



Chapter 13

Development of a Dipstick Assay for Soy Allergens: Inexpensive Detection to Control Allergen Exposure

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Abstract

Allergen measurements are used in the food industry and are also routinely performed as part of indoor air quality investigations and occupational health monitoring. In this chapter we describe how to develop a simple, convenient, rapid test to analyze soy allergens that can be used in production environments by non-skilled staff to facilitate immediate corrective action and minimize risk and that can be produced in laboratories not equipped with sophisticated instruments.

The strip assay described consists of a membrane that is bonded to an adhesive support where an absorbent pad is also attached to absorb excess reagents. The membrane is stripped with specific antibodies against soy allergens in this case (test line) and a goat anti-rabbit IgG antibody (control line). The strip is exposed to a mix of sample and gold-conjugated specific antibody. Colored bands are read out visually or by densitometry.

Key words Lateral flow assay, Strip assay, Rapid test, Allergen, Monitoring

1 Introduction

Allergen measurements are used in the food industry and are also routinely performed as part of indoor air quality investigations and occupational health monitoring [1, 2], and for standardization of allergenic products.

There are several different screening methods available for allergen testing and each has its advantages and disadvantages. Traditionally food and environmental allergen analysis relies on immunological methods such as enzyme-linked immunosorbent assay (ELISA). ELISA has the advantage of being relatively quick and simple to perform and can be used to detect many known allergens; however, it can only detect one allergen at a time, is susceptible to cross-reactivity, and can lead to the generation of false-positive and false-negative results [3]. In food allergen analysis also polymerase chain reaction (PCR) approaches have been used

traditionally. PCR methods are complementary to ELISA methods, and are used to amplify and detect the DNA of an allergen. PCR approaches are highly specific, sensitive to very low levels of allergen, and can be used to detect more than two allergens at once. However, the PCR is limited because some food processing methods can destroy detectable DNA, causing false-negative results. PCR methods are also highly susceptible to food matrix interferences. In addition, DNA methods are not suitable for the detection of certain allergens, such as egg [3].

More sophisticated methods are liquid chromatography–tandem mass spectrometry (LC–MS–MS) and fluorescent multiplex array technology. LC–MS–MS is an alternative method for allergen detection that is highly specific and sensitive, and has the ability to directly analyze multiple allergens in a single analysis [3]. Fluorescent multiplex array technology has also been developed that allows the simultaneous detection of multiple allergens in a single reaction well, with significantly increased sensitivity [4], which is increasingly being used for allergen detection in homes, schools, and occupational health settings [5, 6].

All these techniques described for food allergen analysis or environmental monitoring require the use of a specialized laboratory staffed by skilled personnel. In this chapter we would like to provide tools and tips to develop a simple, convenient, rapid test to analyzed allergens that can be used in production environments by non-skilled staff to facilitate immediate corrective action and minimize risk and that can be produced in laboratories not equipped with sophisticated instruments.

Here, we describe a simple strip assay where a membrane is bonded to a support structure such as a plastic strip for ease of handling the membrane for exposure to the test sample and readout. In this assay, differently than the traditional concept of a lateral flow device, the conjugate is not set in a dry format on a separate pad along the membrane flow path, but the conjugate is added in a liquid format mixed with the sample in an ELISA plate well. Typical components of a traditional lateral flow immunoassay device are a plastic backing card, membrane, sample pad, absorbent pad, conjugate pad, and possibility a wick and/or cover tape. Reagents are dispensed or coated; each material is kept in place in the backing card. Finally card is cut into individual test strips. Manufacturing can be done from completely manual to completely automated. In this chapter we want to explain step by step how to be able to prepare a prototype of a strip test in a common research laboratory housing basic laboratory equipment, but no sophisticated instruments (e.g., dispense platform, laminator, sheet slitters). When we want to jump from research to product development and scaled up to manufacturing, manual procedures should be avoided and automatic

equipment should be used in place. There are several companies that can help you in the way through to full-scale automated manufacture.

2 Materials

2.1 *Diluents and Buffers*

1. Phosphate-buffered saline (PBS): To 1200 mL of physiological saline (0.9% NaCl) add 216 mL of Na_2HPO_4 solution (9.4 g/L) and 72 mL of KH_2PO_4 solution (8.9 g/L). Adjust pH to 7.4.
2. Soy hull low-molecular-weight extract (SHLMWE), in house extract (*see* Ref. [7]).
3. Elution buffer: PBS containing 0.2% bovine serum albumin (BSA) (A7030-100G; Sigma Aldrich) and 0.5% Tween-20 (Sigma Aldrich).
4. Dilution buffer: PBS containing 1% BSA and 0.5% Tween-20 (*see* **Note 1**).

