *et al.* 1992) and *Y. ruckeri* (Argenton *et al.* 1996; Yugueros *et al.* 2001) and have the potential to be used with RT-PCR using mRNA as the template.

Aeromonas salmonicida *ssp.* salmonicida *enrichment culture medium*

Three formulations of enrichment culture media have been developed for the three strains of *A. salmonicida* known to exist in Australia: goldfish, greenback flounder and acheron biovars. Each of the media is tailored for the sensitivities of the target biovar but all rely on a mix of selective agents that is common to all three biovars. In previous work (Carson *et al.*, 2001b), it was established that *A. salmonicida* ssp. *salmonicida*, the exotic strain that causes furunculosis, would not grow well in the formulations developed for the Australian biovars. It was established however, that the core selective agents common to these formulations were found to be well tolerated by a range of *A. salmonicida* ssp. *salmonicida* isolates, indicating that it would be possible to develop a selective medium for this exotic biovar. Development of a medium for *A. salmonicida* ssp. *salmonicida* would contribute directly to establishing surveillance capability, and hence preparedness, for this important exotic pathogen.

Disease forecasting

From field trials, it was established that SEC-PCR-EHA, and its RT-PCR counterpart, could be used to establish presence or absence of disease agent and provide an indication of active infection or a history of exposure. Such data can be used for quarantine assessment and for determining the prevalence of infection in a population of fish. These data are all valuable for disease management and control purposes. More information can be obtained using this technology, particularly as a means of assessing risk and forecasting disease likelihood. Prospective testing will require longitudinal studies to be undertaken where populations are repeatedly tested and the prevalence correlated with subsequent disease episodes. Prior to development of the SEC-PCR-EHA technology, there has not been a convenient or practical basis on which to measure prevalence. If correlation

studies are undertaken it should be possible to construct a forecasting model based on levels of covert infection and the likelihood of disease. This data would provide a useful means of disease avoidance or the basis for better disease management because of pre-emptive intervention.

11. PLANNED OUTCOMES

The project sought to develop a practical system for the detection of covert infections in salmonid fish that was not reliant on the use of stress testing live fish. The purpose of the technology is to provide farmers with a basis of assessing the level of infection in apparently healthy stock and make practical judgements regarding stock management. Determination of pathogen carriage levels provides a rational basis on which to consider treatment, determine stock movement as a prospective purchaser or seller. Stock testing prior to movement is seen as a major contribution towards establishing zones of freedom from disease, both intra-state and inter-state. The technology provides a generic platform for developing and optimising population level screening systems for other bacterial pathogens associated with other farmed aquatic animals.

12. CONCLUSIONS

12.1. Objective 1. *Develop a procedure for extracting bacterial DNA from selective enrichment media that is suitable for PCR and for processing multiple samples.*

- An extraction protocol was developed based on the use of the chaotropic agent guanidinium thiocyanate (GuSCn) in conjunction with glass microfibre filter plates.

- Direct lysis of bacteria in enrichment culture media was achievable using GuSCn and the released nucleic acids bound directly to glass microfibre filters. Using a 96 well plate format, high throughput processing was achieved within one hour with few processing steps.

- The lysis protocol could be used for both Gram positive and Gram negative bacteria and be used for the simultaneous extraction of both RNA and DNA.

- The extracted nucleic acids were suitable for both PCR and RT-PCR amplification.

- PCR quality nucleic acid could be extracted from the enrichment media irrespective of the different types of selective agents used.

12.2. Objective 2. *Determine optimum conditions for the PCR test to maximise specificity and sensitivity of the procedure.*

- PCR cycle number, annealing temperatures and choice of *Taq* polymerase were modified to obtain detection levels as low as 4 femtograms of DNA (or RNA) equal to 1 genome equivalent or 1 cell.

- Specificity of both PCR and RT-PCR was maintained at these high levels of detection and there was no evidence of cross reactions with genotypically or phenotypically similar organisms. Known cross reaction of the *A. salmonicida* 16S rRNA primer set with some strains of *A. hydrophila* and *A. bestiarum* remained using the improved PCR and RT-PCR conditions.

- Detection levels by PCR or RT-PCR of target pathogens in selective enrichment media using the high throughput extraction protocol ranged between 1 and 16 cfu 200μl^{-1} of medium by PCR and 1 and 9 cfu 200μl^{-1} of medium by RT-PCR.

