METHOD 4000

IMMUNOASSAY

1.0 SCOPE AND APPLICATION

1.1 Immunoassay is an analytical technique useful for the separation, detection and quantitation of both organic and inorganic analytes in diverse environmental and waste matrices. Immunoassay methods are used to produce two types of quantitative results: 1) range-finding or screening results indicative of compliance with an action level, and 2) assay values.

1.2 Commercially-available testing products present immunoassay protocols that are rapid, simple and portable. These products can be used effectively in both laboratory and field settings, and require limited training. These test products substantially increase the number of data points that can be generated within a given time period, and permit an operator to analyze a number of samples simultaneously, within a relatively short period of time. Results are available immediately upon completion of the test, and can assist in the on-site management of personnel and equipment, as well as the data management activities of the laboratory.

1.2.1 A list of validated immunoassay testing products is available from the USEPA Office of Solid Waste.

1.3 Section 11.0 provides a glossary of basic immunoassay terms.

1.3.1 The glossary is not intended to be comprehensive, but to provide basic definitions that will assist in understanding product inserts and publications relating to immunoassay technology.

1.3.2 The performance of test products will vary from manufacturer to manufacturer. The performance claims and limitations of each test product will be provided in the package insert. The package insert of each test product purchased should be read to determine if the performance is acceptable for a given application.

2.0 SUMMARY OF METHOD

2.1 The immunoassay test products available will often vary in both format and chemistry. The characteristics of a specific product are described in the package insert provided by the manufacturer. This summary is, therefore, general in scope, and is intended to provide a general description of the more common elements of these methods.

Immunoassay test products use an antibody molecule to detect and quantitate a substance in a test sample. These testing products combine the specific binding characteristics of an antibody molecule with a detection chemistry that produces a detectable response used for interpretation. In general, antibody molecules specific for the method's intended target are provided at a predefined concentration. A reporter (i.e., signal generating) reagent, composed of the target compound conjugated to a signal producing compound or molecule (e.g., enzymes, chromophores, fluorophores, luminescent compounds, etc.), is also provided. The concentration, affinity, and specificity of the products' antibody influences performance, as does the chemistry of the reporter reagent.

The reporter reagent and antibody molecules of a given product are binding partners, and complex in solution. The addition of a positive sample containing the target substance to this solution results in a competitive binding reaction between the target analyte and the reporter reagent for the antibody sites. The antibody concentration, and therefore binding capacity, is limited to prevent the simultaneous binding of both the reporter and target molecules. The concentration of reporter reagent that can bind to the antibody is inversely proportional to the concentration of substance in the test sample.

Immunoassay methods may be heterogeneous (i.e., requiring a wash or separation step), or homogeneous (i.e., not requiring a separation step). In commonly available heterogeneous testing products, the antibody is immobilized to a solid support such as a disposable test tube, and the bound reporter reagent will be retained after removing the unbound contents of the tube by washing. Therefore, a negative sample results in the retention of more reporter molecules than a positive sample. The analysis of a standard containing a known concentration results in the immobilization of a proportional concentration of reporter reagent. A positive sample (i.e., containing a higher concentration than the standard) results in the immobilization of fewer reporter molecules than the standard, and a negative sample (i.e., containing less than the standard) will immobilize more.

2.2 A chemistry of the detection of the immobilized reporter is used for interpretation of results. The reporter molecule may be a conjugate of the target molecule and a directly detectable chromophore, fluorophore, or other specie, or conjugated to an enzyme that will act upon a substrate to produce the detectable response. Immunoassay testing products have a quantitative basis, and will produce a signal that is dependant on the concentration of analyte present in the sample. For environmental immunoassay methods, the signal produced is exponentially related to the concentration of the compounds present. Many immunoassay methods use enzymes to develop chromogenic response, and are termed enzyme immunoassays. Assays that generate a chromogenic response are analyzed photometrically, and use the principles of Beer's Law (Absorbance = Extinction Coefficient x Concentration x Path Length) to determine the concentration of analyte in a sample.

Immunoassay methods can provide quantitative data when configured with a series of reference standards that are analyzed and used to construct a standard curve. The signal generated from the analysis of a test sample is used to determine concentration by interpolation from the standard curve. Alternatively, these testing products can be configured to determine if a sample is positive or negative relative to a single standard.

Individual immunoassay testing products are reviewed and accepted by the EPA-OSW for the detection of sample analytes in specified matrices. A variety of testing products, produced by several different developers, may be available for the same compound(s) and matrices. Each of theses methods have been formulated using independently developed reagents that may result in significantly different performance characteristics and limitations.

The performance of the immunoassay testing products ultimately relates to the characteristics of the antibody, reporter molecule, and sample processing chemistry. The dose-response characteristics of a method, the position of the standard relative to the claimed action level, and the stated cross-reactivity characteristics of the selected test product, provide relevant information regarding the performance and recognition profile of the selected test product.