2.2 *Strip Components*

1. Direct cast-backed nitrocellulose nitrate membrane (UniSart CN95 19,910 Sartorius Stedim Biotech, Goettingen, Germany) (*see* **Note 2**).
2. Capture antibody test line: Rabbit anti-soy hull low-molecular-weight extract (SHLMWE) polyclonal antibodies (*see* Ref. [7]).
3. Capture antibody control line: Goat anti-rabbit IgG antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).
4. Adhesive support: Sealing tape for multiwell plates (polyolefin sealing tape, acrylate adhesive) (Nunc®).
5. Absorbent pad: Filter paper grade 470, pore size 20–30 μm (Schleicher & Schuell, Dassel, Germany).

2.3 *Assay Assembly Components*

Airborne sample collection, extract production, and antibody conjugation are out of the scope of this chapter.

1. Samples: Collect airborne samples in glass microfiber filters and elute them in elution buffer overnight at 4 °C as previously reported [7]. Store eluates at –20 °C. Dilute eluates to be tested in the strip assay at 1:100 in dilution buffer (*see* **Note 3**).
2. Standards: Prepare three solutions of SHLMWE at the following concentrations: 1.56, 6.25, and 25 ng/mL in dilution buffer.
3. Ninety-six-well microplate (Costar®; Corning, NY).

4. Gold-conjugated antibody: Conjugate a portion of rabbit anti-SHLMWE polyclonal antibody to 40 nm colloidal gold particles using passive adsorption (*see Note 4*). Check optical density (OD) of the concentrated conjugate. Store conjugate at 4 °C. Make up daily conjugate working solution with a final OD of 2 by diluting concentrated conjugated antibody in dilution buffer.

3 Methods

Wear gloves. Diligently follow all waste disposal regulations when disposing waste materials. Carry out all procedures at room temperature unless otherwise stated.

3.1 Strip Assembly

1. Cut a rectangular piece of nitrocellulose membrane of 10 × 1.3 (W × H cm) (*see Notes 5 and 6*).
2. Prepare capture antibody solution for **test line**: Prepare rabbit anti-SHLMWE polyclonal antibodies at a final concentration of 1 mg/mL in PBS, pH 7.2 (*see Notes 7 and 8*).
3. Prepare capture antibody solution for **control line**: Prepare goat anti-rabbit IgG antibody at a final concentration of 500 µg/mL in PBS, pH 7.2.
4. Perform hand stripping of the test line by pipetting 30 µL of the capture antibody solution for test line following an imaginary straight line at 0.3 cm from the membrane origin (*see Fig. 1, Note 9*).
5. Perform hand stripping of the control line by pipetting 30 µL of the capture antibody solution for control line following an imaginary straight line at 0.6 cm from the membrane origin (*see Fig. 1, Note 10*).
6. Dry membranes for 1 h at 37 °C in an oven (*see Note 11*).



Fig. 1 Manual stripping process. Test line positioned at 0.3 cm from the membrane origin and control line at 0.6 cm from the membrane origin

