Hitachi Chemical Diagnostics, Inc.
Allergy Monograph Series

Allergen Standardization Program
Introduction

When in vitro diagnosis of allergy was introduced in the 1970s, the need for greater knowledge about the materials and chemistry affecting the reading and measurement of specific IgE antibodies became obvious. However, not until the 1990s did the research and knowledge on a significant number of common allergens and extract compositions become available. Now, a number of highly sophisticated technologies and the delineation of the molecular characterization of allergens allow the manufacturers of in vitro diagnostics to implement these advances in their product lines. It is now possible to provide reproducible lots of the most common allergenic extracts that retain all potential antigens, and exclude irrelevant materials under conditions which preserve the biological activity. The new knowledge and methods are all combined in the quality system developed by Hitachi Chemical Diagnostics, Inc. New lots of the allergen-containing reagent are standardized according to strict in-house quality control systems giving the clinical laboratory and referring physician the best opportunity to obtain reliable and reproducible test results to the benefit of the allergic patient.

CLA® Specific IgE Assay

CLA is a diagnostic test for the simultaneous detection of specific IgE antibodies to different allergens. It uses a panel format as opposed to single allergen detection. The test chamber (Pette) contains up to 36 individual allergens coupled to a cotton thread solid phase. The test is performed by drawing the patient sera into the chamber for incubation followed by a buffer wash. A secondary antibody, anti-human IgE-HRP, is incubated in the chamber followed by a second buffer wash. The substrate is a chemiluminescent reagent. The luminescent signal is read in Hitachi Chemical Diagnostics’ CLA-1™ Luminometer to determine the concentration of IgE specific antibody attached to the solid phase.

CLA® Allergen Specific Reagents

The importance of the antibody and conjugate...
The analytical performance profile of an immunoassay system is highly dependent on the affinity and specificity of the secondary antibody as well as the optimal conditions of the conjugation procedure. The CLA test uses a polyclonal anti-IgE antibody conjugated to Horseradish peroxidase (anti IgE-HRP). The conjugation has been optimized according to: oxidant concentration, HRP concentration, HRP antibody ratio, coupling time, reductant used, and time of addition. The quality of the conjugate formation is monitored by HPLC, which allows the operator to consistently stop the reaction to achieve conjugates with molecular weights of 200 to 400 kDa.
The resulting in-house conjugate has a high signal with very low background. Graph 1 (below) shows the importance of monitoring and controlling the process in-house versus using a commercial vendor of the conjugate. The graphs depict a conjugate run with a negative serum sample to demonstrate the background (non-specific binding). It is clearly observed that the commercial vendor conjugate increases the non-specific binding which may result in false positive test results in the low-end. Graph 2 (below) shows the results of two conjugates run with a positive sample.

**Non-specific binding with commercial and in-house conjugates**

**Graph 1**

**Correlation between the in-house and commercial conjugates**

**Graph 2**

A significantly lower background and higher signal were seen with the in-house conjugate.
The importance of the allergen-containing reagent

The allergen is a critical component in any allergy test. It is one of the major sources of discrepancy in the results seen between different in vitro diagnostic tests and skin testing and is also one of the main sources of reproducibility problems between lots. The causes of variation include the raw material source, extraction procedure and the coupling procedure.

Thus, characterization and standardization of the allergen-containing reagents require the use of the best raw materials containing all-important allergenic proteins. Well-characterized extracts will improve lot to lot reproducibility and provide a standard of reference for manufacturing. Clinical and analytical performance of the assay can be compared with the available documentation on the molecular composition of allergenic extracts. This in turn will allow a comprehensive evaluation of the overall diagnostic performance of the CLA test in the clinical laboratory. The following outlines the allergen quality program used by Hitachi Chemical Diagnostics.

Literature search: Before initiation of the standardization work, a search is done for each allergen to identify major and minor allergens, extraction procedures, and epidemiological studies.

Raw materials: The raw material selected for each allergen determines the quality of the finished product. Given the variability in raw materials available from vendors, an in depth investigation is performed for each allergen. Raw materials are purchased from different vendors. Different species, collection sites, and collection years are tested when available. Various preparation methods of the crude extract are tested. The raw materials are extracted and analyzed by SDS-PAGE to determine the presence of major and minor allergens. Often a significant difference is observed between raw materials purchased from different vendors. The SDS-PAGE above (Figure 1) shows an example of the difference found in raw materials from different vendors for Kentucky Blue Grass. The vendors tested were Crystal Labs, New England Antigenics, Biopol and a Standardized extract from Bayer.

The major allergens for Kentucky Blue Grass are Poa p 1a (35.8 K), Poa p 1b (33K), Poa p IX (55, 36, 34, 32, 28-29, 12K). The SDS-PAGE showed the absence of most major protein bands in two of the raw materials tested.
Another example is shown in the SDS-PAGE picture to the left (Figure 2). A significant difference in protein pattern may also be noted between different species of the same allergens. This picture demonstrates the difference between two different species of Tuna: Yellow Fin and Albacore.

Based on these raw material studies, the source material representing the best epitope variety of the allergen is selected for further characterization.

**Optimal pH and buffer for extraction:** Once a raw material is selected, the optimal extraction method is investigated. Several buffers, pHs, and additives such as EDTA are tested. Sometimes a large difference in signal may be observed when different extraction methods are used. The graph below (Graph 3) shows an example of the response of Short Ragweed extracted with Phosphate Buffer with and without EDTA tested with different serum samples.