The precision, and ultimately the sensitivity of an immunoassay method, is a function of the signal-to-noise characteristics of its dose-response curve, and its operational consistency. Methods having a high slope and low non-specific signal generation produce the most sensitive and precise methods. Signal imprecision applied to a dose-response curve having a shallow slope exhibits

proportionally greater imprecision in the calculated concentration than would a method having a steeper slope. In an action level testing product, this would cause the reference standard to be positioned further from the action level, increasing the incidence of false positive results. Similarly, a method having less non-specific signal generation (higher signal-to-noise ratio) will be more sensitive and precise when other characteristics (i.e., dose-response slope) are held constant.

Immunoassay methods are used to detect contamination at a specific concentration below the claimed detection level for the test product. For example, an immunoassay used to detect PCB contamination in soil at 1 ppm will include a standard preparation containing less than 1 ppm. The reference preparation concentration is positioned to minimize the incidence of false negative results at the claimed detection level. For remediation and monitoring applications, where action levels of interest are defined, immunoassay methods should exhibit a negligible incidence of false negative results, and minimal false positives.

For a single point action level test, the concentration of analyte relative to the action level is selected by the developer, and is influenced by the precision (i.e., intra-assay, inter-person, inter-lot, inter-day, etc.), sample matrix interferences and other performance characteristics and limitations of the basic method. The concentration of analyte in the reference materials should be less than, but close to, the claimed action level. The concentration selected for the standard defines the concentration that will produce a 50% incidence of false positive results by the test product. While this issue is one representing limited liability to the operator, it is a practical issue that often requires attention. An immunoassay method for the detection of 1 ppm of PCB using a standard containing 0.8 ppm of PCB will experience a 50% false positive incidence in samples containing 0.8 ppm of PCB, and some incidence of false positive results in a sample containing between 0.8 and 1 ppm. A similar immunoassay that uses a standard containing 0.4 ppm will experience a 50% false positive incidence in samples containing 0.4 ppm of PCB, and some incidence of false positive results in a sample containing between 0.4 and 1 ppm. The closer the standard concentration is to the action level, the better the overall performance.

2.3 Cross reactivity characteristics illustrate the specificity of the underlying immunochemistry. The antibody molecules used by a test product bind to a target compound and then participate in the process of generating the signal used for interpretation. Antibody molecules bind by conformational complimentarity. These molecules can be exquisitely specific, and can differentiate subtle differences in the structure of a compound. The binding characteristics of reagents in different test products can vary, and influence the recognition profile and incidence of false results obtained by the method. Immunoassay methods should detect the target analytes claimed by the test product and exhibit limited recognition for compounds and substances not specified.

3.0 INTERFERENCES

3.1 Non-target analytes may bind with the antibody present, producing a false-positive result. These non-target analytes may be similar to the target analytes, or they may be chemically dissimilar co-contaminants. During evaluation of each test product for RCRA testing applications, studies were conducted to determine these "cross-reactive" constituents. At a minimum, these studies evaluated the response of the test product to all other similar RCRA analytes in that analyte class, as well as for selected lists of non-RCRA analytes. This testing scheme is designed to ensure that all other similar RCRA analytes and likely co-contaminants are evaluated during cross-reactivity testing. The results of these studies are presented in each method in tabular form, providing separate data sets for each test product evaluated.

3.2 Interference in the binding of an antibody to its target compound, or reporter molecule reagent, may occur when testing sample matrices with confounding contaminants or circumstances (e.g., oil, pH, temperature, some solvents). Immunoassay products contain sample processing technology that has been developed and validated for use with specified matrices. Interferences incurred from the testing of incompatible matrices may prevent the testing product from meeting its performance claims, and increase the number of false positive or false negative results. Individual immunoassay products designate the intended sample matrices.

3.3 Immunoassay products differ in shelf-life and storage requirements. Test products that are operated outside of the shelf-life and storage temperature recommendations may not provide the claimed performance.

3.4 Some test products have designated temperature ranges for operation. When these products are used, all tests must be performed within the specified operating temperature limits, or else false negative/positive results may exceed performance claims.

4.0 APPARATUS AND MATERIALS

4.1 Each test product will specify the apparatus and materials provided, as well as any additional apparatus and materials necessary for performance of the test.

5.0 REAGENTS

5.1 The two basic reagents used in immunoassay analysis are the antibody (e.g., anti-PCP) and reporter conjugate reagent (e.g., PCP molecules bound to an enzyme).