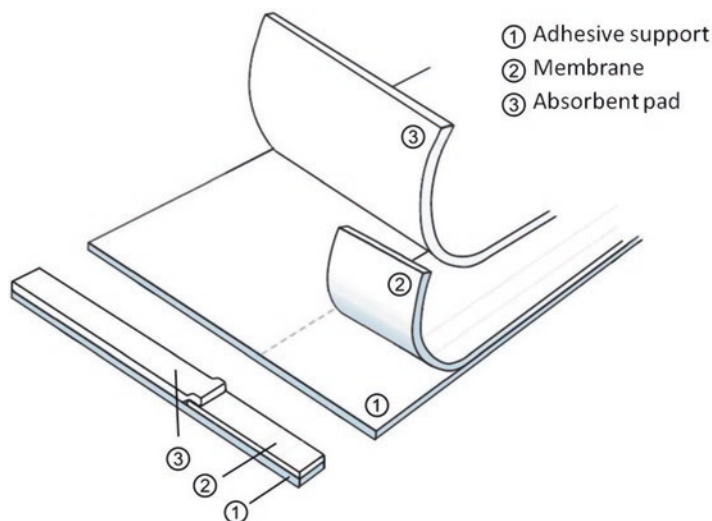


Fig. 2 Scheme of strip assembly

7. Cut an adhesive support of the following dimensions: 10×3.6 (W $\times$ H cm).
8. Put membrane on top of the adhesive support starting at the origin, as shown in Fig. 2 (see Fig. 2, Note 6).
9. Cut an absorbent pad of the following dimensions: 10×2.5 (W $\times$ H cm) (see Notes 5 and 12).
10. Place absorbent pad on top of the adhesive support and overlapping the membrane by 2 mm as shown in Fig. 2 (see Fig. 2).
11. Cut vertically in strips of 4 mm of width by a guillotine (see Notes 13 and 14).
12. Store strips in a zipped bag with a silica gel pouch (desiccant to absorb moisture) at 4 °C.

3.2 Assay Assembly

1. Add 20 μ L of the three standard solutions of SHLMWE or diluted samples to the corresponding well.
2. Add on top 20 μ L of conjugate working solution (diluted rabbit anti-SHLMWE polyclonal antibody gold-conjugated solution; OD 2).
3. Mix gently by repeatedly pipetting up and down.
4. Insert a test strip in each well to allow allergen-antibody complexes to diffuse across the membrane and react with the antibodies immobilized on the test and control lines. If allergen-antibody complexes react with the immobilized antibodies a red-purple line appears.
5. Remove the test strips from the well after 30 min.
6. Read them **immediately** by naked eye and/or by densitometry (Fig. 3).



Fig. 3 A positive test with the test and control line colored after having been inserted in the well

3.3 Result Analysis by Naked Eye

After the liquid reagent flows through the test and control lines a colored line could be observed with naked eye.

1. Results should be read on the side of the membrane to which capture reagents are applied.
2. A visible red-purple line has to appear at the control line for the test to be valid.
3. Reader can rank sample results as negative (–), positive (+), or double positive (++) by comparing the intensity of the test line appeared with the intensity of the line obtained with a three-point standard curve of known concentrations (1.56, 6.25, and 25 ng/mL) of SHLMWE.

3.4 Result Analysis by Densitometry

1. A visible red-purple line has to appear at the control line for the test to be valid (Fig. 3).
2. Scan the side of the membrane to which capture reagents are applied with a flatbed scanner (Epson Expression 1600).
3. Analyze line intensity by densitometry with Image J 1.45 s (NIH, USA) (*see* **Note 15**) or a similar software.

3.5 Densitometry with Image J 1.45 s

1. Open the image file using File>Open in ImageJ.
2. The analysis requires the image to be a grayscale image. The simplest method to convert to grayscale is to go to Image>Type>8-bit.
3. Choose the Rectangular Selection tool from the toolbar. Draw a rectangle around the first strip. ImageJ assumes that your

lanes run vertically (so individual bands are horizontal), so your rectangle should be tall and narrow to enclose a single strip.

4. After drawing the rectangle over your first strip, press the 1 key (command +1 on Mac) or go to Analyze>Gels>Select First Lane to set the rectangle in place. The selection will now be highlighted and have a 1 in the middle of it.
5. Use your mouse to click and hold the rectangle on the first strip and drag it over to the next strip. Press 2 or go to Analyze>Gels>Select Next Lane.
6. Repeat **step 5** for each strip to set the rectangle in place.
7. After you have set the rectangle on the last strip press 3 or go to Analyze>Gels>Plot Lanes to draw a profile plot of each membrane.
8. The profile plot represents the relative density of the contents of the rectangle over each membrane. The rectangles are arranged top to bottom on the profile plot. Higher peaks represent darker bands.
9. Images of immunoassays always have some background signal, so the peaks do not reach down to the baseline of the profile plot. It will be necessary to close the peak so that we can measure its size. Choose the Straight Line tool and draw a line across the base of the peak. This step requires some subjective judgment to decide where the peak ends and the background noise begins.
10. When each peak has been closed off at the base, select the Wand tool and click inside the first peak. Repeat this for each peak and measurements should pop up in the Results window.
11. The values from the Results window can be moved to a spreadsheet program by selecting Edit>Copy All in the Results window (*see Note 16*).

