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## Single-dilution enzyme-linked immunosorbent assay for quantification of antigen-specific salmonid antibody

Stewart W. Alcorn, Ronald J. Pascho

**Abstract.** An enzyme-linked immunosorbent assay (ELISA) was developed on the basis of testing a single dilution of serum to quantify the level of antibody to the p57 protein of *Renibacterium salmoninarum* in sockeye salmon (*Oncorhynchus nerka*). The levels of antibody were interpolated from a standard curve constructed by relating the optical densities (OD) produced by several dilutions of a high-titer rainbow trout (*O. mykiss*) antiserum to the p57 protein. The ELISA OD values produced by as many as 36 test sera on each microplate were compared with the standard curve to calculate the antigen-specific antibody activity. Repeated measurements of 36 samples on 3 microplates on each of 6 assay dates indicated that the mean intraassay coefficient of variation (CV) was 6.68% (range, 0–23%) and the mean interassay CV was 8.29% (range, 4–16%). The antibody levels determined for the serum sample from 24 sockeye salmon vaccinated with a recombinant p57 protein generally were correlated with the levels determined by endpoint titration ( $r^2 = 0.936$ ) and with results from another ELISA that was based on extrapolation of antibody levels from a standard curve ( $r^2 = 0.956$ ). The single-dilution antibody ELISA described here increases the number of samples that can be tested on each microplate compared with immunoassays based on analysis of several dilutions of each test serum. It includes controls for interassay standardization and can be used to test fish weighing <3 g.

Regular monitoring of the health of fish reared in intensive aquaculture is an important step toward reducing the introduction and spread of infectious diseases.<sup>32</sup> The immune system of fish may be adversely affected by changes in physiologic processes resulting from an inattention to water quality or fish rearing density,<sup>4,30</sup> diet formulation,<sup>7,21,40</sup> or fish handling methods.<sup>4,5</sup>

Serum antibody levels are an indicator of the overall immune system activity of a fish. The amount of antibody can be estimated using a variety of assays, including agglutination, radial immunodiffusion, or neutralization reactions, although the sensitivity of these tests is limited.<sup>1,27,31,41</sup> Monoclonal antibodies to fish antibody<sup>3,11,37</sup> have been adapted to the enzyme-linked immunosorbent assay (ELISA) and used to quantify low levels of specific antibody activity.<sup>2,9,24,38</sup> When estimating fish antibody levels in an ELISA, several dilutions of each serum sample are usually analyzed to arrive at an endpoint dilution or units of antibody.<sup>2,8,20,24,26,41</sup> Replicate analyses of several dilutions of each test serum, however, can limit the number of fish evaluated on each ELISA microplate and increase reagent costs. In an effort to analyze greater numbers of fish on each microplate, the optical density (OD) of a single dilution of test serum has been used for antibody concentration comparisons.<sup>13,15,18,38</sup> However,

because of nonrandom sources of error such as slight differences among reagent preparations or individual microplates, direct comparison of OD values is valid only for samples assayed on the same microplate. A method of standardization is required for comparisons between samples tested among assay microplates on the same day (intraassay), between assay dates (inter-assay), or between samples tested in separate laboratories. Although several investigations have utilized a single dilution of 1 or more control sera for standardization of test sample results,<sup>19,23,36</sup> comparisons of ELISA standardization methods have shown that the optimal method of determining antibody concentrations is by reference to a standard curve.<sup>6,12,25</sup>

As part of a study examining the immune functions of groups of sockeye salmon (*Oncorhynchus nerka*) reared at 8 or 12 C throughout their life cycle, a sensitive method was required to quantify specific-antibody levels for large numbers of fish that were sampled twice per year over 3 years. The ELISA was selected for this study because it offered the potential to test individual sera from large numbers of fish at each life stage in a timely manner, without compromising sensitivity. A measure of standardization was required so that the effects of season and the fish's life stage on the humoral immune response could be determined. Here, we describe the development of an ELISA to quantify the concentration of serum antibody to the 57-kD protein (p57) of the salmonid pathogen *Renibacterium salmoninarum* by reference to a standard curve. In contrast to other ELISAs for fish antibody that rely

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on a standard curve, the antibody units in the ELISA reported here were calculated by testing a single serum dilution. The assay allowed up to 36 samples to be tested in duplicate on each microplate. We also describe the relative performance of the single dilution method when compared with 2 other ELISAs for fish antibody that rely on analyzing multiple dilutions of each test serum.