5.1.1 The formation of antibodies to haptenic molecules (i.e., most environmental contaminants) is induced by the derivatization and coupling of molecules of the target analytes to large carrier molecules such as albumin, hemocyanin or thyroglobulin. The increased size and complexity of the immunogen (antigen) conjugate, once injected, is sufficient to stimulate the immune system to produce an antibody response. The effectiveness of the immunogen in producing antibodies having the prerequisite binding characteristics and recognition profile is influenced by the surface density of the chemical groups on the carrier molecule, the nature of the bridge chemistry used, the point of attachment, the immunization protocol, immunogen concentration, adjuvants (i.e., immune response stimulants), and the species of the host animal.

5.1.2 An enzyme-reporter conjugate reagent is synthesized by coupling a target analyte or derivative of a target analyte to an enzyme, such as horseradish peroxidase. Enzymes enhance the sensitivity of the method by action on a substrate and the production and catalytic amplification of the detection signal. A single enzyme molecule used in immunoassay methods will convert approximately 10^6 molecules of a target analyte into a detectable product within one minute at ambient temperature.

5.2 Each test product will specify the reagents provided, as well as any additional reagents necessary for performance of the test.

6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1 Testing of solid waste by immunoassay requires production of a reproducible, particulate free leachate. It is critical that this leachate be produced using a solvent that allows the reproducible extraction and recovery of the target analytes, and is compatible with the antibody/enzyme conjugate of the immunoassay system used. Buffers, detergents, and solvents, used together or in combination, have been used effectively for extraction. Filtration of particulate matter may be integrated into the immunoassay test, or accomplished as a separate step within the protocol.

6.2 The immunoassay test products included in SW-846 methods will provide explicit waste- or medium-specific directions for handling samples and extraction of target analytes.

6.3 See the introductory material to this chapter, Organic Analytes, Section 4.1.

7.0 PROCEDURE

7.1 The specific procedure for each immunoassay test product is supplied by the manufacturer in the package insert.

7.2 The recognition characteristics, sensitivity, detection ranges(s), effective operating temperature, interferences and cross-reactivity of the test will depend on the product being used.

7.3 Immunoassay methods include both a sample processing and immunoassay component. It should be noted that the immunochemical reagents and sample processing components supplied with each product is specific to each manufacturer. Methods available from different manufacturers for the same compound and application may have significantly different performance characteristics.

8.0 QUALITY CONTROL

8.1 The performance of the tests cited in the immunoassay methods in this manual has been reviewed, and found to be consistent with the claims that are made in the manufacturer's literature. In order to meet this performance expectation, the analyst must:

- Follow the manufacturer's instructions for the test product being used,
- Use test products before the specified expiration date,
- Use reagents only with the test products for which they are designated,
- Use the test products within their specified storage temperature and operating temperature limits.

8.2 It is important to evaluate the performance claims and limitation provided with each testing product to determine its application to a specific matrix and testing program.

8.3 Refer to Chapter One for standard quality control procedures.

9.0 METHOD PERFORMANCE

9.1 A false negative is defined as a negative response for a sample containing the target analytes at or above the stated action level. False negative rate is measured by analyzing split

samples using both the test product and a separate reference method. False negative data are provided in each method for each test product evaluated.

9.2 A false positive is defined as a positive response for a sample that contains analytes below the specified action level. Like false negatives, false positive rates are measured by analyzing split samples with both the test product and a separate reference method. False positive data are provided in each method for each test product evaluated.

9.3 Cross-reactivity and recognition profile data are provided at the end of each method in tabular form, providing separate data sets for each test product evaluated. Using these data, the analyst can evaluate if contaminants are present which are likely to produce a false positive response, and the magnitude of that response.

9.4 For single-point tests, sensitivity data are provided demonstrating the concentration of target analyte(s) that can be detected with greater than 95% confidence.

9.5 Data are provided demonstrating the bias of the testing products accepted. These data may be from:

- serial dilution of samples (i.e., is the recovery of target analyte a function of concentration?),
- sample recovery studies, and
- studies correlating the results of the testing product with a reference method.

9.6 Data are provided demonstrating that the extraction efficiency of the test being evaluated correlates with that of the referenced method.

10.0 REFERENCES

1. S.B. Friedman, "Doing Immunoassays in the Field", *Chemtech*, December 1992, pp 732-737.

2. Roitt, L., Brosstoff, J., Male, M., (eds.), Immunology, J.B. Lippincott Co., Philadelphia, Pennsylvania, 1989

3. Stites, Daniel P., Terr, Abba I., (eds.), Basic and Clinical Immunology, Appleton and Lange, Norwalk, Conneticut, 1991

4. Odell, W.D. and Daughaday, W.H., Principles of Competitive Protein-Binding Assays, J.B. Lippincott Co., Philadelphia, Pennsylvania, 1971