4 Notes

1. Conjugate diluent/antigen diluent contains Tween-20. Detergent is required for uniform capillary flow. The addition of a nonspecific protein such as bovine serum albumins (BSA) can be used to stabilize the capture reagent.
2. Choosing a membrane is one of the most critical steps of the strip development. Membrane characteristics will determine in a great extent the strip performance (i.e., response time, background, sensitivity). The UniSart® CN 95 is a large-pore-size (around 15 µm pore size) cellulose nitrate membrane with a

capillary speed of around 100 s/4 cm that is directly cast onto a 100 μm clear polyester backing and with a binding capacity for proteins of 28 μg IgG/ cm^2 . Choosing a fast lateral speed membrane, like the UniSart® CN 95, generates less background and gives a shorter time to signal on the test strip, but gives lower sensitivities. Choosing a backed membrane offers two advantages: they are easier to handle and the polyester film acts as a barrier to chemical contamination from adhesives used to stick membranes to cards during strip assembly.

3. Optimal dilution was chosen in order to minimize the “hook effect” throughout the range of concentrations likely to be encountered in environmental samples. The hook effect, also known as prozone effect, describes an artifact seen when the analyte is in very high concentration, beyond the dynamic range of the method. This excess amount cannot be measured accurately and yields lower signal than expected in the assay (*see* Ref. [8]).
4. You can perform conjugation by yourself or alternatively contract this service. There are several companies that offer gold colloidal conjugation and assure a good-quality conjugate.
5. Be very careful with strip and all its component dimensions (i.e., nitrocellulose membrane; absorbent pad). They are crucial in the strip performance. Strip performance can also be modified by the test line and control line position from origin.
6. Always manipulate membrane and absorbent pad gently. Do not exert pressure on them in order to avoid damaging their structure. Wear gloves at all times and do not handle them with tweezers as it would leave marks on it. If tweezers are required choose a blunt flat-tip tweezers.
7. **Capture reagent diluent** should be always buffered to achieve consistency and pH must be around 7.0–7.5 as most antibodies have their isoelectric points between 5.5 and 7.5. Detergents (i.e., Tween® 20, Triton™ X-100) should be avoided or used at concentrations <0.01% in capture buffers. These chaotropic agents may interfere the molecular interaction between the protein and the nitrocellulose membrane. We will observe that the signal may start to develop, but as soon as the capture reagent will start to detach, signal will start to disappear.
8. The reagent dispensing rate ($\mu\text{L}/\text{cm}$) and the concentration of capture reagent to be dispensed should be optimized.
9. For **stripping reagents** onto the membrane by hand there are several tips that can be handy. We recommend using low-volume pipettes (i.e., P10) even though you have to pipette several times to cover all the membrane width. That would help you to have a narrower line as the width of the protein

line is mainly determined by the contact zone between the dispensed liquid and the membrane. If capture reagent is applied with a higher volume pipette (i.e., P20, P100) the reagent is spread out over a wider area, resulting in a broader line with less color intensity and potentially achieving a lower sensitivity. In order to dispense antibody following a straight line we found it useful to create a template on a paper with the test and control lines drawn with a permanent pen at 0.3–0.6 cm from the origin, respectively. To start stripping reagents place your membrane on top of your template (*see* Fig. 1) and guide your dispensing by looking at the drawn lines that show through. Avoid using a ruler to dispense as you can damage the membrane structure by exerting too much pressure. It is also advisable to avoid drawing lines at the backing film of the membrane. Ink may smear when getting wet even if a permanent ink is used.

10. To prevent you from touching the already dispensed test line rotate the membrane 180° before dispensing the control line. Before rotating the membrane do a little mark on one corner to know the membrane orientation.
11. **Blocking:** Normally it is not necessary to block the membrane after applying the capture reagent. The presence of nonspecific proteins such as BSA in the sample and conjugate diluents is frequently sufficient to prevent the detector reagent and analyte from nonspecific binding.
12. Absorbent pad's bed volume will control the volume of sample analyzed and depends on absorbent pad characteristics and dimensions.
13. Avoid exerting pressure on the strip while cutting with the guillotine.
14. Discard one-half of strip at each side border as stripping could be less homogenous near the borders.
15. ImageJ is a public-domain, Java-based image-processing program developed at the National Institutes of Health that can be used to compare the density (or intensity) of bands on an immune test strip. You have to obtain a digital image of your strips in .tif, .jpg, .png, or other image formats. You can use a flatbed scanner, and the appropriate scanner software to save a well-exposed good-quality image.
16. The values calculated by ImageJ are essentially arbitrary numbers; they only have meaning within the context of the set of peaks that you selected on the image you have been working on. They do not have units. The usual procedure is to express the density of the selected bands relative to some standard band that you also selected during this process.

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