## Materials and methods

### Fish

Broodyear 1994 sockeye salmon of the Lake Wenatchee (Washington) stock were reared from eggs at the Western Fisheries Research Center (US Geological Survey) in Seattle, Washington. The average weight of the experimental fish was approximately 45 g unless otherwise noted. Fish were maintained at 8 or 12 C in sand-filtered, Lake Washington water that had been treated with ultraviolet (UV) light and were fed a commercial diet *ad libitum* 3 days/wk.

Broodyear 1993 rainbow trout (*O. mykiss*) of the Shasta (California) strain, averaging approximately 500 g, were obtained as juvenile fish and maintained at 15 C as described above. Rainbow trout of the Mt. Lassen (California) strain were obtained as broodyear 1992 eggs and reared to approximately 500 g in ambient treated Lake Washington water.

### Blood and tissue processing

Fish were anesthetized in 200 ppm triclanemethane sulfonate,<sup>a</sup> and blood was collected from the caudal vessel with a syringe. The blood was allowed to clot overnight at 4 C and then centrifuged at  $2,000 \times g$  for 20 min at 4 C. All sera were stored at  $-80$  C for later testing by an ELISA for fish antibody. The sera from 45 Mt. Lassen strain rainbow trout were used to prepare a normal serum pool. Sera were diluted for ELISA in 0.05 M Tris-buffered saline (TBS, pH 8.0) containing 1% Tween 20 and 3% bovine serum albumin (T-TBS-BSA).

When necessary, fish were also tested for the presence of a soluble antigenic fraction of *R. salmoninarum* by an ELISA (BKD-ELISA). The kidney and spleen were pooled and diluted 1:8 in 0.01 M phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20. Following homogenization, each tissue pool was heated at 100 C for 15 min. Processed tissue homogenates were centrifuged at  $8,800 \times g$  for 6 min at 4 C, and the supernatant was used for testing in the BKD-ELISA.

### Monoclonal antibody

Mouse hybridoma clone 1.14 producing antibody to salmonid IgM (monoclonal antibody [MAb] 1.14)<sup>11</sup> was grown in a serum-free medium.<sup>b</sup> Hybridoma culture supernatant was used as the source of secondary antibody for antibody ELISAs I and II.

### Recombinant p57 protein

A purified recombinant 57-kD protein of *R. salmoninarum* (r-p57)<sup>c</sup> was produced by cloning the DNA sequence coding

for the mature p57 protein into a *Escherichia coli* protein expression system.<sup>4,10</sup> Following expression, the purity of the recovered recombinant protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (data not shown).

### Vaccination of salmonids with r-p57 protein

Rainbow trout and sockeye salmon were injected with the r-p57 protein to provide serum standards or test sera for the antibody ELISA. Following injection, fish were maintained at the appropriate temperature in sand-filtered, UV light-treated Lake Washington water.

*Serum standards.* Shasta strain rainbow trout were injected intraperitoneally with 100  $\mu$ g of r-p57 protein emulsified in Freund's complete adjuvant. Fish were maintained at 15 C following injection. Forty-two days after the primary injection, each fish was given a booster injection intraperitoneally of 20  $\mu$ g r-p57 protein emulsified in Freund's incomplete adjuvant (FIA). All fish were exsanguinated 70 days after the initial injection.

*Normal and test sera.* Normal serum samples were obtained from 20 sockeye salmon from each temperature group. Other fish from the 8 C and 12 C temperature groups were injected intraperitoneally with 50  $\mu$ g of r-p57 protein emulsified in FIA. Following injection, fish were maintained at the appropriate temperature. At 5, 6, 7, 8, and 9 wk after injection, serum was collected from 20 injected fish from each temperature group.

*Low-responder serum pool.* Sockeye salmon were injected intraperitoneally with 50  $\mu$ g of r-p57 protein emulsified in FIA. Fish were maintained at 15 C, and sera was collected from 10 fish at 5, 6, 7, and 8 weeks after injection. Individual serum samples were assigned to the low-responder pool on the basis of testing a 1:100 dilution in antibody ELISA I; samples with a mean OD between 0.2 and 0.6 were assigned to the low-responder pool.

### BKD-ELISA

The BKD-ELISA used to measure the prevalence and levels of an antigenic fraction of *R. salmoninarum* in the kidney-spleen tissue homogenates was a double-antibody sandwich ELISA<sup>29</sup> with modifications described elsewhere.<sup>28</sup> Tissue homogenates that produced a mean BKD-ELISA OD of  $\leq 0.088$  were categorized as *R. salmoninarum* negative.

