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THE NEXT GENERATION

Assay protocol for the quantitative determination of
Endothelin (1-21) in saliva
using the BIOMEDICA Endothelin ELISA cat.no. BI-20052

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FOR DILUTION OF SALIVA-AN ADDITIONAL VIAL OF PB-BUFFER IS REQUIRED.
PB-Buffer (Cat.no BI-9M) should be ordered additionally-the buffer is for

1. Introduction

This assay protocol describes the measurement of Endothelin in human saliva, using the Biomedica Endothelin ELISA. cat.no. BI-20052.

The presence of immunoreactive Endothelin in human saliva was first published in 1991 ⁽¹⁾. Endothelin has been measured in saliva using HPLC techniques ⁽²⁾. Recently, a publication described the successful application of the Biomedica Endothelin ELISA for saliva measurement ⁽³⁾. It was shown that salivary Endothelin concentrations were raised in patients with chronic heart failure and indicated progression of disease severity through each New York Heart Association Functional Class. Furthermore, Endothelin concentrations in saliva seem to discriminate between controls and patients with mild symptoms of chronic heart failure.

These findings suggest that measurement of Endothelin in saliva may be a simple, non-invasive method to assist in the diagnosis and assessment of disease severity in patients with suspected and established congestive heart failure.

2. Additional material and equipment required

- Standard ELISA equipment:
 - Distilled water
 - Variable volume pipettes in the range of 50 μ l to 1000 μ l
 - Multichannel or multipipette
 - Manual or automatic microwell washer
 - ELISA reader equipped with 450 nm filter
 - Graph paper or software for calculation of results
- Sarstedt Salivettes.

3. Reagent and sample preparation - performance of the assay

3.1. Sample collection

Last meal or cigarette should have been consumed at least one hour before sample collection.

1. Rinse mouth with water, wait 10 minutes.
2. Put cotton roll of the Sarstedt Salivette tube into the mouth, chew for 30 sec., keep in mouth for two additional minutes.
3. Place the cotton roll into the flat bottom upper tube of the Salivette, seal with the stopper and centrifuge for 3 minutes at 1000 rcf (rotational centrifugal force = g).
4. Remove the flat bottom tube from the Salivette and pipette the clear saliva from the bottom of the V-tube, aliquot and store at -20°C .

3.2. Assay procedure

We recommend duplicates for all values.

1. Redissolve the Endothelin stock (amber vial with red cap) in 2 ml of PB-Buffer and leave for 30 min. at room temperature ($18-26^{\circ}\text{C}$), shake well. The Endothelin stock then contains approximately 10 fmol/ml Endothelin (exact value is stated on the label). Reconstituted ET stock-standard is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles.
Do not use the plasma-standards 0 - 5 (white caps) and controls (yellow caps)!
2. Prepare a serial dilution of the Endothelin stock standard solution with PB-Buffer down to approx. 0.6 fmol/ml (e.g. 10 / 5 / 2.5 / 1.25 / 0.625 fmol/ml). PB-Buffer is used as a zero standard.
3. Collect the saliva samples according to the instructions above. Dilute saliva samples 1+4 with PB-Buffer, mix well.
4. Redissolve detection antibody (green cap) in 5.5 ml of assay buffer and leave at room temperature ($18-26^{\circ}\text{C}$) for 30 min., shake well. Reconstituted antibody is stable at -20°C until expiry date stated on the label. Avoid repeated freeze-thaw cycles!
 - Mark positions for blank, ET stock-standards, samples on the protocol sheet supplied.
 - Take microtiter strips out of the alu bag and mark as appropriate. Mark 2 wells as blank.Store unused strips with the desiccant at 4°C in the plastic bag supplied. Strips are stable until expiry date stated on the label.
5. Dilute washing buffer concentrate to 1000 ml (add the 100 ml of concentrate to 900 ml of distilled water) mix well, avoid formation of foam. Crystals in the buffer concentrate will dissolve at room temperature. The diluted washing buffer is stable at 4°C until expiry date stated on the label.
 - All reagents and samples must have room temperature ($18-26^{\circ}\text{C}$) before used in the assay, this usually takes about 30 min.
6. Pipette 200 μ l of standards and samples in the respective wells.
7. Add 50 μ l detection antibody (green) to all wells except blank, mix well.
8. Cover strips with plastic film and incubate overnight (16-24 hours) at room temperature ($18-26^{\circ}\text{C}$).

Make sure all wells are sealed well with the film to avoid evaporation.

9. Discard contents of the wells and wash 5x with 350 μ l diluted washing buffer. Make sure that residual buffer is removed completely after the last wash, e.g. by inverting the plate and tapping firmly on absorbent paper.
10. Add 200 μ l conjugate (red) to all wells.
11. Cover strips with plastic film and incubate for 1 hour at 37°C in an incubator/shaker.
If no shaker is available, incubate for 3 hours at 37°C without shaking.
12. Discard contents of the wells and wash 5x with 350 μ l diluted washing buffer. Make sure that residual buffer is removed completely after the last wash, e.g. by inverting the plate and tapping firmly on absorbent paper.
13. Add 200 μ l substrate to all wells and incubate for 30 min. at room temperature (18-26°C) in the dark.
14. Add 50 μ l stop solution to all wells, shake well.
15. Determine absorption immediately with an ELISA reader at 450 nm against 690 or 620 nm as reference.
If the correction wavelength of 620 or 690 nm is not available read only at 450 nm.

4. Assay characteristics

Five human saliva samples were spiked with 5 fmol/ml Endothelin each.

The recovery results of these samples were as follows:

Sample	fmol/ml	Recovery
1	4.7	95%
2	5.1	102%
3	5.2	104%
4	3.9	79%
5	5.1	103
Mean recovery		104 %

5. References

1. Lam et al., Immunoreactive endothelin in human plasma, urine, milk, and saliva.
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2. Denver et al., Salivary endothelin concentrations in the assessment of chronic heart failure.
The Lancet (2000) 355, 468-469
3. Whitson et al., Salivary endothelin and its response to postural changes in humans.
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