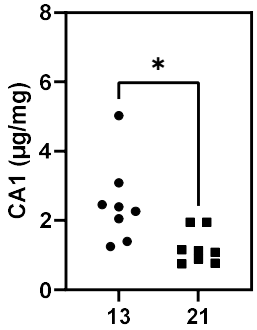
INTRODUCTION

Carbonic anhydrase 1 (CA1) is a cytosolic enzyme, expressed in gill, which plays a key role in acid-base regulation and salt transport. Consistent with literature reports (ref 1), we found that levels of CA1 in gill extracts decreased when rainbow trout were acclimated at 21°C, relative to 13°C (below).



PRINCIPLE OF THE ASSAY

The assay uses polyclonal antibodies generated against recombinant Rainbow Trout CA1. The antibodies also recognize Atlantic salmon CA1. Standards and diluted samples (100 µl) are incubated in anti-CA1 coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If CA1 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 CA1 is present, a blue color develops. Color development is stopped after 20-minutes by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of CA1 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-CA1 coated plate (12 x 8-well strips)
- HRP conjugate stock.
- CA1 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 stock vials 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 plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl 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.

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

DILUENT

The diluent is formulated for measurement of CA1 in trout and salmon gill extracts. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of recombinant Rainbow Trout CA1 in a stabilizing matrix. Reconstitute it with 200 µl of deionized water, gently mix, and prepare the 100 ng/ml standard as described on the vial label.
2. Label seven polypropylene tubes as 50, 25, 12.5, 6.25, 3.13, 1.56 and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 100 ng/ml CA1 standard into the tube labeled 12.5 ng/ml and mix. This provides the 50 ng/ml CA1 standard.
4. Similarly prepare the 12.5 to 1.56 ng/ml standards by two-fold serial dilution.

The CA1 stock should be used within 30 min of reconstitution and then discarded.

HRP CONJUGATE

The HRP conjugate is provided as a 2x stock. Prior to use it must be diluted with one volume of YD50-1 diluent. For each 8-well strip, mix 0.5 ml of 2x stock with 0.5 ml of YD50-1. Use 100 µl of the diluted HRP conjugate per well.

SAMPLES

We measured CA1 in gill extracts and normalized levels to total protein. Gill extracts were prepared and characterized as described below.

1. Approximately 100 mg of gill filament was homogenized in 0.5 ml of 50 mM NaCl, 10 mM Tris.HCl, 1 mM EDTA, pH 7.4.[2]
2. Homogenates were centrifuged at 15,000 rpm for 2 minutes using a benchtop microfuge.
3. Supernatants were saved and used for testing.
4. Total protein in the supernatants was determined using BCA protein assay.

Supernatants were tested for CA1 after 100-1000-fold dilution with diluent YD50-1 (do not use other diluents). Optimal dilutions should be determined empirically but we suggest testing Rainbow Trout extracts at a 1000-fold dilution and Atlantic salmon extracts at 400-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 appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well).
5. Dispense 100 µl of diluted 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 5 times 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[3] 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 CA1 concentration. We suggest fitting data to a two-site, total and non-specific binding model.
2. Derive the concentration of CA1 in the samples.
3. Multiply the derived concentration by the dilution factor to determine the concentration in the original sample and divide values by the respective total protein concentration (mg/ml).
4. If the absorbance values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

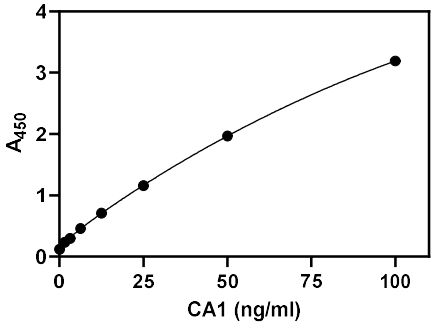
[2] We homogenized samples in 1.5 ml Safe-lock Eppendorf tubes containing 0.1 ml of 1.6 mm stainless steel beads using a Bullet Blender. Homogenization was performed at setting 10 for 1 minute.

[3] 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.

TYPICAL STANDARD CURVE

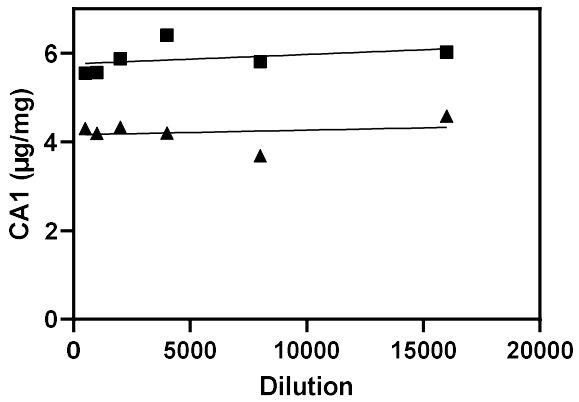
A typical standard curve is shown below. This curve is for illustration only.

CA1 (ng/ml)	A_{450}
100	3.192
50	1.970
25	1.160
12.5	0.710
6.25	0.459
3.16	0.300
1.56	0.235
0	0.121



PERFORMANCE

Linearity: To assess the linearity of the assay, Rainbow Trout gill extracts with CA1 concentrations of 5.9 and 4.2 μg/mg of total protein were serially diluted to give values within range of the assay.



REFERENCES

1. Houston AH and McCarty LS. Carbonic anhydrase (acetazolamide-sensitive esterase) activity in the blood, gill and kidney of the thermally acclimated rainbow trout Salmo gairdneri. J. Exp. Biol. (1978), 73, 15-27.

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