### Antibody ELISAs I and II

Antibody ELISA I was used to optimize certain assay parameters that were subsequently incorporated into the final single-dilution ELISA for fish antibody. Those parameters included selection of the serum standard and optimal dilution for a test serum. The final assay, based on interpolating antibody units from a standard curve, was termed antibody ELISA II.

*Antibody ELISA I.* Commercially prepared wash buffer, antigen coating buffer, azino-bis(3-ethyl benzthiazolinesulfonic acid) (ABTS) peroxidase substrate, and ABTS stop solution<sup>e</sup> were used according to the manufacturer's instructions. All volumes were 200  $\mu$ l/microplate well unless otherwise stated. The washing procedure consisted of rinsing each well 5 times with wash buffer for 30 sec. All dilutions

of fish serum, MAb 1.14 secondary antibody, and a horseradish peroxidase-conjugated goat immunoglobulin to mouse IgG (HRP conjugate)<sup>f</sup> were made in T-TBS-BSA. The optimal working dilutions for the r-p57 protein, the MAb 1.14 secondary antibody, and the HRP conjugate were determined in separate experiments (data not shown). The MAb 1.14 was diluted 1:1,000 in T-TBS-BSA. The HRP conjugate was diluted either 1:2,000 or 1:8,000, depending on the manufacturer's lot.

Microplate<sup>g</sup> well surfaces were coated for 16 hr at 4 C with 1 µg/ml r-p57 protein in coating buffer, then the unbound protein was removed by the wash procedure. Aliquots of each fish serum were placed in the appropriate microplate wells. The microplates were incubated for 2 hr at 15 C before the unbound serum proteins were washed from the wells. The MAb 1.14 secondary antibody was added to each well, the microplates were incubated for 1 hr at 25 C, and the unbound antibody was washed from the wells. The HRP conjugate was then applied to each well, and the microplates were incubated 1 hr at 25 C before the unbound conjugate was removed by the washing procedure. Control wells (blanks) were coated with r-p57 protein, were incubated with T-TBS-BSA only during the fish serum incubation, and received both the secondary antibody and HRP conjugate. Secondary antibody and HRP conjugate control wells were coated with antigen and received only the MAb 1.14 secondary antibody or HRP conjugate at the appropriate steps; in all other steps the wells received T-TBS-BSA. Substrate control wells were not coated with antigen and received only T-TBS-BSA during all subsequent steps. The ABTS substrate was added to each well, and the microplate was incubated for 15 min at 37 C. Color development was stopped by the addition of 50 µl/well of a 4× stop solution. The OD of each well was measured at 405 nm.

**Antibody ELISA II.** Antibody ELISA II was performed by the same method as antibody ELISA I but included 1:400, 1:800, 1:1,600, 1:3,200, and 1:6,400 dilutions of the Shasta strain rainbow trout serum standard in T-TBS-BSA. Each dilution was tested in triplicate on every microplate. The antigen-specific units per milliliter of antibody of test sera were determined by reference to the standard serum dilutions in the antibody ELISA II.

### Rainbow trout serum standard

The serum from each of 14 Shasta strain rainbow trout injected with the r-p57 protein was tested by antibody ELISA I to select a serum standard for antibody ELISA II. The OD values were determined for doubling dilutions of each fish serum sample from 1:50 to 1:25,600 in T-TBS-BSA. The test serum, MAb 1.14 secondary antibody, and HRP conjugate volumes were 100 µl/well. A serum standard for antibody ELISA II was selected for having moderate to high antibody concentration and a linear relation ( $r^2 \geq 0.95$ ) between the mean OD and the serum dilution.

### Test serum dilution

A single dilution for screening serum test samples in antibody ELISA II was selected on the basis of comparing the antibody concentration in sera from sockeye salmon injected with the r-p57 protein with the antibody concentration in sera

from uninjected sockeye salmon (normal serum) using antibody ELISA I. The low-responder serum pool served as a source of antibody to the r-p57 protein. Among 40 normal serum samples from the uninjected sockeye salmon, 10 were selected to serve as negative controls because there was no detectable *R. salmoninarum* antigen in the corresponding kidney samples on the basis of the BKD-ELISA (*R. salmoninarum*-negative controls). Serial doubling dilutions from 1:10 to 1:1,280 and from 1:10 to 1:80 in T-TBS-BSA were made from the low-responder pool and the individual *R. salmoninarum*-negative control sera, respectively. Each dilution of an *R. salmoninarum*-negative serum was tested in duplicate, and each dilution of the low-responder serum pool was tested in triplicate. These data were used to compare the mean OD for a given dilution of the low-responder serum pool with the mean OD for the same dilution of the *R. salmoninarum*-negative control serum.

