

## Technical Report

**Emily M. Chester and Virginia J. Vitzthum. 2017. Protocol for extraction and enzyme immunosorbent assay (ELISA) of progesterone or estradiol from human salivary samples preserved with or without sodium azide (protocol v1.0).**

*(With proper citation of this original source, including the IU Scholarworks permanent URL, protocol may be followed as outlined below or modified for user's needs. Please direct questions to [vitzthum@indiana.edu](mailto:vitzthum@indiana.edu) .)*

**Developed at the Laboratory for Evolutionary Anthropology (EVA Lab), Indiana University, Bloomington.**

**Financial support:** National Science Foundation (PLR 1142201, OPP 1319663); Indiana University, Bloomington

**First application:** Chester, EM, JA Madden, and VJ Vitzthum. 2017. *Lose your cool! Eliminating the cold chain in field collection of salivary samples destined for enzyme-immunoassay of steroid concentrations.* Poster presented at the Animal Behavior Conference, Indiana University, Bloomington, April 6, 2017.

**Abstract:** Field research in remote areas presents many challenges, often including a lack of facilities and resources for maintaining a cold chain to preserve samples at low temperatures until they can be assayed in a laboratory. Cold-chains are necessary for the storage and transport of many types of biological samples (e.g., blood, urine and saliva) in order to reduce degradation and inhibit bacterial and fungal growth.<sup>1</sup> One alternative to a cold chain is to add sodium azide, a potent antimicrobial, to each biological sample.<sup>2</sup> However, sodium azide is incompatible with commercial enzyme immunoassay (EIA) kits that use horseradish peroxidase, an enzyme inactivated by sodium azide.<sup>3</sup> To address this problem, we tested and validated EIA protocols that use an alternative enzyme, alkaline phosphatase. These protocols can be used for the measurement of steroid hormones in salivary samples that have been preserved with sodium azide, thus eliminating the trouble and expense of maintaining a cold chain from the field to the lab.

<sup>1</sup>Bruckner, S, A Albrecht, B Peterson, J Kreyenschmidt, J. (2012) Int. J. Food Sci. Technol. 47, 1639-1646.

<sup>2</sup>Cabrol, L., Quéméneur, M., & Misson, B. (2017) J. Microbiol. Methods. 133, 62-65.

<sup>3</sup>Liu, G., Amin, S., Okuhama, N., Liao, G., & Mingle, L. (2006) Histochem. Cell. Biol. 126, 283-291.

## **PROTOCOL**

### **Preparation of Saliva Samples**

Saliva is thawed on day of extraction at room temperature. Samples are vortexed vigorously for 10 minutes then centrifuged at 3,000 rpm for 10 minutes.

### **Steroid Extraction**

#### **Extraction of Salivary Estradiol**

Saliva sample supernatants (1 ml) are pipetted into 16 X 125 mm salinized glass test tubes. Diethyl ether (9 ml) is added to all tubes, then capped and sealed with parafilm, and vortexed vigorously for 15 min on a multi-tube vortexer. Layers are allowed to settle for 20 minutes, then frozen by immersing tube in a methanol/dry ice bath. Tubes are wiped free of methanol, then the upper organic phase is decanted into a 13 X 100 mm salinized glass tube and evaporated under nitrogen in a 35°C water bath.

Diethyl ether (9 ml) is added again to the aqueous partition and the extraction process repeated, the second organic partition added into the same final test tube, which ensures a more complete transfer of steroids during the extraction. The organic layer is dried under nitrogen, then sides of final test tube are rinsed with 1 ml of ether and the tube is dried completely with nitrogen.

Samples are reconstituted in 250 µl of assay buffer from assay kit, vortexed on multi-tube vortexer for 10 minutes, then transferred to 0.65 ml microcentrifuge tubes. Samples are frozen overnight at -20°C until assayed.

#### **Extraction of Salivary Progesterone**

Procedure for extracting progesterone was identical to salivary estradiol, with the exception that 500 µl saliva supernatant is needed and 4.5 ml of diethyl ether is used twice in the extraction. Samples are reconstituted in 250 µl of assay buffer, resulting in a 2x concentration of sample.

### **Steroid ELISA**

#### **Estradiol Assay**

Salivary estradiol concentrations are determined with a commercially available enzyme immunosorbent assay (ELISA) kit (ADI-901-17417β-Estradiol high sensitivity ELISA kit, Enzo Life Sciences, Farmingdale, NY). During extraction,

samples are concentrated 4X in assay buffer, and then are plated in duplicate for each sample. The standard curve is prepared as per the instructions, but extended to eight standards with 7.8 pg/ml as the lowest value. The cross-reactivity of Estrone is 17.8% and Estriol is 0.9%. The intra-assay variation is 4.2 % and the inter-assay variation is 7.8 %. Measured concentrations are divided by 4 in final report, due to 4x concentration during extraction.

### Progesterone Assay

Salivary progesterone concentrations are determined by ELISA kit (ADI-901-011 Progesterone ELISA kit, Enzo Life Sciences, Farmingdale, NY). During extraction, samples are concentrated 2x in assay buffer, and then are plated in duplicate. The cross-reactivity of 5 $\alpha$ -Pregnane-3,20-dione is 100%, 5-Pregnen-3 $\beta$ -o1-20-one is 3.46%, and Corticosterone is 1.43%. The intra-assay variation is 8.04% and the inter-assay variation is 6.57%. Measured concentrations are divided by 2 in final report, due to 2x concentration during extraction.