**Arbor assay CORT EIA protocol – Ondi Crino 28/04/2023**

**Background - Enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA)**

The CORT EIA is a technique that uses competitive binding to quantify CORT. The 96-well plates are coated with a primary antibody. CORT binds to a polyclonal antibody solution. This in turns binds to the primary antibody on the plate. A solution of CORT conjugate with an enzyme will compete with CORT from the samples to bind to the antibody solution. The higher the level of CORT in your sample, the less of the CORT conjugate with enzyme can bind to the antibody solution (and hence the less that binds to the primary antibody on the plate). A colorless substrate is added that reacts with the enzyme bound to the CORT conjugate that catalyzes the breakdown of the substrate to a colored product. The color of the product is inversely proportional to the amount of CORT in the unknown sample. The more CORT in your sample, the less CORT conjugate with enzyme that can bind and, hence, the less enzyme available to catalyze the breakdown of the substrate.

The color of each well is determined using spectrophotometry. Standard curves of known concentrations of CORT are run on each plate. These standard curves produce optical densities that correspond to known CORT values. The optical densities from each unknown sample are used in an equation generated from the standard curve to calculate the amount of CORT in each unknown sample.

**How to determine sample dilution for EIA**

You need 50ul of sample per well x 3 if samples are assayed in triplicate. Some sample will be ‘lost’ in the tube and unavailable to pipette. Therefore, you need at least 250ul of total reconstituted sample. As a rule, the more plasma you can use in your assays the more accurate your data will be. If you are assaying samples that you suspect have low levels of CORT, then it’s best to select a slightly lower dilution.

To determine optimal sample dilution, first run a serial dilution curve. This will also be used to test assay parallelism.

1. Pool several samples (reconstituted in 250 ul assay buffer) following extraction.
2. Serially dilute as follows:
   1. Para 1 = straight pool
   2. Para 2 = 300ul of Para 1 + 300 ul of assay buffer [1:2] dilution
   3. Para 3 = 300 ul of Para 2 + 300 ul of assay buffer [1:4] dilution
   4. Para 4 = 300 ul of Para 3 + 300 ul of assay buffer [1:8] dilution
   5. Para 5 = 300 ul of Para 4 + 300 ul of assay buffer [1:16] dilution
   6. Para 6 = 300 ul of Para 5 + 300 ul of assay buffer [1:32] dilution
   7. Para 7 = 300 ul of Para 6 + 300 ul of assay buffer [1:64] dilution
3. Repeat if you want to assay several sets of pools (I did two for yolk CORT validation)
4. Following EIA assay, test assay parallelism using ANCOVA with logit OD as dependent variable and LogDilution\*Type as an interaction term. Type = Standard curve or Serial dilution. A non-significant result indicates that the slopes are not significantly different (and hence there is assay parallelism)

DATA$Type <- factor(DATA$NomType)

mod1 <- aov(LogitOD~LogDilution\*Type, data=DATA)

summary(mod1)

**500 pg/ml buffer preparation (Green Top; will be used as interassay standard)**

The interassay standard is used to compare results between 96-well plates. The coefficient you calculate from these samples is necessary for publication.

1. Make buffer assay that is 500 pg/ml.

2. Combine multiple tubes of CORT standard provided in kit (100,000 pg/ml).

3. Dilute 1.25ml CORT standard into 500mL of assay buffer.

4. Aliquot 400μl into Eppendorf tubes for external interassay standard.

5. This must be run on every plate.

6. Dispose of the remainder of aliquot after use.

**EIA protocol - This is the most simple assay set up**

***Reagent preparation***

Reagents should be brought to room temperature before use.

1. Assay Buffer

Prepare a [1:5] dilution with 10ml of assay buffer and 40ml of ddH20.

2. Wash Buffer

Prepare a [1:20] dilution with 20ml of assay buffer and 380ml of ddH20.

***Standard Curve – prepared in test tubes***

**The standard curve generates optical density readings against known amounts of CORT. The optical densities generated from unknown samples are then used in an equation generated from the standard curve to calculate the amount of CORT. Every plate should have a standard curve.**

Tube 1: 50μl of 100,000pg/ml stock + 450μl assay buffer (concentration: 10,000 pg/ml)

Tube 2: 250μl (from Tube 1) + 250μl assay buffer (concentration: 5,000 pg/ml)

Tube 3: 250μl (from Tube 2) + 250μl assay buffer (concentration: 2,500 pg/ml)

Tube 4: 250μl (from Tube 3) + 250μl assay buffer (concentration: 1,250 pg/ml)

Tube 5: 250μl (from Tube 4) + 250μl assay buffer (concentration: 625 pg/ml)

Tube 6: 250μl (from Tube 5) + 250μl assay buffer (concentration: 312.5 pg/ml)

Tube 7: 250μl (from Tube 6) + 250μl assay buffer (concentration: 156.25 pg/ml)

Tube 8: 250μl (from Tube 7) + 250μl assay buffer (concentration: 78.125 pg/ml)

Tube 9: 250μl (from Tube 8) + 250μl assay buffer (concentration: 39.063 pg/ml)

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | NSB | NSB | NSB | Standard 7 | Standard 7 | Standard 7 |  |  |  |  |  |  |
| B | B0 | B0 | B0 | Standard 8 | Standard 8 | Standard 8 |  |  |  |  |  |  |
| C | Standard 1 | Standard 1 | Standard 1 | Standard 9 | Standard 9 | Standard 9 |  |  |  |  |  |  |
| D | Standard 2 | Standard 2 | Standard 2 | Green Top | Green Top | Green Top |  |  |  |  |  |  |
| E | Standard 3 | Standard 3 | Standard 3 | Sample 1 | Sample 1 | Sample 1 |  |  |  |  |  |  |
| F | Standard 4 | Standard 4 | Standard 4 | Sample 2 | Sample 2 | Sample 2 |  |  |  |  |  |  |
| G | Standard 5 | Standard 5 | Standard 5 | Etc. |  |  |  |  |  | EE | EE | EE |
| H | Standard 6 | Standard 6 | Standard 6 |  |  |  |  |  |  | ES | ES | ES |

***Assay Procedure for half volumes (see plate set-up)***

1. Prepare standard curve as above. Use within one hour of preparation.
2. Pipette 75μl of assay buffer into NSB wells.
3. Pipette 50μl of assay buffer into B0 wells.
4. Pipette 50μl of each standard into wells.
5. Pipette 50μl of each sample into wells.
6. Pipette 25μl of conjugate into all wells using repeat pipette.
7. Pipette 25μl of antibody into all wells except NSB wells using repeat pipette.
8. Cover plate with plate sealer.
9. Incubate plate for 1 hour at room temperature (no heat) at 750rpm.
10. **Tape the well strips to the plate before emptying.** Vigorously empty plate into sink. Rinse four times with 200μl of wash solution for each wash. After final wash, tap plate firmly on paper towels to get rid of any remaining fluid. **You must put a layer of Kim Wipes over the top of the paper towels.** This prevents fibers from the paper towels from adhering to the wells of your plates which will affect the optical density readings. The tapping should sound loud. You will not break the plate. Make sure plate appears dry and that there are no bubbles in the wells. Remove the tape.
11. Add 100μl of TMB Substrate solution to every well using repeat pipette.
12. Incubate at room temperature on the counter for 30 minutes (no shaking).
13. Add 50μl of stop solution to all wells using repeat pipette.
14. Read on plate reader at 450nm asap.