Title: tPA ELISA SOP

Approvals:
Preparer:_____Kari Britt_____ Date _____ 09Jun09_____
Reviewer:_____Bob O'Brien_____ Date _____ 09Jun09_____

1. **Purpose:**
   1.1. To determine the concentration of tPA in a sample.
2. **Scope:**
   2.1. Applies to determining the concentration of tPA in a sample using the IMUBIND tPA ELISA kit from American Diagnostica.
3. **Responsibilities:**
   3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.
4. **References:**
   4.1. IMUBIND tPA ELISA kit instructions
   4.2. plate reader SOP
5. **Definitions:** N/A
6. **Precautions:**
   6.1. Plasma is of human origin and should be treated as Biosafety Level 2. Dispose of waste in biohazard containers.
7. **Materials:**
   7.1. IMUBIND tPA ELISA kit from American Diagnostica (cat# 860)
      7.1.1. Microtest strips coated with anti-tPA IgG coat and non- immune IgG
      7.1.2. PET buffer
      7.1.3. tPA depleted plasma
      7.1.4. tPA antigen standard plasma
      7.1.5. Conjugate -HRP labeled anti-tPA Fab fragments
   7.2. TMB (3, 3', 5, 5' – Tetramethylbenzidine) substrate
   7.3. disposable reagent reservoirs or weigh boats
   7.4. micropipettors (P-20, P-100 or 200, multichannel) and tips
   7.5. lab towels
   7.6. microfuge tubes
   7.7. microtiter plate reader operable at 630nm
8. **Procedure:**
   8.1. **Sample and Reagent Preparation**
      8.1.1. **PET buffer:**
         8.1.1.1. Dissolve the contents of the PET-buffer vial in 1L ± 50mL of water.
         8.1.1.2. Stir until dissolved, approximately 15 minutes.
         8.1.1.3. Label as: PET buffer, Store: 2-8°, Dispose: Drain, [Date], [Inititials].
      8.1.2. **Prepare Detection Antibody Conjugate:**
      8.1.3. Dilute the 100X Detection Antibody Conjugate with PET buffer according to the following instructions.

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8.1.3.1. Determine the total number of samples and standards that will be assayed.
8.1.3.2. Determine the final volume of diluted antibody conjugate by multiplying the total number of samples and standards by 55µl.
8.1.3.3. Determine the volume of 100X Detection Antibody Conjugate to use by dividing the final volume of diluted antibody conjugate by 100.
8.1.3.4. Determine the volume of PET buffer to use by subtracting the volume of 100X Detection Antibody Conjugate from the final volume of dilute antibody conjugate.
8.1.3.5. Place the volume of PET buffer needed into a test tube.
8.1.3.6. Add the volume of 100X Detection Antibody Conjugate to the PET buffer. Be sure to dispense the 100X Detection Antibody Conjugate below the surface of the PET buffer and to rinse the inside of the pipet tip by pipetting up and down several times with the tip below the surface of the PET buffer.
8.1.3.7. Close the top of the test tube securely and invert several times to mix.
8.1.3.8. Label the test tube as: Diluted Antibody Conjugate in PET buffer for tPA ELISA, Store: 2-8°C, Dispose: Drain, [Date], [Initials].

8.1.4. **tPA Antigen standard:**

8.1.4.1. Aseptically add 0.5mL of ultrapure water to a vile containing tPA Plasma Standard (30ng/mL).
8.1.4.2. Aseptically add 0.5 mL of ultrapure water to a vile containing tPA Depleted Plasma Standard (0ng/mL).
8.1.4.3. Agitate both vials gently for 5 minutes.
8.1.4.4. Mix the tPA Plasma Standard and the tPA Depleted Plasma Standard in microfuge tubes according to the table below.

Concentration ng/mL	tPA Plasma Standard µL	tPA depleted plasma µL
0	0	30
10	10	20
20	20	10
30	30	0

8.1.4.5. Label the tubes as: tPA standard for ELISA, [Concentration], Store: -20°C, Dispose: Drain, [Date], [Initials].

8.1.5. **CHO samples:**

8.1.5.1. If not done already, centrifuge the samples to remove cells from the media and remove the supernatant to a new tube. Use the supernatant in the assay. This step is not necessary if the cells were removed while performing a previous SOP or if the sample has been eluted from a chromatography column.

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8.1.5.2. In a new microfuge tube, dilute the samples 1:10 by combining 10µl of sample with 90µl PET buffer.