- The detection levels in the enrichment culture were achieved by a combination of nucleic acid concentration in the microfibre extraction plates, high template test volume, and high concentration of the PCR facilitator,

bovine serum albumin, used at a concentration of 1.5 μg μl^{-1} in the PCR reaction mix.

12.3. **Objective 3.** *Optimise PCR conditions to incorporate a PCR protection system to prevent false positive reactions arising from DNA contamination.*

- The isopsoralen IP-10 was successfully incorporated into the PCR format as a post-amplification procedure to inactivate amplicon.

- Using a concentration of 40 μg ml^{-1} of IP-10, PCR sensitivity was not compromised and it was possible to inactivate at least 6×10^7 amplicons.

- The level of inactivation is sufficient to control aerosol and low level contamination, but is unlikely to be sufficiently robust for major events.

- Activation of IP-10 was achieved simply using a UV transilluminator operating at 300 nm together with sample cooling using a flexible ice brick. Inactivation could also be achieved using a UV crosslinker operating at 365nm with an output of 9.5 mW/cm^2.

- Short amplicons, ranging in length from 145 bp to 288 bp and A+T ratios between 54% and 45% respectively could be adequately inactivated using IP-10.

12.4. **Objective 4.** *Develop a test procedure based on enzyme hybridization assay (EHA) that will verify any positive PCR reactions using a secondary confirmatory gene probe and in a format suitable for testing multiple samples.*

- A biphasic PCR system based on the NucleoLink™ format was implemented for a one-tube test that incorporates an amplicon verification step based on enzyme linked hybridization with a secondary species specific DNA probe. Nucleic acid extraction and PCR-EHA are both performed in a 96 well format and are integrated for high throughput processing of samples.

- The system was implemented for all four target pathogens and detection levels ranged from 4-16 cfu 200 μl^{-1} of enrichment medium. This level of sensitivity is more than sufficient to detect low numbers of target pathogen in the selective enrichment media.

- The assay system was also optimised for use with the RT-PCR format; the detection level in this format ranged from 3-9 cfu 200μl^{-1} of enrichment medium for the four target pathogens.

- Based on extensive testing with specific pathogen free fish, cut-off values for the EHA verification system were established and set at 1.2 times the negative control after 60 minutes or 1.4 times the negative control after 18 hours development.

12.5.   Objective 5. *Optimise the culture conditions and PCR detection process to ensure the minimum test time between sample collection and test result.*

- A high throughput format was implemented for all stages of the SEC-PCR-EHA test. Using industry standard 8x12 well format plates, 96 samples could be processed simultaneously from DNA extraction, PCR through to probe hybridization to verify PCR positive results.

- Culture enrichment required 5 days incubation to obtain best results while the detection and verification hybridization assay could be completed within 24 hours.

- With the appearance of *Aeromonas salmonicida* biovar *acheron,* a new atypical biovar and the cause of disease in farmed Atlantic salmon, a new selective enrichment medium, HK3C was formulated for this strain. Development of this medium complements earlier formulations for the goldfish and greenback flounder biovars of *A. salmonicida.*

12.6.   Objective 6. *Test populations of salmonids with the optimised SEC-PCR-EHA system to verify test performance and obtain baseline data on carrier prevalence.*

- Validation trials of the SEC-PCR-EHA system were undertaken for the four target pathogens using specific pathogen free stock as well as fish with known recent histories of disease.

- Prevalence levels of covertly infected fish were obtained for the four target pathogens but some difficulties were encountered reconciling PCR positive/enrichment culture negative findings. In such cases, PCR reactions were weak and either remained so during the culture incubation period or disappeared during incubation, suggesting that the PCR-EHA was detecting viable but non-culturable cells or cell remnants containing target pathogen DNA.

- A RT-PCR targeting 16S ribosomal RNA was implemented as a means of establishing if target pathogens present in a sample are viable or non-viable.

While reducing the number of PCR positive reactions/enrichment culture negative findings, complete reconciliation was not possible, probably because ribosomal RNA is a stable species of nucleic acid relative to messenger RNA.

- DNA based PCR can be used for testing populations of fish for evidence of any exposure to the target pathogen and would be suitable for quarantine and freedom from disease investigations. RT-PCR is more suitable for assessing carriage of live cells and would be used for screening populations of fish with a known history of infection.

- Testing of fish populations with SEC-PCR-EHA has provided useful data to fish farms either as a means of determining freedom from disease, assessing levels of exposure following a disease episode, or as a means of determining the effect of vaccination on carriage levels.

