**ESTRADIOL (E2) EIA – Double Ab Protocol**

Updated: 10/10/18 KJF

**Assay Preparation**

1. Warm reagents and goat-anti rabbit (GAR) coated plate to room temperature (23oC) on slide warmer for at least 30 min (will take longer if running multiple plates!). Prepare plate map while reagents are coming to room temperature.
2. Prepare standards
   1. Standard values used are: 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, and 1.95 pg/well
   2. Dilute standard working stock (1000 pg/well) serially 1:2 according to table. Note that the 1000 pg/well does not get plated on the assay.
   3. Make a “zero” which will be used for the blanks and 0 pg/well – it’s only EIA buffer – you need 250uL/plate.
3. Dilute HRP
   1. Prepare HRP dilution @ 1:200,000 by adding the appropriate amount of HRP to the matching amount of EIA buffer according to the table.
   2. Remember to remove the volume of EIA buffer that you will be adding of HRP so the total volume is even.
4. Dilute antibody (Ab)
   1. Prepare Ab dilution @ 1:250,000 by adding the appropriate amount of Ab to the matching amount of EIA buffer according to the table.
   2. Remember to remove the volume of EIA buffer that you will be adding of Ab so the total volume is even.
5. Prepare controls
   1. Dilute Control 1 and Control 2 from 1:6 to 1:6.
   2. Control 3 is Control 2 at 1:2.

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| **# of plates** | **Standards** | | **HRP @ 1:200,000** | | **Antibody @ 1:250,000** | |
| **1000 pg/well (uL)** | **EIA buffer/tube (uL)** | **1:5000 HRP (uL)** | **EIA buffer (mL)** | **1:5000 Ab (uL)** | **EIA buffer (mL)** |
| 1 | 150 | 150 | 150 | 6 | 120 | 6 |
| 2 | 200 | 200 | 300 | 12 | 240 | 12 |
| 3 | 250 | 250 | 450 | 18 | 360 | 18 |
| 4 | 300 | 300 | 600 | 24 | 480 | 24 |
| 5 | 350 | 350 | \*\*Only put ≤4 plates worth in each amber vial.\*\* | | \*\*Only put ≤4 plates worth in each amber vial.\*\* | |
| 6 | 400 | 400 |

**Plate Loading**

1. **DO NOT WASH PLATE!**
2. Add 50 µL of EIA buffer to all wells
3. Pipet 70 µL EIA Buffer to Blank wells
4. Pipet 20 µL of standards, controls, and samples per well as quickly and accurately as possible, according to plate map
5. Add 50 µL of prepared Estradiol HRP to all wells
6. Add 50 µL of prepared Estradiol antibody to all wells, **EXCEPT FOR BLANKS!**
7. Gently tap plate to make sure liquids are at the bottom of the wells
8. Cover plate with plate sealer and **place on plate shaker for 2 hours** at room temperature

**Substrate**

1. Wash the plate wash solution using plate washer
2. Blot plates on paper towel to remove excess moisture – can be left upside down for up to 20 minutes.
3. Add 100 µL TMB to each well carefully, avoiding splashing
4. Cover plate with plate sealer and incubate at room temperature for ~30 minutes on slide warmer
5. Add 50 µL of 1N HCl to each well to stop development. Will change color from blue to yellow.
6. Read plate using “Estradiol Double Ab assay protocol” in Gen5 program.