

STANDARD OPERATING PROCEDURE  
**General Use of a Magnetic Particle-Based ELISA Kit for the Determination  
of Chlorpyrifos in Water**

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**KEY WORDS**

Enzyme-Linked Immunosorbent Assay, paramagnetic particle-based tube kit,  
chlorpyrifos

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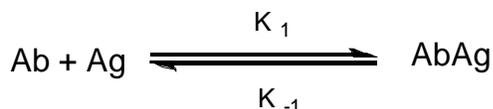
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#### **1.0 INTRODUCTION**

An Enzyme-Linked Immunosorbent Assay (ELISA) is a physical assay, characterized by the Law of Mass Action, which relies on the reaction of a target analyte (or antigen, Ag) with a selective antibody (Ab) to give a product (AbAg) that can be measured:



In a competitive, magnetic particle-based ELISA, the antibody is bound to particles that are suspended in solution. When a test sample containing an antigen and an antigen-enzyme conjugate are added, competitive inhibition occurs between the antigen-enzyme conjugate and an unlabeled antigen. After the formation of an immune complex from an antigen-antibody binding, the reagents are separated by a washing, after which a color substrate is added to the immune complex. If the antigen-enzyme conjugate is in excess, a color change will occur. If the unlabeled antigen is in excess, there will be little to no change in color. Color development is inversely proportional to the chlorpyrifos concentration, such that darker color signifies lower concentration and lighter color is indicative of higher concentration.

ELISA has become an important and cost effective alternative data gathering and screening method for the determination of pesticides in environmental matrices, particularly for the analysis of large numbers of samples. For routine testing or screening of pesticides, for example, ELISA analysis may cost as much as 75% less than comparable GC/HPLC analysis. The California Department of Pesticide Regulation (CDPR) routinely uses commercial ELISA test kits as a screening or data gathering method for monitoring pesticide residues in compliance monitoring as well as research studies. This SOP describes the procedures for the general use of a commercial paramagnetic particle-based ELISA tube kit, and outlines specific performance assessment guidelines, including a summary of the materials, methods, calculations, and statistical procedures necessary to quantitatively measure and evaluate method detection limit, method accuracy, method precision, method bias, reproducibility, cross-reactivity, and matrix effects.

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#### **1.1 Purpose**

To provide standardized instruction for the use and evaluation of paramagnetic particle-based ELISA kits.

#### **1.2 Definitions**

- 1.2.1 **Accuracy-** the proximity of a measured value to the "true" value.
- 1.2.2 **Antibody-** a protein that selectively recognizes and binds to a target analyte or group of structurally related analytes.
- 1.2.3 **Antigen-** a substance that can elicit the formation of antibodies and react specifically with the antibodies produced.
- 1.2.4 **Calibrators-** standard solutions having discrete compositions. Making measurements with any analytical method or instrument requires calibration to ensure the accuracy of the measurement. ELISA utilizes a working curve method for calibration, i.e., standards or *calibrators* of known composition are used to generate a standard or *calibration* curve, from which all subsequent measurements are calculated.
- 1.2.5 **Competitive Immunoassay-** An immunoassay format in which the (unlabeled) antigen in the unknown sample competes directly with labeled antigen to bind with antibodies. After incubation, the amount of labeled antigen bound to the antibody site is then measured. In a competitive immunoassay, the response will be inversely proportional to the concentration of antigen in the unknown. Hence, a strong response (darker color) indicates a lower concentration of antigen in the unknown, because less was available to compete with the labeled antigen.
- 1.2.6 **Conjugate-** the coupling of a hapten to a carrier protein, such as an enzyme.
- 1.2.7 **Control-** A sample used as a standard of comparison in a controlled experiment.