- During field trials, a new strain of *A. hydrophila* was isolated which cross reacted with the *vap*A primer set, previously thought to be specific for *A. salmonicida*. This finding reduces the confidence that can be placed in this primer and limits its usefulness as a tool for first line screening for *A. salmonicida*.

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APPENDIX 1: Intellectual Property

The intellectual property arising from this research is as listed:

1. Development of a high-throughput combined· DNA+RNA extraction system from enrichment culture media.

2. Development methodology and formulation of a selective enrichment medium for *Aeromonas salmonicida* biovar *acheron*.

3. Optimisation of a one tube biphasic PCR and enzyme hybridization assay suitable for use with DNA PCR and RT-PCR.

4. Copyright in this report.

APPENDIX 2: Staff

Staff engaged on the project:

Principal Investigator

Dr Jeremy Carson   Fish Health Unit, Tasmanian Aquaculture Fisheries Institute, University of Tasmania, Launceston

Research Scientist

Ms Teresa Wilson   Fish Health Unit, Tasmanian Aquaculture Fisheries Institute, University of Tasmania, Launceston

APPENDIX 3: Formulae of media, buffers and reagents

Media

Shieh's marine medium for *Tenacibaculum maritimum* (adapted from Song *et al.* 1988)

Peptone (Oxoid L37)	5.0 g
Sodium acetate	0.01 g
Sodium pyruvate	0.1 g
Citric acid	0.01 g
Yeast Extract	0.5 g
Sea water	900 ml
Distilled water	100 ml
pH (after autoclaving)	7.5-7.8
Autoclave	121°C/15min

HK3C basal medium for *Aeromonas salmonicida*

Proprietary formulation. Commercial-in-confidence

Buffers and Reagents

Note: RO water – reagent grade water prepared by reverse osmosis with a conductivity of <2µS

1 M 1-Methylimidazole (1-MeIm) (10 ml)

797.1 µl 1-MeIm MW 82,1 g mol^{-1} - d = 1,030 kg l^{-1}

6 ml 18 Mohm water

pH is adjusted to 7.0 with HCl

The volume is adjusted to 10 ml with 18 Mohm water. ***1-MeIm is strongly corrosive**

DIAPOPS buffer with 10 mg ml^{-1} BSA

Dissolve 0.05 g BSA Fraction V in 4500 µl RO water

Filter sterilise through a 0.2 µm filter and store at 4°C

For one strip:

Add 120 µl DIAPOPS buffer to 1080 µl of 10mg 900 µl^{-1} BSA just before use.

10 x DIAPOPS buffer (100 ml)

12.70 g TRIS-HCl (157.56 g mol^{-1}) Final concentration: 0.8 M

2.36 g TRIS base (121.1 g mol^{-1}) Final concentration: 0.2 M

8.76 g NaCl (58.44 g mol^{-1}) Final concentration: 1.5 M

The volume is adjusted to 99 ml with 18Mohm water.

Autoclave at 121°C for 30 minutes and then add

1 ml Concentrated Tween 20 Final concentration: 1% (v/v)

The pH must be checked and adjusted to 7.5 with 1 M NaOH.

1 M Diethanolamine (pH 9.8) with 1 mM $MgCl_2$ (1000 ml)

95.6 ml Diethanolamine, MW = 105.14 g mol^{-1}, d = 1.10 kg l^{-1}.

800 ml RO water

pH is adjusted to 9.8 with 10 M NaOH or 10 M HCl

Then add:

0.2033 g $MgCl_2$, 6 H_2O MW = 203.30 g $mole^{-1}$

The volume is adjusted to 1000 ml with RO water.

Diethanolamine buffer can be stored at 4°C.

DMIX for 3 μl RNA elute.

Ingredient	Volume
DNase (1 U/ μl)	0.5 μl
[a]10 mM $MnCl_2$	0.4 μl
[b]100 mM $CaCl_2$ / 900 mM Tris-HCl, pH 8.0	0.16 μl
Total	**1.06 μl**

[a] 10mM $MnCl_2$

Dilute 1 M MnCl stock to 10 mM using 18 Mohm water. Add DEPC to a final concentration of 0.2% mix and stand overnight. Autoclave at 121°C for 30 minutes.

