INTRODUCTION

Troponin-C, a 17 kDa protein, is part of the troponin ITC-complex that regulates muscle contraction. It is expressed as two isoforms. One in fast twitch skeletal muscle (STNC), the other in cardiac and slow-twitch skeletal muscle (CTNC). This assay allows measurement of salmon and trout CTNC. Diseases that damage heart or slow-twitch skeletal muscle cause release of CTNC into serum. As shown in Figure 1, we found that serum CTNC levels were significantly increased in Atlantic salmon with pancreatic disease.

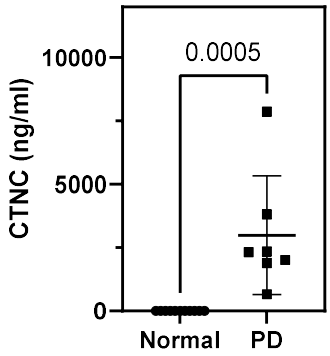


Figure 1. CTNC levels in serum from healthy salmon (3.0±1.5 ng/ml, mean±SD, n=13) and salmon with pancreatic disease (3378±2342 ng/ml, mean±SD, n=7).

PRINCIPLE OF THE ASSAY

The assay[1] uses two different CTNC antibodies, one for solid phase immobilization and one, conjugated to horseradish peroxidase (HRP), for detection. Standards and diluted samples (100 µl) are incubated in antibody coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If CTNC molecules are present, they are sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If CTNC 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 CTNC is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-CTNC coated plate (12 x 8-well strips)
- HRP conjugate stock **Store ≤ -20°C**
- CTNC standard **Store ≤ -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: TNID50-1, 2 x 50 ml **Store ≤ -20°C**
- 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
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

The CTNC stock, HRP conjugate stock, and diluent must be stored 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. Kits 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. First, pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate[2]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl aliquots to the appropriate wells of the ELISA 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.

[1] This ELISA was developed during a Scottish Aquaculture Innovation Centre sponsored collaboration involving scientists from Benchmark Genetics, Cooke Aquaculture, Life Diagnostics, Moredun Research Institute, and The University of Edinburgh

[2] Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

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 specially formulated for measurement of CTNC in salmon serum. It is provided ready to use. DO NOT substitute other buffers. Thaw at room temperature, or in lukewarm water, and mix gently before use. Refreeze unused diluent at -20°C if future use is intended.

STANDARD

1. The stock is lyophilized. Reconstitute it with the volume of diluent indicated on the vial label and prepare the 10 ng/ml standard as instructed.
2. Label seven polypropylene tubes as 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 10 ng/ml CTNC standard into the tube labeled 5 ng/ml and mix. This provides the 5 ng/ml CTNC standard.
4. Similarly prepare the 2.5 to 0.156 ng/ml standards by two-fold serial dilution.

Unused reconstituted CTNC stock should be frozen at or below -20°C.

HRP CONJUGATE

The HRP diluent is provided as a concentrated stock. Approximately five minutes prior to use dilute it with diluent TNID50-1 as detailed on the stock vial label.

SAMPLES

In studies at Life Diagnostics, we found CTNC levels ranging from ~3 ng/ml in serum from healthy salmon to >7 μg/ml in serum from salmon with pancreatic disease. To increase the possibility of obtaining values within range of the standard curve we recommend that each sample be evaluated at dilutions of 100-fold and 2000-fold. To avoid matrix effects do not use dilutions less than 50-fold.

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 μl 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 μl/well).
5. Dispense 100 μl of 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 μl/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 μl 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 μl 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 nm with a plate reader within 5 minutes.

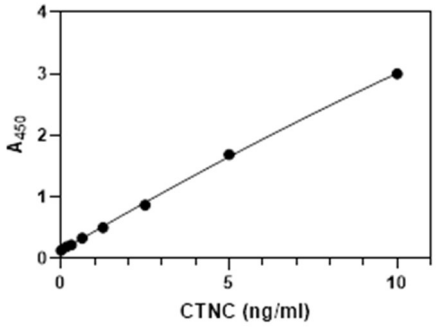
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus CTNC concentration.
2. Fit the standard curve using graphing software. We typically fit to a two-site, total and nonspecific binding model, or a second order polynomial (quadratic) equation.
3. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
4. If the A_{450} values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with absorbance at 450 nm on the Y-axis against CTNC concentrations on the X-axis is shown below. This curve is for illustration only.

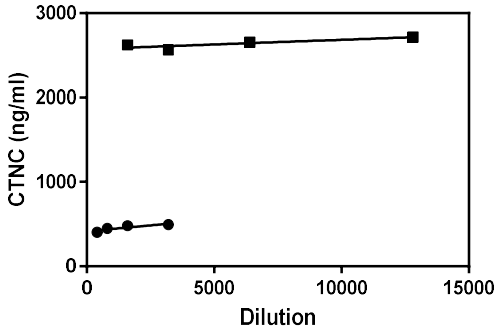
CTNC (ng/ml)	A_{450}
10	3.002
5	1.692
2.5	0.871
1.25	0.506
0.625	0.334
0.3125	0.226
0.156	0.194
0	0.136



Life Diagnostics, Inc.
124 Turner Lane, West Chester, PA 19380
610-431-7707 - info@lifediagnostics.com.com – www.lifediagnostics.com

PERFORMANCE

Linearity: To assess the linearity of the assay, two serum samples sample containing CTNC at concentrations of 443 and 2641 ng/ml were serially diluted to produce values within the dynamic range of the assay.



Specificity: The assay recognizes CTNC from mammals, salmon, and rainbow trout. Although not investigated. The antibodies used in the kit do not recognize fast-twitch skeletal muscle troponin-C from mammals or salmon.

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For technical assistance please email us at info@lifediagnostics.com

Life Diagnostics, Inc.
124 Turner Lane, West Chester, PA 19380
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