5. Ishikawa, E., Kawai, T., Miyai, K. (eds.), Enzyme Immunoassay, Igaku-Shoin, Tokyo, Japan, 1981

6. Tijssen, P. (ed.), Practice and Theory of Enzyme Immunoassays, Volume 15, Elsevier, NY, NY, 1985

7. Butler, John E. (ed.), Immunochemistry of Solid-Phase Immunoassay, CRC Press, Boca Raton, Florida, 1991

8. Ngo, T.T., Lenhoff, H.M., Enzyme-Mediated Immunoassay, Plenum Press, New York, 1985

9. 510K of the Federal Food, Drug and Cosmetics Act, Section 21, CFR 807.87

11.0 GLOSSARY OF TERMS

Antigen	A molecule that induces the formation of an antibody.
Antibody	A binding protein which is produced in response to an antigen, and which has the ability to bond with the antigen that stimulated its production.
B Lymphocyte (B Cell)	A type of lymphocyte that, upon stimulation, differentiates into an antibody-secreting plasma cell.
% BO	A quantitative expression of the sensitivity of an immunoassay, calculated as $(OD_{sample}/OD_{blank})x100$
Carrier	An immunogenic substance that, when coupled to a hapten, renders the hapten immunogenic.
Competitive Immunoassay	An immunoassay method involving an *in-vitro* competitive binding reaction.
Cross-Reactivity	The relative concentration of an untargeted compound that would produce a response equivalent to a specified concentration of the targeted compound. In a semi-quantitative immunoassay, it provides an indication of the concentration of cross-reactant that would produce a positive response. Cross-reactivity for individual compounds is often calculated as the ratio of target analyte concentration to the cross-reacting compound concentration at 50% inhibition of the immunoassay's maximum signal X 100%.
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 $\log_{10}$ 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.
ELISA	*Enzyme Linked Immunosorbent Assay* is an enzyme immunoassay method that uses an immobilized reagent (*e.g.*,antibody adsorbed to a plastic tube), 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.
Enzyme Conjugate	A molecule produced by the coupling of an enzyme molecule to a targeted analyte that is responsible for acting upon a substrate to produce a detectable signal.
Enzyme Immunoassay	An immunoassay method that uses an enzyme conjugate reagent to generate the signal used for interpretation of results. The enzyme mediated response may take the form of a chromogenic, fluorogenic, chemiluminescent or potentiometric reaction. (*see Immunoassay and ELISA*)
False Negatives	A negative interpretation of the method containing the target analytes at or above the detection level. Ideally, an immunoassay test product included in an SW-846 method should produce no false negatives. The maximum permissible false negative rate is 5%, as measured by analyzing split samples using both the test product and a reference method.
False Positives	A positive interpretation for a sample is defined as a positive response for a sample that contains analytes below the action level.
Hapten	A substance that cannot directly induce an immune response (e.g., antibody production), but can bind to the products of an immune response (e.g., antibody) when that response is induced by an alternate mechanism. Chemical contaminants of the environment are haptens.
Hapten-Carrier Conjugate	The coupling of a non-immunogenic molecule (e.g., targeted analyte) to an immunogenic substance (e.g., bovine serum albumin, keyhole limpet hemocyanin) for the purpose of stimulating an immune response.
Heterogeneous Immunoassay Methods	Immunoassay methods that include steps for the separation of substances that become bound to the antibody from those that remain free in solution.
Homogeneous Immunoassay Methods	Immunoassay methods that do not require the separation of bound and free substances, but that utilize antibody molecules that can bind and directly modulate the signal produced by the reporter molecule (e.g., enzyme conjugate).
Immunoassay	An analytical technique that uses an antibody molecule as a binding agent in the detection and quantitation of substances in a sample. (*see Enzyme Immunoassay and ELISA*)
Immunogen	A substance having a minimum size and complexity, and that is sufficiently foreign to a genetically competent host to stimulate an immune response.

Ligand	The molecule, ion or group that forms a complex with another molecule.
Lymphocytes	One of the five classes of white blood cells found in the circulatory system of vertebrates. A mononuclear cell 7-12 µm in diameter containing a nucleus with densely packed chromatin and a small rim of cytoplasm.
Monoclonal Antibodies	Identical copies of antibody molecules that have a common set of binding characteristics.
Optical Density (OD)	Synonymous with *Absorbance*, Optical Density is the amount of light being absorbed at a given frequency, as given by the following equation: $OD = \log I_o - \log I$, where: I_o is the intensity of the incident light, I is the intensity of the transmitted light
Polyclonal Antibodies	A group of antibody molecules that differ in amino acid composition and sequence, and that exhibit binding characteristics. Polyclonal antibodies are produced from a simulation of multiple clones of lymphocytes.
Substrate	Reagents that produce detectable signal when acted upon by enzyme conjugate.