[b] 100 mM $Cacl_2$, 900 mM Tris-HCl, pH 8.0

Add DEPC to 0.2% to solution To 100 mM $CaCl_2$, add DEPC to a final concentration of 0.2% mix and stand overnight. Autoclave at 121°C for 30 minutes. Add 10 μl of 100 mM $CaCl_2$ to 90 μl of sterile DNase and RNase free 1 M Tris-HCl, pH 8.0.

Elution Buffer

DNA elution: 10mM TRIS HCL, pH 8.0.

RNA elution: DEPC treated 18 MΩ water.

Lysis Buffer L6

Dissolve 30g GuSCN* (guanidinium isothiocyanate) in 25ml (0.1M) TRIS HCl, pH 6.4 (dissolve by heating to 60-65°C while shaking).

Add 5.5ml 0.2M EDTA and 700µl Triton X-100 (Sigma, cat#T8787) to this and vortex.

For extracting RNA, add β-mercaptoethanol to a final concentration of 1% just before use.

Store buffer at room temperature in the dark. Note: The shelf-life of the buffer is four weeks if used for DNA extraction and two weeks for RNA extraction.

Lysis Buffer L2

Dissolve 30g GuSCN* in 25ml (0.1M) TRIS HCl, pH 6.4 (dissolve by heating to 60-65°C while shaking).

Store at room temperature, in the dark; shelf life is three weeks.

*Because of the risk of cyanide fumes, prepare GuSCN in fume hood.

0.2 M NaOH, 0.1% Tween 20 (100 ml)

0.8 g NaOH pellets, final concentration: 0.2 M

The volume is adjusted to 100 ml with RO water

Just before use Tween 20 is added to a final concentration of 0.1%

0.4 M NaOH and 0.25% Tween 20 (100 ml)

1.6 g NaOH pellets, final concentration: 0.4 M

100 ml with 18Mohm water

Buffer is autoclaved for 30 minutes at 121°C

Just before use Tween 20 is added to a final concentration of 0.25%.

Saline-EDTA

0.15M NaCl

0.1M EDTA

pH 8

0.5 x SSC, 0.1% Tween 20 (100 ml)

2.5 ml 20 x SSC

0.1 ml Tween 20

The volume is adjusted to 100 ml with RO water.

The buffer can be stored at room temperature.

20XSSC

3M NaCl

0.3M Sodium citrate

Prepared in RO water, pH adjusted to 7.0

TAE electrophoresis buffer (x1)

40mM TRIS base

40mM glacial acetic acid

1mM EDTA, pH 8.0

APPENDIX 4: Workshop notes

In fulfilment of one of the project milestones, a technology transfer workshop was held for fish health scientists. The workshop consisted of a one-day epidemiology lecture given by Dr Chris Baldock of AusVet Animal Health Services followed by three days laboratory and theory sessions.

PROCEDURE 1: Binding of solid-phase primer to NucleoLink™ tubes

(a) BACKGROUND

Generally either the reverse or forward PCR primer can be used as the solid-phase primer. However, if an internal hybridization probe has previously been identified, the primer that the probe recognises must be chosen as the solid-phase primer. The appropriate primer is modified by the addition of a 10 base thymine linker and is phosphorylated at the 5' end in order to facilitate covalent binding to the surface of the NucleoLink tubes. Once modified in this way, the primer is referred to as the solid-phase primer.

(b) MATERIALS SUPPLIED

Binding

NucleoLink PCR-ELISA tubes	1 strip
1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)	3.26 mg
	in tube A
	one tube per 2 strips
1M 1-methylimidazole (1-Melm)	17 µl in tube B
	one tube per 2 strips
18 Mohm water	841 µl
1000 ng µl⁻¹ solid-phase primer	0.85 µl

Denature and washing

0.4 M NaOH	15 ml pre-warmed in tube C
50% Tween 20	
Sterile RO water	
10 x DIAPOPS buffer	

(c) PROCEDURE

1. Only ever second person has tube A and B. If you do, prepare 10 mM EDC in 10 mM 1-MeIm by adding 17 µl from tube B to tube A, vortex to dissolve. Add 8.5 µl from tube A to the aliquot of 18 Mohm water provided, pass tube A on and the second person is to take 8.5 µl from tube A and add it to their 18 Mohm water. Add 0.85 µl of the solid phase primer (final concentration of 1ng µl^{-1}) to the water, EDC and 1 MeIm mix and vortex. Pipette 100 µl of this solution into each NucleoLink well.

