Steroid Hormone Assays – Method Details

Testosterone

96-well ELISA plates were coated overnight at 4 ˚C (100µL 7.6µg/mL DARS (Donkey Anti Rabbit Serum IgG) prepared in house from the Scottish Antibody Production Unit, UK) in sodium bicarbonate coating buffer (pH 9.6). Plates were washed twice with wash buffer (TBS-T, 300µL) and dried before blocking (220µL; 0.5% BSA in PBS, pH 7.4) for 1hr at room temperature. After 2 washes, standards (T1500, Sigma Aldrich, UK), QC's (#480, Bio-Rad Laboratories, UK) and samples (16µL) were added in duplicate to wells with 84µL of T-HRP (1/20,000; 12-03, ASTRA Biotech, Germany) and 50µL of T-Ab (1/200,000; R3S07-259, Meridian Life Science, USA) (both HRP and Ab diluted in assay buffer = 0.1% BSA PBS + 250ng/ml Cortisol). Plates were incubated for 2hrs at 28˚C with shaking. Plates were washed 4 times before the addition of 120µL of TMB (ES022, EMB Millipore, USA) for 10mins in the dark with shaking. The reaction was stopped with 80µL of 1N Sulphuric Acid. The plate was then read at 450nm for analysis using SoftMax Pro (Version 7.1, Molecular Devices).

Cross-reactivities: 100% Testosterone, 69.5% DHT, 19.7% α-Androsten-3β-17β-diol, 4.6% 3β-Androstanediol, 9.3% Dihydroandrosterone, 6.8% Androstenedione, 13.7% 3α-Androstanediol, 2.5% 11-Ketotestosterone. All other steroids tested gave <1% cross reactivity.

The intra-assay %CV was 4.3%. The inter-assay %CV was 16.5%.

The limit of detection is 0.16ng/mL.

Publications:

Wilson KS, Li D, Valentine I, McNeilly A, Girling S, Li R, Zhou Y, Vanhaecke L, Duncan WC, Wauters J (2022) The novel use of urinary androgens to optimise detection of the fertile window in giant pandas. *Reproduction & Fertility* **3:3** pp122-132 https://doi.org/10.1530/RAF-22-0031

Thorburn J, Cole G, Naylor A, Garbett A, Wilson K, James M, Dodd J, Houghton JDR, Collins PC (2023) Preliminary insight into the reproductive traits of the flapper skate *Dipturus intermedius* using in-field ultrasonography and circulating hormone concentrations. Endangered Species Research **52** pp97-111 https://doi.org/10.3354/esr01264

Cortisol

96-well ELISA plates were coated overnight at 4 ˚C (100µL 5µg/mL Goat Anti Mouse IgG; A008, Arbor Assays, USA) in sodium bicarbonate coating buffer (pH 9.6). Plates were washed twice with wash buffer (TBS-T, 300µL) and dried before blocking (220µL; 0.5% BSA in PBS, pH 7.4) for 1hr at room temperature. After 2 washes, standards (H4001, Sigma Aldrich, UK), QC's (H4001, Sigma Aldrich, UK) and samples (20µL) were added in duplicate to wells with 80µL of C-HRP (1/4,000; 12-01, ASTRA Biotech, Germany) and 50µL of C-Ab (0.176µg/mL; 10-10, ASTRA Biotech, Germany) (both HRP and Ab diluted in assay buffer = 0.1% BSA PBS). Plates were incubated for 2hrs at 28˚C with shaking. Plates were washed 4 times before the addition of 120µL of TMB (ES022, EMB Millipore, USA) for 10mins in the dark with shaking. The reaction was stopped with 80µL of 1N Sulphuric Acid. The plate was then read at 450nm for analysis using SoftMax Pro (Version 7.1, Molecular Devices).

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Cross-reactivities: 100% Cortisol, 1.6% Corticosterone, 1.2% 11-deoxycortisol. All other steroids tested gave <1% cross reactivity.

The intra-assay %CV was 3.6%. The inter-assay %CV was 10.5%.

The limit of detection is 0.2ng/mL.

Publications:

Wauters J, Wilson KS, Cools T, Vancsok C, Bouts T, Mulot B, Leclerc A, Haapakoski M, Kok J, Kuhne R, Ochs A, Duncan WC, Girling SJ, Hildebrandt TB, Zhou Q, Li R, Zhou Y, Cai K, Liu Y, Hou R, Rae M, Valentine I, Vanhaecke L, Li D (2023) Pregnancy length and health in giant pandas: what can metabolic and urinary endocrine markers unveil? *Theriogenology Wild* **3** 100063 https://doi.org/10.1016/j.therwi.2023.100063

Progesterone

96-well ELISA plates were coated overnight at 4 ˚C (100µL 5µg/mL Goat Anti Mouse IgG; A008, Arbor Assays, USA) in sodium bicarbonate coating buffer (pH 9.6). Plates were washed twice with wash buffer (TBS-T, 300µL) and dried before blocking (220µL; 0.5% BSA in PBS, pH 7.4) for 1hr at room temperature. After 2 washes, standards (P8783, Sigma Aldrich, UK), QC's (#480, Bio-Rad Laboratories, UK) and samples (20µL) were added in duplicate to wells with 80µL of P4-HRP (1/10,000; 12-02, ASTRA Biotech, Germany) and 50µL of P4-Ab (0.024µg/mL; 10-04, ASTRA Biotech, Germany) (both HRP and Ab diluted in assay buffer = 0.1% BSA PBS + 250ng/ml Cortisol). Plates were incubated for 2hrs at 28˚C with shaking. Plates were washed 4 times before the addition of 120µL of TMB (ES022, EMB Millipore, USA) for 10mins in the dark with shaking. The reaction was stopped with 80µL of 1N Sulphuric Acid. The plate was then read at 450nm for analysis using SoftMax Pro (Version 7.1, Molecular Devices).

Cross-reactivities: 100% Progesterone, 48.2% 5β-pregnane-3,20-dione, 32.1% pregnanolone, 8.5% 5α-pregnane-3α-ol-20-one, 6.9% 5α-pregnane-3,6-ol-20-dione, 6.2% 17α-hydroxyprogesterone, 5.9% 5α-pregnane-3β-1,20-dione, 5.1% pregnenolone, 1.7% 17α-hydroxypregnanolone, 1.2% 21-hydroxyprogesterone. All other steroids tested gave <1% cross reactivity.

The intra-assay %CV was 4.7%. The inter-assay %CV was 10.0%.

The limit of detection is 0.1ng/mL.

Publications:

Thorburn J, Cole G, Naylor A, Garbett A, Wilson K, James M, Dodd J, Houghton JDR, Collins PC (2023) Preliminary insight into the reproductive traits of the flapper skate *Dipturus intermedius* using in-field ultrasonography and circulating hormone concentrations. Endangered Species Research **52** pp97-111 https://doi.org/10.3354/esr01264