### Antibody ELISA II characterization

Determinations of assay precision of antibody ELISA II and a comparison of the antibody ELISA II results with those of 2 other measures of antibody activity were performed.

**Assay precision.** To determine the microplate-to-microplate and day-to-day variability of antibody ELISA II, 34 sockeye salmon sera were analyzed on each of 6 days. The 34 samples were selected from a group of sera previously analyzed by ELISA II and represented the range of ODs that could be obtained from testing a 1:50 dilution. Daily test samples were assigned to the same wells on each of 3 microplates. Samples were tested in duplicate on each microplate. Sufficient volumes of individual sera and dilutions of the rainbow trout serum standard used to calculate the standard curve were prepared for all assays and then aliquoted and stored at -80 C until the day of use. To minimize variation, microplates were from the same manufacturer's lot and were loaded by the same person.

**Assay comparison.** Sera from 24 sockeye salmon vaccinated with r-p57 protein were tested at doubling dilutions from 1:25 to 1:3,200. Ten samples required retesting at doubling dilutions for 1:6.25 to 1:50 to obtain ODs within the linear range of the titer curve for calculation of the units per milliliter of antibody by the corresponding OD value ( $OD_C$ ). Dilution series of the sockeye salmon sera were distributed among 5 microplates. Included on each microplate was a 1:25 dilution of normal Mt. Lassen strain rainbow trout serum and doubling dilutions of the rainbow trout serum standard from 1:100 to 1:6,400. The units per milliliter of antibody to the r-p57 protein in each serum were calculated from the mean OD of a 1:50 dilution as described for antibody ELISA II. The OD values determined by antibody ELISA I were used to calculate the endpoint dilution and the units per milliliter of antibody by  $OD_C$ .<sup>2,31</sup>

### Data analyses

For antibody ELISA II, the 1:400, 1:800, 1:1,600, 1:3,200, and 1:6,400 dilutions of the rainbow trout serum standard were assigned 160, 80, 40, 20, and 10 units/ml antibody, respectively. A standard curve was created for each plate by plotting the log-linear relationship between the mean OD

value of each dilution of rainbow trout serum standard to the assigned units per milliliter of antibody activity. The units per milliliter of antibody activity of each test serum was determined by reference to the standard curve on the same microplate and by multiplying the value by the inverse of the dilution factor. The standard curve was extrapolated for individual test samples whose average OD values were less than the mean OD of the greatest dilution of the serum standard, i.e., test samples that had between 0 and 500 units/ml at a 1:50 dilution. Test samples in this range were not retested at a lower dilution because they were generally non-responders, and nonspecific binding of serum proteins may falsely increase the number of units per milliliter of antibody. The standard curve was not extrapolated for test samples with average OD values greater than the mean OD of the lowest dilution of the serum standard, i.e., test samples with >8,000 units/ml at a 1:50 dilution. Test sera with this level of reactivity were retested at greater dilutions. The results of the entire microplate were disregarded if the coefficient of determination ( $r^2$ ) between the assigned units per milliliter of antibody activity and OD for the 5 points of the standard curve was below 0.95.

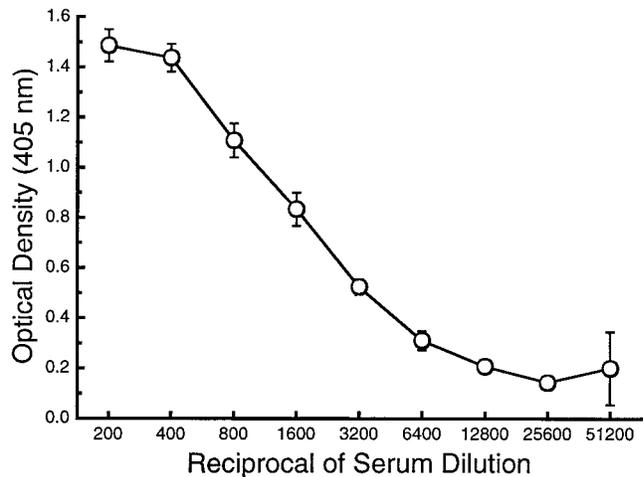
As measures of the precision of antibody ELISA II, the effect of day-to-day and microplate-to-microplate variation on the calculated units per milliliter of antibody activity were determined by analysis of variance.

