**8-Isoprostane ELISA Measurement in Urine Samples**

Alm Lab

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**Special Materials and Equipment**

* 8-Isoprostane ELISA kit (Detroit R&D, Inc.)
* 2N Sulfuric acid solution
* 25 mM Triphenylphosphine (TPP) solution
* Plate reader with 450 nm filter
* Multichannel pipette and pipette tips
* Plastic reservoirs

**Preparations**

*Wash Buffer*

1. Mix the solution with a stir bar, applying low heat until a clear colorless solution is obtained.
2. Dilute the entire contents of the Wash Buffer Concentrate (25 mL) with 225 mL of deionized water to yield a final volume of 250 mL of Wash Buffer 1X.
3. Store Wash Buffer at 4 degrees.

*Sample Dilution Buffer*

1. Dilute the entire contents of the Sample Dilution Stock Buffer (25 mL) with 225 mL of deionized water to yield a final volume of 250 mL of Sample Dilution Buffer 1X.

*Standards*

1. Label 5 Eppendorf tubes as Standard 1 to 5.
2. Prepare standards 1 to 6 by mixing the following solutions:

|  |  |  |  |
| --- | --- | --- | --- |
| Standards | Final Concentration (pg/mL) | Add Sample Dilution Buffer (mL) | Serial Dilutions Procedure |
| No. 6 | 5,000 | 1.998 mL | 2 uL of stock solution |
| No. 5 | 1,000 | 1.6 mL | Add 0.4 mL of No. 6 |
| No. 4 | 500 | 1 mL | Add 1 mL of No. 5 |
| No. 3 | 100 | 1.6 mL | Add 0.4 mL of No. 4 |
| No. 2 | 50 | 1 mL | Add 1 mL of No.3 |
| No. 1 | 10 | 1.6 mL | Add 0.4 mL of No. 2 |

**Same day Preparations**

*ELISA reagents*

1. Allow reagents to equilibrate to room temperature before proceeding with the assay.

*Plate map*

1. Draw plate map. Each plate must contain a minimum of three blanks (BL), three maximum binding wells (BO), and a six-point standard curve (S1-S6). Each control should be assayed in triplicate, and each sewage sample should be assayed in six replicates.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| BL | BL | BL |  |  |  |  |  |  |  |  |  |
| BO | BO | BO |  |  |  |  |  |  |  |  |  |
| Std. 1 | Std. 1 | Std. 1 |  |  |  |  |  |  |  |  |  |
| Std. 2 | Std. 2 | Std. 2 |  |  |  |  |  |  |  |  |  |
| Std. 3 | Std. 3 | Std. 3 |  |  |  |  |  |  |  |  |  |
| Std. 4 | Std. 4 | Std. 4 |  |  |  |  |  |  |  |  |  |
| Std. 5 | Std. 5 | Std. 5 |  |  |  |  |  |  |  |  |  |
| Std. 6 | Std. 6 | Std. 6 |  |  |  |  |  |  |  |  |  |

*HRP Conjugate*

1. Note: HRP Conjugate must be used the same day and cannot be stored for later use.
2. Calculate how much HRP Conjugate you need and dilute it accordingly:

|  |  |  |
| --- | --- | --- |
| # Strips in plate | 8-isoprostane-HRP conjugate | HRP buffer 1X |
| 12  (full plate) | 12 **uL** (full vial) | 12 **mL** |
| 6  (half a plate) | 6 **uL** | 6 **mL** |
| 3 | 3 **uL** | 3 **mL** |
| 1 | 1 **uL** | 1 **mL** |

*Urine samples*

1. In a 50-ml conical tube add:
   1. 20 mL of **Sample Dilution Buffer** (1:5 dilution)
   2. 100 microliters of **TPP** 25 mM (final concentration 0.1 mM).
   3. 5 ml of urine.
2. Mix by inverting tube a few times.
3. Spin samples down for 30 seconds at 4000 rpm to precipitate TPP.

**8-Isoprostane-2Fa ELISA**

1. Load 200 uL of **Sample Dilution Buffer** into the blank (BL) wells. Load 100 uL of **Sample Dilution Buffer** into the maximum binding (BO) wells.
2. Load 100 uL of each **standard** into the appropriate wells.
3. Load 100 uL of each **sample** into the appropriate wells.
4. Load 100 uL of the **diluted 8-isoprostanes-HRP conjugate** into the BO wells, the standard wells, and the sample wells. DO NOT add HRP conjugate into the BL wells.
5. Incubate the plate at room temperature for two hours.
6. Wash the plate three times with 400 microliters of the diluted **Wash Buffer** per well.
7. After the last wash, pat the plate dry onto paper towels.
8. Add 200 uL of **TMB substrate** to all wells (including BL wells).
9. Incubate the plate at room temperature for 15-30 minutes*. Keep incubation time constant throughout study.*
10. Add 50 uL of **2N Sulfuric Acid** to all wells.
11. Read the plate at 450 nm.

**References**

Detroit R&D, Inc. Manual 8-Isoprostane ELISA kit

Santos, J.M. et al. (2015) Could sewage epidemiology be a strategy to assess lifestyle and wellness of a large scale population?

Santos, J.M. et al. (2016) Differential BPA levels in sewage wastewater effluents from metro Detroit communities

Yan, Z. et al. (2010) A significant proportion of F2-isoprostanes in human urine are excreted as glucuronide conjugates