

INTRODUCTION

Cathelicidins are small antimicrobial proteins. Expression levels increase in Rainbow Trout (ref 1) and Atlantic Salmon (ref 2) during bacterial infections.

PRINCIPLE OF THE ASSAY

The assay uses a polyclonal antibody that recognizes Rainbow Trout and Atlantic Salmon Cathelicidin-2 (referred to here as Cathelicidin). Unconjugated antibody is coated on wells of a microtiter plate and used for capture. Horseradish peroxidase (HRP) conjugated antibody is used for detection. Standards and diluted samples (100 μ l) are incubated in the antibody coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 μ l) is added and incubated for 45 minutes. If Cathelicidin molecules are present, they are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If Cathelicidin is present, a blue color develops. Color development is stopped by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of Cathelicidin is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-Cathelicidin coated plate (12 x 8-well strips)
- 2x HRP conjugate, 7 ml
- Cathelicidin stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: YD50-1, 2 x 50 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes or 96-well polystyrene plates
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

Store the standard vial at -20°C. The remainder of the kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. The kit will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is conducted with a complete understanding of the instructions and with adherence to good laboratory practice.
3. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate¹. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 μ l aliquots to the wells of the antibody-coated plate.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

WASH SOLUTION

The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 4°C for one week.

DILUENT

The diluent is formulated for measurement of Cathelicidin in trout and salmon serum or plasma. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. Reconstitute it with 200 μ l of deionized water, gently mix, and prepare the 40 ng/ml standard as described on the vial label.
 2. Label seven polypropylene tubes as 20, 10, 5, 2.5, 1.25, 0.625, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
 3. Pipette 0.25 ml of the 40 ng/ml Cathelicidin standard into the tube labeled 20 ng/ml and mix. This provides the 20 ng/ml Cathelicidin standard.
 4. Similarly prepare the 10 – 0.625 ng/ml standards by two-fold serial dilution.
- Discard the stock after use.

¹ Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

HRP CONJUGATE

For each 8-well strip used in the assay, mix 0.5 ml of 2x HRP conjugate with 0.5 ml of YD50-1 diluent. Use 100 μ l per well.

SAMPLES

Trout. We found Cathelicidin levels ranging from approximately 1 μ g/ml in plasma from healthy fish to approximately 120 μ g/ml in plasma from fish infected with *Flavobacterium psychrophilum*. Because of the wide range, optimal dilutions must be determined empirically. However, we suggest that samples initially be tested at a 1000-fold dilution.

Salmon. We found that Cathelicidin levels ranged from approximately 0.3 μ g/ml in plasma from healthy salmon to approximately 1.2 μ g/ml in plasma from salmon with pancreatic disease. We suggest testing salmon plasma at a 100-fold dilution.

Ideally, dilutions should be performed in polystyrene 96-well plates (not provided). This allows quick and easy transfer of diluted samples to the antibody-coated plate using 8 or 12-channel multi-pipettors.

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 4°C.
2. Dispense 100 μ l of standards and samples into the wells.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 μ l/well).
5. Dispense 100 μ l of 1x HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 μ l/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 μ l of TMB into each well.
10. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
11. After 20 minutes, stop the reaction by adding 100 μ l of Stop solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read absorbance at 450 nm² with a plate reader within 5 minutes.

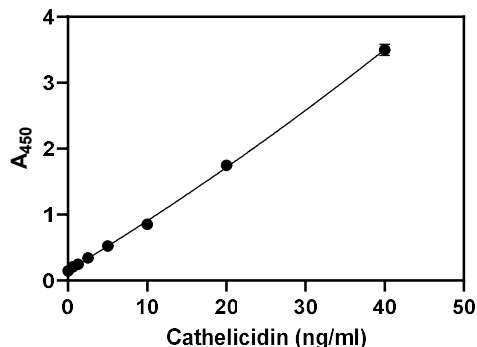
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the Cathelicidin concentration.
2. Fit the standard curve using graphing software. We suggest using a second order polynomial (quadratic) equation.
3. Derive the concentration of Cathelicidin in the samples.
4. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
5. If the absorbance values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve is shown below. This curve is for illustration only.

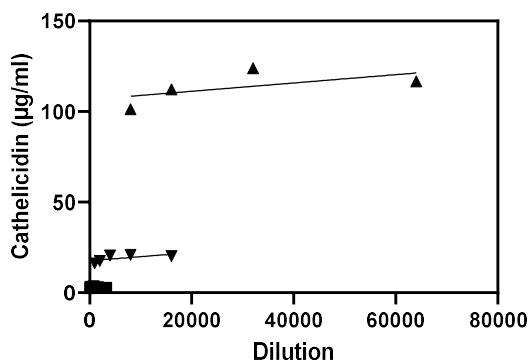
Cathelicidin (ng/ml)	A ₄₅₀
40	3.498
20	1.748
10	0.853
5	0.524
2.5	0.341
1.25	0.245
0.625	0.207
0	0.144



² If absorbance of the high standard is ≥ 4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.

PERFORMANCE

Linearity: To assess the linearity of the assay, three rainbow trout plasma samples with cathelicidin concentrations of 3.1, 19.1, and 113.6 $\mu\text{g/ml}$ were serially diluted to produce values within the dynamic range of the assay.



REFERENCES

1. Furlan M, et al. Induced expression of cathelicidins in trout (*Oncorhynchus mykiss*) challenged with four different bacterial pathogens. *J Pep Sci.* 2018;24:e3089. <https://doi.org/10.1002/psc.3089>
2. McGrath L, O'Keefe J and Slattery O. Antimicrobial peptide gene expression in Atlantic salmon (*Salmo salar*) seven days post-challenge with *Neoparamoeba perurans*. *Developmental and Comparative Immunology* 127 (2022) 104287

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