For the assay comparison, an endpoint dilution titer was defined as the reciprocal of the dilution of a given sockeye salmon serum that produced a mean OD value greater than or equal to the threshold OD value. The threshold OD value was twice the mean of the OD values produced by the normal Mt. Lassen strain rainbow trout serum samples from all plates.<sup>31</sup> The units per milliliter by OD<sub>c</sub> antibody were calculated for test sera after the mean OD values for both the test sera and rainbow trout serum standard dilutions were corrected by subtracting the appropriate mean control serum OD value.<sup>2</sup> A curve describing the relationship of the OD value to the dilution of the rainbow trout serum standard was determined. The inflection point of the curve was determined by log/logit analysis. The inflection point was considered to represent the 50% endpoint dilution of the rainbow trout standard serum and the corresponding OD value (OD<sub>c</sub>). Regression analysis was used to determine the dilution of each test serum at the OD<sub>c</sub> of the standard curve from the same microplate. Only the linear portion of the test serum curve was used for analysis. When the mean OD value for the lowest dilution of a test sample was below the OD<sub>c</sub> value, the sample was assigned a value for units per milliliter by OD<sub>c</sub> of 0.0. The distribution of units per milliliter of antibody activity was compared with the endpoint dilution titer distribution and the units per milliliter by OD<sub>c</sub> distribution by regression analysis.

## Results

### ELISA development

**Rainbow trout serum standard.** Each of the injected Shasta strain rainbow trout produced a measurable antibody response to the r-p57 protein. Maximum OD values typically occurred at serum dilutions between



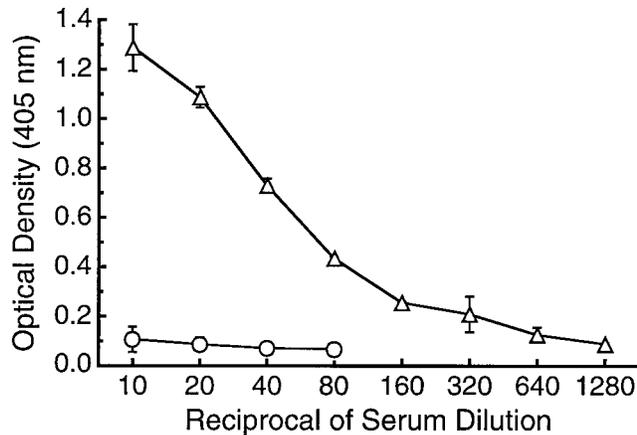
**Figure 1.** Antibody ELISA I OD values for dilutions of serum from a rainbow trout hyperimmunized with the recombinant p57 protein. Data points are means ( $\pm$ SD) for 4 separate measurements. The standard curve used in antibody ELISA II is represented by the 1:400–1:6,400 dilutions.

1:50 and 1:3,200. The serum from 1 fish was selected as a standard for antibody ELISA II based on a linear relationship between the  $\log_2$  of the serum dilution and the mean OD for dilutions between 1:400 and 1:6,400 ( $r^2 = 0.983$ ; Fig. 1).

**Test serum dilution.** A single dilution for screening test sera was chosen based on 2 criteria: (1) the lowest dilution at which the mean OD value produced by the *R. salmoninarum*-negative control sera was not different from the mean OD value of the blanks and (2) the dilution at which the mean OD value of the low-responder serum pool was at least 5 times greater than the mean OD value for the *R. salmoninarum*-negative control sera. At a dilution of 1:40, all of the *R. salmoninarum*-negative sera produced mean OD values that were not different from those produced by the blanks ( $P > 0.05$ ). The mean OD value for the low-responder serum pool was at least 6-fold greater than the mean OD value of *R. salmoninarum*-negative sera when diluted 1:10, 1:20, 1:40, or 1:80 (Fig. 2). On the basis of these data and for convenience of sample processing, a 1:50 dilution was selected to be the standard dilution for screening serum samples from test fish for antibody ELISA II.