2. Seal strip with tape and incubate at 50°C for at least 5 hours.

3. After incubation, add 75 µl of the 50% Tween 20 to tube C.

4. Remove the contents of the NucleoLink wells and wash wells three times with 150 µl of 0.4 M NaOH, 0.25% Tween 20, incubate for 15 minutes at 50°C and was three more times. * **Select medium or low speed for the electronic pipette to avoid amplicon aerosols at this step.**
   EMPTY WELLS BY STRIKING AGAINST A FOLDED TOWEL SEVERAL TIMES

5. Prepare 100 ml of 1 x DIAPOPS buffer from 10 x stock solution.

6. Rinse the wells three times with DIAPOPS buffer, soak for 5 minutes and rinse three more times.
   EMPTY WELLS BY STRIKING AGAINST A FOLDED TOWEL SEVERAL TIMES

7. Place strip into a clip seal bag and place at 4°C overnight, the strips should not be sealed.

PROCEDURE 2: Extraction of nucleic acids using a 96-well glass microfibre plate and a vacuum manifold

(a) BACKGROUND

The nucleic acid extraction technique uses guanidinium thiocyanate (GuSCN) to lyse the bacterial cell walls and bind the nucleic acids to the glass microfibre. Ethanol is used to wash the nucleic acids, acetone is used to dry the glass microfibre and TRIS buffer is used as an elution buffer. This method can be used to extract RNA and DNA, however when extracting RNA β-mercaptoethanol is added to the GuSCN and the ethanol and elution water are treated with DEPC. For this demonstration we will be concerned with DNA only.

(b) MATERIALS SUPPLIED

Decimal dilution of bacteria for DNA extraction

Polypropylene tubes containing dilution medium

Culture of *A. salmonicida* for DNA extraction

Blood agar plate

Extraction

Whatman GF/B glass microfibre filter plate

Lysis buffer L6	Stored at 4°C
Lysis buffer L2	Stored at 4°C
70% Ethanol	Stored at 4°C

Acetone

TRIS buffer, pH 8, 10mM

(c) PROCEDURE

1. With a sterile loop inoculate tube N with a very small amount of *A. salmonicida*. The suspension should be very faintly turbid.
2. Prepare decimal dilutions by adding 500 µl from tube N to tube 10^{-1}.
3. Repeat this until tube 10^{-7}, place tubes in the fridge.
4. Label the blood agar plate as in figure 1.
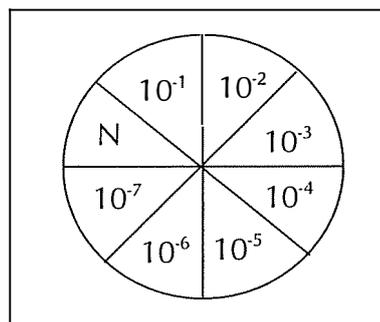
Figure 1

5. Transfer a 20 µl drop from the appropriate tube onto the appropriate section of the agar plate. Leave the plate upside down for the whole procedure. Once the inocula are absorbed, invert the plate and incubate at 25°C.
6. Place TRIS buffer in heat block, set heat block to 80°C.

7. Set up vacuum extraction system as shown in figure 2.

Figure 2
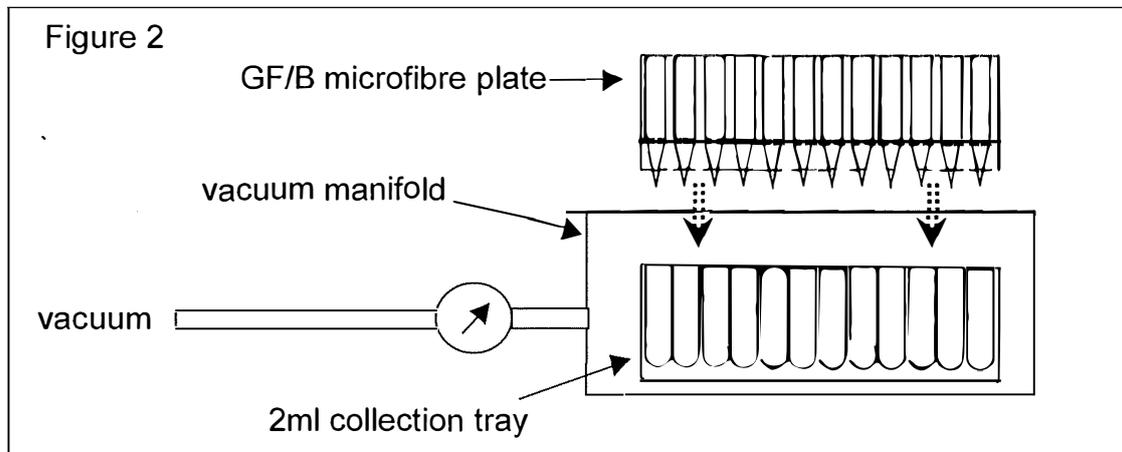
8. Add 100 µl of RO water to each filter well to be used.