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- 1.2.8 **Cross-reactivity-** the ability of compounds that are structurally related to the target molecule to bind to the target-specific antibody.
- 1.2.9 **Dose-Response Curve-** Representation of the signal generated by an immunoassay (y axis) plotted against the concentration of the target compound (x axis) in a series of standards of known concentration. When plotting a competitive immunoassay in a rectilinear format, the dose-response will have a hyperbolic character. When the log of concentration is used, the plot assumes a sigmoidal shape, and when the log of signal is plotted against the logit transformation of concentration, a straight-line plot is produced.
- 1.2.10 **ELISA-** an immunoassay format in which the hapten and coating antigen compete for the specific antibody and the amount of bound antibody is detected by an enzyme labeled second antibody. ELISA uses an immobilized reagent (e.g., antibody adsorbed to a plastic plate or to magnetic particles), to facilitate the separation of targeted analytes (antibody-bound components) from non-target substances (free reaction components) using a washing step, and an enzyme conjugate to generate the signal used for the interpretation of results.
- 1.2.11 **Hapten-** Pesticides and other small molecules are not themselves large enough to elicit an antigenic response, but when conjugated to a large molecule (such as a protein) they can stimulate the formation of antibodies. Conjugation is accomplished by adding functional groups to the target analyte that will facilitate its binding to a carrier protein. These groups, called spacer arms, contain functional groups such as -OH, -COOH, -SH, or -NH<sub>2</sub>. The combination of a target analyte with a spacer arm is called a hapten.
- 1.2.12 **Immunoassay-** a biochemical test that measures the level of a substance (analyte) in a liquid, using the reaction of an antibody or antibodies to its antigen.
- 1.2.13 **Matrix Blank-** a representative field sample that is free of analyte. Blanks are managed analytically in a manner analogous to that of ordinary field samples.

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- 1.2.14 **Matrix effects-** non-specific reactions (interferences) between one or more ELISA components (e.g., antibody, conjugate, target analyte) and constituents of the substance containing the target analyte (e.g., surface water, soil or plant extract), which may lead to inferior test results.
- 1.2.15 **Negative control-** A control sample that is known to be devoid of the antigen of interest. For the analysis of the Strategic Diagnostics ELISA kits, the negative control is DI (distilled, deionized) water.
- 1.2.16 **Precision-** the proximity of a value to the mean of a series of measured values obtained from repeated measurement of the same sample, without reference to its agreement with the "true" value.
- 1.2.17 **Samples-** subsets of a representative population or matrix, e.g., a collection of water or sediment from a defined field or study site.
- 1.2.18 **Signal to Noise Ratio-** a measure of signal strength relative to background noise. The ratio compares the level of a desired signal (such as from a spectrophotometric light source) to the level of background noise. The higher the ratio, the less obtrusive the background noise is.
- 1.2.19 **Signal-** an electric current or electromagnetic field used to transmit data from one point to another.
- 1.2.20 **Target analyte-** the compound for which the ELISA has been developed.

## **2.0 MATERIALS**

### **2.1 Chlorpyrifos RapidAssay ELISA kit (Strategic Diagnostics, Inc., Newark, N.J.):**

- a). Chlorpyrifos antibody (mouse monoclonal antichlorpyrifos) coupled paramagnetic particles in buffered saline.
- b). Chlorpyrifos horseradish peroxidase (HRP) labeled enzyme conjugate.
- c). Color solution (hydrogen peroxide and 3,3',5,5'-tetra-methylbenzidine).
- d). Stopping solution (0.5% sulfuric acid).

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- e). Washing solution (deionized water).
  - f). Standards (0, 0.22, 1.0, and 3.0  $\mu\text{g/L}$ -1)
  - g). Control (1.8  $\mu\text{g/L}$ -1).
  - h). 100 disposable polystyrene test tubes
- 2.2** Single wavelength, benchtop RPA-I photometric analyzer (Strategic Diagnostics, Inc., Newark, N.J.).
  - 2.3** Two-piece (a tube rack and a magnetic base unit) 60-position magnetic separation rack (Strategic Diagnostics, Inc., Newark, N.J.)
  - 2.4** Eppendorf Research Pro Adjustable-Volume (100-1000  $\mu\text{L}$ ) Pipette (Brinkman Instruments, Inc., Westbury, NY).
  - 2.5** Eppendorf Repeater 4780 repeating pipette (Brinkman Instruments, Inc., Westbury, NY).
  - 2.6** Digital Timer (for timing incubations).
  - 2.7** Fisher Vortex Mixer (Fisher Scientific, Pittsburgh, PA).

## **3.0 PROCEDURE**

### **3.1 ELISA Procedure and Spectrophotometric Measurement**

- 3.1.1 All ELISA calibrators/samples are run in duplicate. Label 12 disposable polystyrene test tubes for the calibrators (zero, 0.22, 1.0, 3.0 ppm), the control (1.8 ppm), and the negative control (DI water). Label duplicate test tubes for each unknown using representative field sample numbers. Place all tubes sequentially in the separation rack.
- 3.1.2 Add 250  $\mu\text{L}$  of DI water to the labeled negative control tubes and 250  $\mu\text{L}$  of the appropriate calibrator to the labeled calibrator tubes.
- 3.1.3 Add 250  $\mu\text{L}$  of unknowns to be analyzed to their respective tubes.
- 3.1.4 Add 250  $\mu\text{L}$  of HRP-labeled enzyme conjugate to each labeled tube (calibrators, controls, and samples).