### Antibody ELISA II characterization

**Assay precision.** For a given test day, 34 sockeye salmon sera were tested on each of 3 microplates. The daily microplate-to-microplate coefficient of variation (CV) among all of the sera tested ranged from 0 to 23%, with a mean ( $\pm$ SD) CV of  $6.68\% \pm 4.62\%$ . Among the 18 microplates from all 6 test days, the CV for the daily grand mean of any 1 sample was between 4% and 14%. Whereas the interassay variation for a

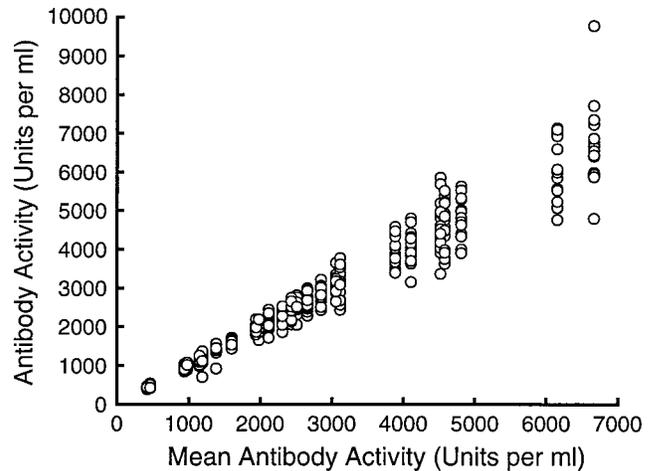


**Figure 2.** OD values produced in antibody ELISA I by serum from sockeye salmon categorized as *R. salmoninarum* negative (○) and from a low-responder serum pool (△). Data points are means ( $\pm$ SD) for 10 *R. salmoninarum*-negative fish tested in duplicate and for the low-responder pool tested in triplicate.

given sample was generally proportional to the relative magnitude of its antibody activity (Fig. 3), the mean CV was  $8.29\% \pm 2.99\%$ . When a mean antibody activity value based on all of the samples tested on a given microplate was assigned to each microplate, there was an intraassay effect of assay plate ( $P = 0.0207$ ) and an interassay effect of assay date ( $P = 0.0004$ ).

To further examine the microplate-to-microplate variation, the data for each sample were normalized by conversion to a percentage of the daily mean prior to statistical analysis, i.e., a mean antibody activity value for each sample was calculated daily on the basis of triplicate testing and then the antibody activity value calculated for each replicate was converted to a percentage of the daily mean. The first microplate loaded on each assay date had the greatest units per milliliter of antibody activity for most of the samples. The samples tested on that microplate were 102.1% of the daily mean on average, whereas the samples tested on the other 2 microplates were 98.7% and 99.1% of the mean. The variation was considered to be related to equipment because all microplates were treated equally each day. The increase in the calculated units per milliliter of antibody activity for most samples on the first microplate did not affect the ranking of the samples by relative antibody concentration.

The effect of the assay date was predominantly due to the variation occurring among the samples tested on the first day. The mean units per milliliter of antibody activity of all of the samples tested on the first day was an average of 92.5% of the mean units per milliliter of antibody activity for each of the other assay dates. Removal of the first assay date's results from the data eliminated the day effect ( $P = 0.2691$ ).



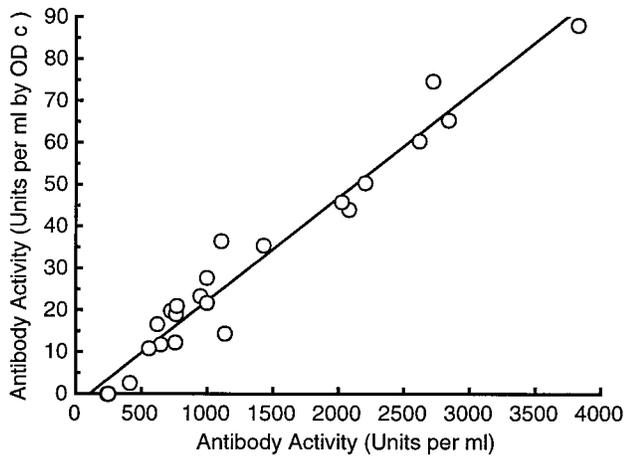
**Figure 3.** Precision of antibody ELISA II as determined by repeated measurements of 34 sockeye serum samples (for clarity, only data from 25 representative samples are shown). Data points are grand means for 18 duplicate measurements (x axis) or means for 2 values (y axis).

To further examine the day-to-day variation in calculated antibody activity, the sockeye salmon sera were ranked daily based on the mean antibody activity for 3 determinations. These data were then compared for the 6 assay dates. Two sera were eliminated from the ranking because their daily mean OD values were outside the range of the standard curve. Among the 6 assay dates, 22% (7/32) of the sera did not change their relative position in the ranking. A change of 1 position, up or down, occurred at least once with 63% (20/32) of the sera. About 13% (4/32) of the sera changed 2 positions, and 1 serum sample increased 3 positions in the ranking at least once over the 6 test days.