9. Clear wells using minimum vacuum.

10. Add 500 µl of Lysis Buffer 1 to each well followed by 200 µl of sample.

11. Let stand at room temperature for 10 to 15 minutes.

12. Clear wells using minimum vacuum. This is very important as Lysis Buffer 1 contains the detergent Triton X which will foam if too much vacuum is applied and many cause cross-contamination.

13. Add 200 µl of Lysis Buffer 2 to each well and repeat.

14. Carefully lift microfibre plate from vacuum manifold and place a clean 2 ml collection tray into manifold.

15. Increase vacuum pressure to about 10" Hg and wash wells five times with 200 µl of cold ethanol.

16. Wash wells once with 100 µl acetone. Once no acetone is visible in the well, run vacuum for a further 5 minutes.

17. Before eluting the DNA with pre-heated TRIS buffer, carefully lift the microfibre plate from vacuum manifold and check that no acetone remains on the drip directions. If some acetone is remaining, dab drip directors with a sterile tissue and apply vacuum for a further 2 minutes.

18. Carefully replace the 2ml collection tray with a 1ml packing tray and place a sterile microtitre tray on top of the packing tray.

19. Add 35µl of pre-heater TRIS buffer into each well and leave to stand for 5 to 10 minutes.

20. Elute DNA using minimum vacuum.

21. Place a further 20 µl of TRIS buffer into each well, allow to stand for 1 minute and repeat the elution.

22. Carefully remove the microfibre plate from the manifold and check the drip directors for TRIS buffer. If significant drips remain carefully replace the plate and apply vacuum for a further 5 minutes.

23. Remove microfibre plate from manifold and take out the microtitre tray that now contains DNA. Seal microtitre tray with plate sealing tape and store at 4°C overnight.

PROCEDURE 3: Biphasic PCR

(a) BACKGROUND

An *Aeromonas salmonicida* 16S PCR will be performed to demonstrate the PCR-EHA.

The PCR master-mix used for biphasic PCR has the same ingredients as a conventional PCR. To 'force' amplification to use the bound reverse primers, the amount of reverse primer added to the master-mix is reduced. In most systems a primer ratio of 1:8 is optimal. If DNA amplification with a primer ratio of 1:1 is used the efficiency of the solid-phase DNA amplification will be low.

(b) MATERIALS SUPPLIED

DIAPOPS buffer

11mg ml^{-1} Fraction V BSA

x10 PCR Buffer

dNTPs

50 mM MgCl$_2$

P-forward (40 pM µl^{-1})

P-reverse (5 pM µl^{-1})

10% Tween 20

50% Glycerol

IP10   Stored at 4°C in the dark

Taq   Stored at –20°C

18 Mohm Water

Positive control   100 fg *Aeromonas salmonicida* DNA

(c) PROCEDURE

1. Block wells with 150µl of freshly prepared DIAPOPS buffer with 10 mg ml^{-1} Fraction V BSA.

2. Seal tubes with tape and shake strips at room temperature for 1 hour.
    EMPTY WELLS BY STRIKING AGAINST A FOLDED TOWEL SEVERAL TIMES

3. Prepare *PCR reaction mix adding the IP10 last. Prepare enough reaction mix to set up eight NucleoLink tubes as shown in Figure 3.

*PCR reaction mix	Final concentration	Volume (µl)
x10 PCR Buffer	x1	2.00
dNTPs	2 µM each	1.60
50 mM MgCl$_2$	1.375 mM	0.55
P-forward (40pM µl^{-1})	1 µM	0.50
P-reverse (5pM µl^{-1})	0.0625 µM	0.25
10% Tween 20	0.1%	0.20
50% Glycerol	10%	4.00
Water		8.60
Taq	2U/ reaction	0.10
4mg ml^{-1} IP10	35 µg ml^{-1}	0.20
		Total 18µl + 2µl template

4. Retrieve from the fridge DNA extracted using vacuum system.

5. Remove BSA from NucleoLink wells and add 18µl of PCR reaction mix to each well. Add 2µl of the appropriate template (Figure 3) to the wells.