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- 3.1.5 Add 500  $\mu$ L of chlorpyrifos antibody coupled paramagnetic particles to each labeled tube (calibrators, controls, and samples).
- 3.1.6 Place each tube in the vortex mixer for approximately 1-2 seconds.
- 3.1.7 Incubate the mixture for 15 minutes at room temperature.
- 3.1.8 At the end of the incubation period, connect the tube rack containing calibrators and unknowns to the magnetic base unit and allow the magnetic particles that are in solution to separate for 2 minutes.
- 3.1.9 Holding the base and the rack together, slowly invert the rack and decant the contents of the tubes into a sink.
- 3.1.10 While still inverted, gently blot the tubes on an absorbent pad (such as paper towels) to remove excess solution from the lips of the tubes.
- 3.1.11 Add 1 ml of washing solution and allow to stand for 2 minutes.
- 3.1.12 Decant and blot the tubes dry as in Step 3.1.10 above.
- 3.1.13 Repeat the wash step two more times.
- 3.1.14 After tubes were thoroughly washed, remove the tube rack from the magnetic base.
- 3.1.15 Add 500  $\mu$ L of color solution to all tubes.
- 3.1.16 Place each tube in the vortex mixer for approximately 1-2 seconds.
- 3.1.17 Incubate at room temperature for 20 minutes.
- 3.1.18 After incubation, add 500  $\mu$ L of stopping solution to each tube.
- 3.1.19 Read results at 450 nm on the RPA-I photospectrometer.

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#### **4.0 CALCULATIONS**

##### **4.1 Analysis of Spectrophotometric Measurements**

4.1.1 From measured absorbances, calculate the mean absorbance value (B) for the calibrators and unknowns.

4.1.2 Calculate B/B<sub>0</sub> for each of the calibrators/unknowns:

$$B/B_0 = (\text{mean absorbance of calibrators or unknowns}) / (\text{mean absorbance of negative control})$$

The term B/B<sub>0</sub> normalizes measured absorbances (B) with respect to background values (B<sub>0</sub>).

4.1.3 Construct a standard curve by plotting the B/B<sub>0</sub> for each standard on a vertical logit (Y) axis versus the corresponding chlorpyrifos concentration on a horizontal logarithmic (X) axis:

$$\text{logit} (B/B_0) = m(\ln C) + b \quad (1)$$

*where*

*m* = slope

*b* = intercept

*C* = concentration of chlorpyrifos in unknown

*and*

$$\text{logit} (B/B_0) = \ln [(B/B_0)/(1 - (B/B_0))] \quad (2)$$

The calibration curve should have a correlation coefficient  $r > 0.99$  ( $R^2 > 0.98$ ). If not, repeat the measurements. If replicate measurements fail, it will be necessary to repeat the preparation and

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analysis of calibrators per Section 3 above.

- 4.1.4 %B/Bo for controls and samples will then yield levels in ppb of chlorpyrifos directly by solving the resulting equation of the line for ln C:

$$\ln C = ([\logit (B/B_0)] - b)/m \quad (3)$$

- 4.1.5 The manufacturer (Strategic Diagnostics, Inc., Newark, N.J.) defines the limit of detection (LOD) as 90% B/Bo. The LOD may also be obtained directly from assay results by subtracting three times the standard deviation (SD) of the negative control from its mean absorbance (MABS):

$$\text{LOD (\%B/Bo)} = \text{MABS}_{\text{negative control}} - 3 \cdot \text{SD}_{\text{negative control}} \quad (4)$$

which gives the LOD in terms of % B/Bo. Substitute LOD (%B/Bo) into the equation of the line generated above to convert to units of concentration. Alternatively, the minimum concentration of substance that can be measured may be expressed in terms of the method detection limit (MDL). The MDL is defined as the minimum concentration of substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. The MDL is determined by multiplying the appropriate one-tailed 99% t-statistic by the standard deviation ( $\sigma$ ) obtained from a minimum of three replicates (seven recommended) of a matrix spike subsample containing the analyte of interest at a concentration one to five times the estimated MDL:

$$\text{MDL} = t_{(n-1, \alpha = 0.99)} (\text{standard deviation}) \quad (5)$$

The t-statistic is obtained from standard reference tables or from the table below.