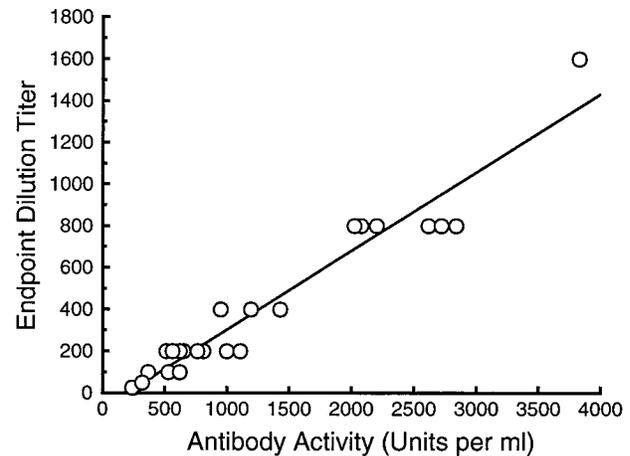
**Assay comparison.** On the basis of antibody ELISA II, the levels of specific antibody to the r-p57 protein in the test sera ranged from 241 to 3,830 units/ml. Antibody titers calculated for the same sera on the basis of an endpoint dilution ranged from 25 to 1,600. Antibody levels based on comparison of the  $OD_C$  of the standard curve ranged from undetectable to 109 units/ml by  $OD_C$ . Three sera categorized as having an undetectable level of antibody by the  $OD_C$  method had 241, 248, and 253 units/ml by antibody ELISA II. The distribution of antibody activity units in serum correlated with the distribution of units per milliliter by  $OD_C$  (Fig. 4;  $r^2 = 0.956$ ) and generally correlated with the distribution of endpoint dilutions (Fig. 5;  $r^2 = 0.936$ ).

## Discussion

An ELISA was used to measure the specific antibody response of sockeye salmon to a protein antigen of *R. salmoninarum*. The goal in developing this antibody ELISA was to improve the efficiency and ver-



**Figure 4.** Correlation between serum antibody levels derived by the standard curve method of antibody ELISA II with those calculated from the same data on the basis of comparison to 50% of the maximum OD values produced by dilutions of the serum standard (OD<sub>c</sub>). For the standard curve method, the antibody activity units per milliliter were calculated on the basis of the mean OD produced by a 1:50 dilution. Each data point represents an individual fish.



**Figure 5.** Correlation between serum antibody levels derived by the standard curve method of antibody ELISA II with those calculated from the same data on the basis of an endpoint dilution. For the standard curve method, the antibody activity units per milliliter were calculated on the basis of the mean OD produced by a 1:50 dilution. Each data point represents an individual fish.

satility by which levels of specific antibody are quantified in fish without sacrificing the sensitivity offered by an immunoassay. The p57 protein of *R. salmoninarum* was used in this study because it is a major factor in the pathogenesis of the bacterium,<sup>39</sup> and the importance of the humoral response to this protein by fish during an infection is poorly understood.<sup>16</sup> Whereas other ELISAs have been reported for measuring the levels of serum antibody in salmonids, a unique advantage of the method described here is that salmonid antibody levels were estimated from a standard curve on the basis of analyzing a single dilution of serum.<sup>2,33</sup> Serum antibody concentrations calculated on the basis of the single-dilution assay were correlated with those for the same samples tested by the multiple dilution methods. These data suggested that quantification of serum antibody concentrations from a single dilution was as accurate as quantification by the other methods. By reducing the testing to a single sample for each fish, gains can be made in efficiency, cost savings, and in the ability to test very small fish, such as those used in vaccine trials. These improvements can be important, because the variability in antibody levels among fish in response to a treatment or a change in environmental condition can be very great, requiring the testing of many fish for an accurate profile.<sup>27,36</sup>

A 1:50 dilution was chosen for the test sera based on the lack of nonspecific protein binding, the convenience of processing samples at that dilution, and the economical use of the original sample. Typically, 500  $\mu$ l of each diluted test serum was used for testing, so only 10  $\mu$ l of whole serum was required to measure the antibody response of a fish; in most instances, a

suitable sample was obtained from juvenile salmonids weighing <3 g. In addition, the ability of this ELISA to distinguish the weak-responder serum pool from sera of *R. salmoninarum*-negative fish suggested that diluting the serum will not affect the ability to identify fish that have responded weakly to the p57 protein.