6. Seal strip with Tape 8 (Nunc) run in a thermocycler with the following program:

    1. 94°C for 3 minutes

    2. 94°C for 45 seconds

    3. 60°C for 45 seconds

    4. 72°C for 1 minute

    5. Repeat 2-4 35 times

    6. 72°C for 5 minutes

    7. End

7. After cycling place strip in a frame and place into a UV crosslinker and irradiate for 900 seconds at 365nm, UV intensity 9.6 mW/cm^2.

8. Remove Tape 8 and <u>discard.</u> Transfer liquid PCR product into a spare microtitre tray and run a confirmatory agarose gel to verify amplification

Figure 3: PCR-ELISA format

Well #	N	-2	-4	-5	-6	-7	+C	-C
	1	2	3	4	5	6	7	8
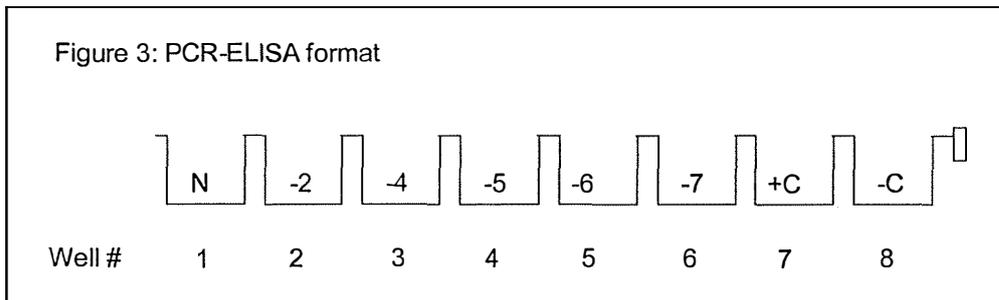
PROCEDURE 4: Hybridization with biotin labelled internal probe

(a) BACKGROUND

During biphasic PCR, DNA amplification has occurred in both the liquid and solid-phase. The biotin labelled internal probe is used to detect any amplified product that is attached to the covalently bound solid-phase primer. This internal probe also adds another level of specificity as it has been designed to target *Aeromonas salmonicida*.

(b) MATERIALS SUPPLIED

<u>Denaturation</u>
0.2 M freshly prepared NaOH
50% Tween 20
DIAPOPS buffer

<u>Hybridization</u>

10 mg ml^{-1} salmon sperm DNA	Stored at –20°C
Biotinylated probe (50 pmol µl^{-1})	Stored at –20°C

20 x SSC
100 x Denhardt's
10% Tween 20
0.5 x SSC + 0.1% Tween 20 (wash buffer)

Detection

Streptavidin alkaline phosphotase conjugate

1M Diethanolamine (pH 9.8) with 1 mM MgCl$_2$

p-Nitrophenyl phosphate (pNPP)

(c) PROCEDURE

1. Denature solid phase amplicons with freshly prepared NaOH + 0.1% Tween 20. To prepare, dispense 15 ml of 0.2 M NaOH into a plastic tray and add 30 µl of 50% Tween 20 to the NaOH. Wash strip three times with 150 µl of this solution, incubate for 5-10 minutes and then wash three more times.

   EMPTY WELLS BY STRIKING AGAINST A FOLDED TOWEL SEVERAL TIMES

2. Wash the empty wells three times, soak for 5 minutes and wash three times with 150µl DIAPOPS buffer at room temperature.

   EMPTY WELLS BY STRIKING AGAINST A FOLDED TOWEL SEVERAL TIMES

3. Detect amplicon with a biotinylated internal probe. Denature salmon sperm DNA and biotinylated probe by boiling for 6 minutes and cool rapidly to 4°C for at least 2 minutes. Add DNA to hybridisation base and dispense 100 µl into each well.

DENATURE	VOLUME (µl)
10mg ml^{-1} salmon sperm DNA	8
Biotinylated probe (50 pmol µl^{-1})	0.8

HYBRIDISATION BASE	VOLUME (µl)
20XSSC	240
100xDenhardt's solution	40
10% Tween 20	8.0
RO water	503.2
Total	800

4. Incubate strips at 50°C for at least 1 hour.

5. Wash the emptied wells three times in 0.5xSSC, 0.1% Tween 20. Soak for 15 minutes at 50°C and wash three more times at room temperature.