The method of choice for this allergen was Phosphate Buffer with EDTA. Other extraction conditions tested rendered lower results.
Extraction procedures: Several extraction procedures are investigated for each allergen. Some of the procedures tested are: hot extraction, overnight extraction, bead beater treatments, ammonium sulfate precipitation and protein purification. The graph below (Graph 4) shows an example of the difference in low-end sensitivity detection for Mountain Cedar extracted by two different methods.

A significant increase in low-end sensitivity was seen with the ammonium sulfate precipitation method as compared to the overnight extraction.

The SDS-PAGE to the right (Figure 3) illustrates the difference in the protein profile of tuna extracted with the following methods: raw, cooked and canned material.

The response of patient sera to the three different extraction methods was different. Some patient sera reacted to the raw extract but not to the cooked and canned, whereas some patient sera reacted to the cooked and not the raw material. These results indicate the importance of choosing the right extraction procedure for in vitro as well as in vivo allergy testing.
**Optimization of coupling procedure:** Once the raw material and the extraction procedure is selected, the coupling conditions are optimized. Different buffers, pHs, and concentrations are tested to couple the allergen to the cotton thread solid phase. Sometimes differences in signal are seen with the coupling methods used that are specific to the allergen and to the sera tested. The graph below (Graph 5) illustrates the difference in response seen for a patient allergic to Japanese Cedar, coupled at different pHs.

For the patient tested, the best coupling pH was 7.2. However, other patients reacted better to Japanese Cedar coupled at 8.5. The optimal procedure is selected based on a large number of serum samples tested.
**Dilution curves of positive sera:** When the allergen is optimized, a dilution curve of high positive responding sera is tested to determine linearity and low-end sensitivity. The graph below (Graph 6) shows an example of a dilution curve for Cockroach (German, American and a mix of both).

![Graph 6: Dilution curves for Cockroach allergens](image-url)
**Extract characterization:** Characterization of each one of the allergens is a major part of this quality control system. For each extract the following analyses are done: protein determination, SDS-PAGE, western blot analysis with several positive and negative samples, and capillary electrophoresis. An example of this analysis for English Plantain is shown below (Figure 4).

The SDS-PAGE shows the protein pattern for the allergen as well as the major proteins. It is the first step to ensure the presence of the proteins needed. The capillary electrophoresis is a blueprint of the allergen composition that is easy and fast to perform. The western blots with different positive sera show the immunological activity of the proteins. The western blots are also used to document the presence of major and minor allergens. A representative group of positive sera that reacts to different proteins are selected for each allergen.
Extract and pette stability: Stability at the extract and pette level is determined for each allergen once the extraction and coupling procedures are optimized.

In-house clinical studies: Performance studies are undertaken for each standardized allergen using sera from allergic patients. The CLA test response is compared to Pharmacia® CAP. The sensitivity, specificity and efficiency with a reference to CAP are calculated. The results of such a study with Burning Bush are shown below (Table 1).

![Table 1]

Documentation and validation: Per ISO 9001 and FDA regulations, a validation is done for each allergen. All the documentation needed for manufacturing and quality assurance is completed before the transfer to Manufacturing.
Sera library for quality assurance: Positive sera reactive to different protein bands are selected for each allergen. The serum is selected by performing western blot analysis so the protein response for each serum is known. The sera is purchased in large volumes and transferred to the Quality Assurance department. The table below (Table 2) is an example of the sera selected for Egg White.

<table>
<thead>
<tr>
<th>Sera</th>
<th>CAP IU'S Class</th>
<th>CLA Class</th>
<th>Lysozyme</th>
<th>Trypsin Inhibitor 36kD</th>
<th>Conalbumin</th>
<th>Ovomucoid 46kD</th>
<th>Lysozyme 14kD</th>
</tr>
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<tbody>
<tr>
<td>Sample A</td>
<td>27 - 4</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample B</td>
<td>26.8 - 4</td>
<td>4</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample C</td>
<td>4.27 - 3</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample D</td>
<td>4.11 - 3</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample E</td>
<td>1.36 - 2</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample F</td>
<td>1.34 - 2</td>
<td>2</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample G</td>
<td>1.26 - 2</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample H</td>
<td>0.86 - 2</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample I</td>
<td>0.43 - 1</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample J</td>
<td>0.27 - 1/0</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sample K</td>
<td>0.11 - 1/0</td>
<td>1/0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2

Each sera has a Pharmacia CAP and a CLA Class value, as well as the response of the sera to the different allergenic proteins in Egg White. This detailed information on each of the quality assurance samples allows the manufacturing group to spot allergens deficient in important proteins.

Standardized allergen blueprint: To ascertain consistency and reproducibility between lots, subsequent lots are compared to the blueprint of the established in-house allergen reference standard. A file with detailed information on the extraction, coupling, extract characterization and qualification procedure for each allergen is completed for each allergen reference standard and is available for the Manufacturing group as a quick and easy reference for manufacturing standardized allergens. Upon completion of the standardization of each allergen, a multifunctional team assures transfer and implementation to Manufacturing and Quality Assurance.

To date more than 50 of the most common allergens have gone through the standardization procedure (a list is available upon request).

Summary: The Allergen Standardization Program has aided HCD in the development of a scientifically well-characterized product. It has also aided in the understanding of allergens that have not been studied in detail and for which information on allergenic proteins was not previously available. The program incorporates and conforms to the guidelines published by the NACCLS. The new in-house conjugate in combination with the Allergen Standardization Program has enhanced the quality of the CLA Allergy Test as well as the reproducibility between reagent and allergen lots.