A different antibody ELISA was developed to measure antibody responses by coho salmon (*O. kisutch*) to *R. salmoninarum*.<sup>33</sup> For that assay, the microplate well surfaces were coated with whole *R. salmoninarum* cells. Although that antibody ELISA was more sensitive than a conventional agglutination assay, the authors reported problems with false positives thought to be due to cross-reactive antibodies. By utilizing a recombinant version of the p57 protein instead of whole bacteria, the antibody ELISA described here should be more selective in which fish antibodies will bind to the antigen. Also, the use of a monoclonal mouse antibody for the detection of the bound fish antibody as opposed to polyclonal rabbit sera may reduce false positives due to direct binding of the secondary antibody to other fish serum proteins that may remain after sample incubation.

The results of an ELISA for antibody may be reported in several forms, such as OD values,<sup>18,36</sup> or as arbitrary units on the basis of the reactivities of standards or dilution of the test serum.<sup>2,6</sup> OD values for the antibody ELISA II were transformed into antibody units on the basis of the standard curve. By using the same reference serum to form a standard curve for each microplate, results can be standardized among individual samples (fish) from large groups whose analysis required several microplates or even multiple assay dates.<sup>25</sup> The analysis of assay precision revealed

that various nonrandom sources of error, such as the differences among microplates or the day the ELISA was performed, formed a much larger proportion of the total variability when the data were not standardized. When the ODs of the test sera were not transformed into antibody units, those components accounted for about 78% of the variance. In contrast, when the data were standardized only 30% of the variance could be attributed to the nonrandom sources of error. The results of triplicate testing of individual samples on each of 6 dates demonstrated that reproducible results could be obtained when samples were analyzed on different microplates during a given run or when analyzed on different assay dates. The intraassay variation was similar to that reported by others.<sup>6,14,34</sup>

These results also suggest that a proportion of the total variability in a multiplate ELISA can be controlled by ensuring that samples representing each test group are assigned to every microplate. This practice will help to eliminate the microplate-to-microplate effect by spreading random variations due to slight differences in microplates evenly across all treatment groups. In addition, a positive control sample can be used for quality control on each microplate. If the calculated units per milliliter of antibody activity of the positive control sample falls outside a predetermined range, the results of the entire microplate should be discarded. At the time of these experiments, a high-titer rainbow trout anti-p57 antiserum was included on all plates to establish the mean units per milliliter of antibody activity. The interday precision of 14% was used to set the units per milliliter of antibody activity range for the positive control serum for assay plate quality control.

Unfortunately, the correlation between the calculated antibody units and the actual amount of fish antibody is not known. Therefore, the units of antibody activity in a test serum is only comparable to that of other samples derived from dilutions of the same serum standard. However, if the standard serum is from a hyperimmunized fish with suitable reactivity, a sufficient quantity of the standard will be available for many assays. Only about 3  $\mu$ l of the serum standard is required to prepare the dilutions for a single microplate assay. A large pool of antisera from immunized fish could be developed for the standard serum and distributed among interested laboratories to insure that results are comparable.

The single-dilution antibody ELISA described here can be adapted to detect antibodies to a variety of antigens. Similar assays have also been used for the detection of antibodies to multiple antigens.<sup>22,35</sup> Potential capture antigens must be able to adsorb to microplate surfaces. Also, a standard antiserum must be available from a fish species whose antibody is recognized by

the secondary antibody. The Warr 1.14 MAb was originally produced to recognize rainbow trout antibody.<sup>11</sup> It has since been shown to cross-react with the antibody of coho salmon,<sup>17</sup> and in other analyses during this study it reacted with the antibodies of sockeye salmon and chinook salmon (*O. tshawytscha*) (data not shown). This assay was designed to use commercially available ELISA reagents as much as possible and an anti-fish mouse antibody from a hybridoma that is readily available, so that this ELISA could be easily performed in other laboratories.

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### Sources and manufacturers

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- b. Life Technologies, Gaithersburg, MD.
- c. Dianne Baker, University of Washington, Seattle, WA.
- d. pET, Novagen, Madison, WI.
- e. Kirkegaard and Perry Laboratories, Gaithersburg, MD.
- f. Calbiochem-Novabiochem Corp., San Diego, CA.
- g. Costar Corp., Cambridge, MA.

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