   EMPTY WELLS BY STRIKING AGAINST A FOLDED TOWEL SEVERAL TIMES

6. Add 0.5 µl SA-AP to 1 ml DIAPOPS buffer + 2 mM MgCl$_2$.

7. Add 100µl to each well and incubate for one hour at 37°C.

8. Empty wells and wash three times, soak for 5 minutes and wash three times with 150µl DIAPOPS buffer at room temperature.

EMPTY WELLS BY STRIKING AGAINST A FOLDED TOWEL SEVERAL TIMES

9. Prepare 10 mg ml^{-1} pNPP in 1M diethanolamine (pH 9.8) with 1 mM MgCl$_2$ by adding a 20 mg tablet of pNPP to 2 ml of 1 M diethanolamine. Vortex vigorously to dissolve. Add 90 µl to each well.

10. Incubate for 30-60 minutes at room temperature in the dark.

11. Measure OD at 405 nm in an ELISA reader.

12. Leave strip in the dark overnight.

PROCEDURE 5: Absorbance readings after overnight incubation

(a) BACKGROUND

Occasionally very weak positive results are not clearly distinguishable from the background negative control reading after one or two hours. These reactions may become clearer after 15 hours incubation. After this time any weak positive reactions should be at least 1.4 times the value of the negative control.

(b) PROCEDURE

1. Measure OD at 405 nm in an ELISA reader.
2. Compare readings with those taken on the previous day.

APPENDIX 5: Publications and Presentations

Presentations at conferences

Wilson, T. and Carson, J. 2001. Selective enrichment culture PCR-ELISA to detect covert infections in farmed salmonids. S. Battaglene and J. Cobcroft (Edits.). The First Scientific Conference of the Atlantic Salmon Aquaculture Subprogram Handbook, 18-19 July, 2001, CSIRO Marine Laboratories, Hobart. 87pp.

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APPENDIX 6: Abbreviations

1-MELM	1-methylimidazole
ACAM	Australian Collection of Antarctic Microorganisms
at	attograms 10^{-18}g
ATCC	American Type Culture Collection
bp	base pair
BHI	brain heart infusion
BSA	bovine serum albumin
CCR	carbodiimide condensation reaction
CFU	colony forming units
CORT	selective enrichment medium for *Lactococcus garvieae*
DEPC	diethylpyrocarbonate
DIAPOPS	Detection of Immobilised Amplified Product in a One-Phase System
DNA	deoxyribose nucleic acid
cDNA	copy DNA
DPIWE	Department of Primary Industries, Water & Environment
dNTPs	deoxynucleotide triphosphates
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylene-diamine-tetra acetic acid
EHA	enzyme hybridization assay
EI	enrichment index
ELISA	enzyme linked immunosorbent assay
fg	femtograms 10^{-15}g
HK3C	selective enrichment medium for *A. salmonicida* biovar *acheron*
IP-10	Isopsoralen-10, PCR amplicon inactivator
MIC	minimum inhibitory concentration
MPN	most probable number
MSA	marine Shieh's agar
NCFB	National Collection of Food Bacteria
NCIMB	National Collection of Industrial & Marine Bacteria
ng	nanograms 10^{-6}g
OD	optical density
PAAS	probe assay *Aeromonas salmonicida*
PCR	polymerase chain reaction
pg	picograms 10^{-12}g
PLC	product limiting concentration
POSI	selective enrichment medium for *Tenacibaculum maritimum*
POST	selective enrichment medium for *Yersinia ruckeri*
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
RO	reverse osmosis water, $<2\mu$S
SBA	sheep's blood agar
SDS	sodium dodecyl sulphate
SEC	selective enrichment culture
SIF	stress inducible furunculosis
SKDM	selective kidney disease medium
vapA	gene for the surface protein layer (A-protein) virulence factor, *A. salmonicida*
RT-PCR	reverse transcriptase polymerase chain reaction
SEC	selective enrichment culture
SPE	Selective Enrichment Culture PCR Enzyme Hybridization Assay
TAE	TRIS acetate EDTA buffer
Taq	*Thermus aquaticus* DNA polymerase
Triton X-100	t-octylphenoxypolyethoxyethanol
UV	ultraviolet