8.2. **Assay**

8.2.1. Equilibrate all reagents to room temperature before use.

8.2.2. Obtain the number of wells needed for the total number of standards and samples that will be assayed.

8.2.3. Reconstitute wells by adding 50µL ± 1µL of PET buffer to each well. Cover the wells with a laboratory film such as Parafilm, and agitate gently for 3-5 minutes at room temperature.

8.2.4. Add 20µL ± 1µL of each tPA standard and sample to individual wells. Mix gently with the pipettor by aspirating and dispensing 3 times.

8.2.5. Record positions of the standards and samples.

8.2.6. Cover the ELISA wells with laboratory film, and incubate for approximately 1 hour at room temperature while agitating gently.

8.2.7. Add 50µL ± 1µL of the diluted antibody conjugate to the wells. Mix gently with the pipettor by aspirating and dispensing 3 times.

8.2.8. Cover the ELISA wells with a clean sheet of laboratory film and incubate for 15 minutes at room temperature while agitating gently. This will label the bound tPA.

8.2.9. Discard the contents of the wells by turning the wells upside down on a laboratory towel and tapping several times.

8.2.10. Wash the wells four times with PET buffer as follows:

8.2.10.1. Fill each well with approximately 200µl of PET buffer.

8.2.10.2. Turn the wells upside down onto a laboratory towel and tap several times to remove the PET buffer.

8.2.11. After the fourth wash it is VERY important to tap the wells dry until all visible fluid is removed from the wells.

8.2.12. Add 100µL± 1µL TMB substrate solution to each well. Tap the wells gently (right side up) to ensure that the TMB solution settles at the bottom of the wells.

8.2.13. Cove the wells with a clean sheet of laboratory film and incubate at room temperature for 15-60minutes while agitating gently.

8.2.14. Measure the absorbance at 630nm per plate reader SOP.

8.3. **Generate a Standard Curve and Calculate Results**

Note: For detailed directions on how to generate a standard curve using Microsoft's Excel 2007 see section 9 (Attachments). Other appropriate software programs may be used to generate the standard curve.

8.3.1. Plot absorbance at 630nm against the 0, 10, 20, and 30 ng/mL standards.

8.3.2. Fit a linear trendline through the points.

8.3.3. Include the R-squared value and linear equation on the graph.

8.3.4. Use the equation to calculate the concentration of tPA in the wells.

8.3.5. Calculate the concentration of tPA in the culture media by multiplying by the dilution factor of 10.

9. Attachments:

9.1. Figure 1: Standards dilution table located in section 8.2.6.

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9.2. Directions for generating a standard curve using Microsoft's Excel 2007

9.2.1. Open a new spread sheet in Excel 2007.

9.2.2. Enter the concentration data (X axis) from top to bottom into a column starting with 0 and ending with the number value of the highest concentration.

9.2.3. Enter the corresponding abosorbance value (Y axis) generated by the plate reader in the column directly to the right of the column used to enter the concentration data. For example:

X-value	Y-value
ng/mL	Absorbance
0	0
10	0.092
20	0.179
30	0.320

Note: Enter as many standard concentration values as were used in the assay.

9.2.4. Highlight the cells containing number values only.

9.2.5. Click on the "Insert" tab.

9.2.6. Click on "Scatter" in the "Charts" section.

9.2.7. Choose the chart-type at the top of the left column called "Scatter with only Markers" when you mouse over the choice. The chart will appear.

9.2.8. Right click on one of the data points and choose "Add Trendline…". The "Trendline Options" dialog box will appear.

9.2.9. Select "Linear" and check off "Display Equation on chart" and "Display R-squared value on Chart".

9.2.10. Click on "Close". The line, equation and R-squared value will appear in the chart.

9.2.11. To calculate the concentration of tPA in the sample, substitute the absorbance value for "y" in the equation and solve for "x" and multiply by the dilution factor of 10.

10. History:

Name	Date	Amendment
Sonia Wallman	1997	Initial release
Deb Audino	07Jul05	Put into 2005 SOP format. Added a 1:5 dilution of samples. Changed substrate to TMB
Deb Audino	04Nov05	Changed dilution process for the conjugate because the kit was changed. Increased the dilution of the samples to 1:10 from 1:5
Deb Audino	04Apr08	College name change , format history update
Kari Britt	09Jun09	Added directions for making a standard curve using Microsoft Excel 2007.