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<u>Number of Samples:</u>	<u>t-statistic</u>
3	6.96
4	4.54
5	3.75
6	3.36
7	3.14
8	3.00
9	2.90
10	2.82

## 4.2 Quality Control (QC)

The following QC indicators should be reported with ELISA results each time an analysis is performed. All blanks, calibrators, and unknowns should be run in replicate each time an assay is performed. At the minimum, samples should be analyzed in duplicate. For better quality QC results, increase the number of replicates per the needs expressed in the particular study design.

### 4.2.1 Accuracy

Measure calibrators (minimum duplicate samples), calculate the estimated concentration of each unknown using Equation (3), and express as the percent recovery (%R) as the mean estimated (observed) concentrations relative to expected (known) concentrations:

$$\text{Accuracy} = \%R = (C_{\text{observed}} / C_{\text{expected}}) * 100 \quad (6)$$

Acceptable recoveries fall within the range 70 – 120%.

### 4.2.2 Precision

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Precision is determined from the repeated measurement of the same control sample. To obtain this parameter, repeat the spectrophotometric measurement of the 1.8 ppb positive control supplied in the kit at least 4 times. Precision may then be expressed as the relative standard deviation (RSD) or the percent coefficient of variation (%CV):

$$\text{RSD} = \%CV = \left( \frac{\text{standard deviation of control}}{\text{mean conc. of control [ppb]}} \right) * 100 \quad (7)$$

Values of RSD/%CV should not exceed  $\pm 20\%$ .

#### 4.2.3 Reproducibility

Measure sample concentrations (C) in each matrix and then compare observed values to expected values:

$$\text{Reproducibility} = \left( \frac{C_{\text{observed}}}{C_{\text{expected}}} \right) * 100 \quad (8)$$

Percent recovery for each sample should fall within the 80% to 120% range.

#### 4.3 Matrix Effects

Typically, interferences are quantified by comparing a standard curve produced in a control matrix such as distilled or buffered water with a calibration curve generated in the matrix of interest. The ensuing relative slope of a standard calibration curve in a matrix containing interferences is less steep than with the control system. Thus,

$$m_{\text{matrix system}} < m_{\text{control system}} \quad (9)$$

for those matrices having interfering components. An alternative method for quantitatively assessing matrix interferences is also available. Absorbance values for matrix blanks are first normalized with respect to the absorbance of the blank control matrix,

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$$I_m = [ABS_{\text{Blank A}} - ABS_{\text{Blank B}}] / ABS_{\text{Blank A}} \quad (10)$$

where ABS is the mean absorbance determined from experiment, A is the control matrix (e.g., DI or buffered water), and B is the unspiked environmental matrix. The term  $I_m$  is known as the *index of matrix interference*. Upon calculating  $I_m$  for a particular matrix, it is then used to derive a correction factor, N

$$N = [(100 - I_m) / 100] \quad (11)$$

which is subsequently employed for the direct quantitation of the analyte of interest

$$C_x = N C_{\text{measured}} \quad (12)$$

where  $C_x$  is the actual analyte concentration and  $C_{\text{measured}}$  is the analyte concentration determined from the calibration curve. With this approach, the calculated  $I_m$  values can be considered a "true" matrix interference, thus allowing the determination of the analyte in each matrix directly from the calibration curve in the control matrix.

#### **4.5 Bias**

The situation often arises that a kit can react with far more substances that can be measured by full protocol methods, thus generating positive bias for ELISA. To account for potential bias due to matrix effects, measured values are compared to an accepted reference value in a sample of known concentration or by determining the recovery of a known amount of contaminant spiked into a sample, i.e., a matrix spike. Bias due to matrix effects based on a matrix spike is calculated as

$$\text{Bias} = (x_s - x_u) - K \quad (13)$$

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where

$x_S$  = measured value of spiked sample

$x_U$  = measured value of unspiked sample

K = known value of spike in the sample

The percent recovery (%R) is then determined from the following equation:

$$\%R = [(x_S - x_U)/K]*100 \quad (14)$$

## 5.0 REPORTING REQUIREMENTS

Samples yielding measured concentrations less than the LOD are reported as *nd* or "none detected". Samples which yield concentrations greater than the LOD but less than the linear range of the kit (0.22 ppb) are reported as "< 0.22 ppb". If samples yield concentrations greater than the linear range of the kit (3.0ppb), they are reported as "> 3.0 ppb".

## 6.0 REFERENCES

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