VALIDATION OF SANITATION PROCEDURES TO PREVENT THE CROSS CONTACT WITH ALLERGENS DURING THE PROCESSING OF PORK PRODUCTS

A Thesis

by

DAWNA GAIL WINKLER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2009

Major Subject: Animal Science

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Approved by:

Co-Chairs of Committee,	Kerri B. Harris
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ABSTRACT

Validation of Sanitation Procedures to Prevent the Cross Contact with Allergens During the Processing of Pork Products. (August 2009)

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Co-Chairs of Advisory Committee: Dr. Kerri B. Harris
Dr. Margaret D. Hardin

This study was conducted to develop and validate cleaning procedures for different processing equipment of varying complexity and to determine the efficacy of two different allergen tests. Following introduction of selected allergens to processing equipment, two treatments were applied - water wash or scrub/sanitize – and a no clean was also evaluated. The equipment used consisted of a slicer, grinder, injector, vacuum tumbler, and plastic lugs. To introduce the allergen to the slicer, nine ready-to-eat hams were used. One hundred twenty-two kilograms of pork trim were ground, and a milk allergen was incorporated into the meat. The injector was contaminated with a food allergen by injecting boneless pork loins with a marinade containing soy flour. The slicer, grinder, injector, tumbler, and lugs were then subjected to randomized treatments. The results showed that the water wash and scrub/sanitize treatments did not differ significantly among the pieces of equipment tested. This study supported that both water wash and scrub/sanitize treatments can effectively removed allergens to a level below the industry threshold of 5 ppm.

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CHAPTER I

INTRODUCTION

An estimated three to twenty four million Americans are affected by food allergies. This includes 3 to 8% of children and about 1 to 4% of adults who suffer from food allergies *(3, 4, 8, 18, 20)*. On average, food allergies account for approximately 29,000 emergency room visits and an estimated 150-200 deaths each year. Many of the deaths caused by food allergies are from anaphylaxis, a severe allergic reaction *(8)*.

Over the past ten years, there have been 74 meat and poultry Class I and II recalls due to food allergen issues *(24)*. FDA has also issued 77 allergen related recalls over the past five years *(10)*. The economic impact of recalls can be very large and damaging to a company and their customer base.

Recent demands for value-added and more diverse products have resulted in allergen management becoming more difficult for food processors as well as the consumer *(20)*. This complication or difficulty is because food allergens are not destroyed by processing techniques including high temperatures, pH, and proteolysis *(20)*.

In January 2006, the Food Allergen Labeling and Consumer Protection Act of 2004 became effective. This Act amended the Federal Food, Drug, and Cosmetic Act to require that the label of all Food and Drug Administration (FDA) regulated foods,

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This thesis follows the style of *Journal of Food Protection.*

containing ingredients or proteins from a "major food allergen," declare the presence of such allergens on the label. This Act was passed by Congress to make daily life easier for susceptible consumers and their caregivers to recognize and avoid foods that contain the major food allergens *(25)*. As a result of meat and poultry allergen related recalls, USDA's Food Safety and Inspection Service (FSIS) issued Notice 45-05 *(26)*, which required meat and poultry establishments to reassess their food safety programs to ensure that allergens were properly addressed.

CHAPTER II

REVIEW OF LITERATURE

Over 170 foods have been identified to cause allergic reactions; however, there are eight foods that are responsible for approximately 90% of all food allergies, commonly referred to as the "Big Eight" *(3, 4, 8, 20, 23)*. These eight foods include milk, eggs, soy, wheat, peanuts, tree nuts (e.g., almonds, walnuts, pecans, hazelnuts), fish (e.g., salmon, halibut, cod) and shellfish (e.g., shrimp, crab, lobster). The second eight include sesame seeds, sunflower seeds, cottonseed, poppy seed, mollusks, beans other than green beans, peas, and lentils *(3)*. The "Big Eight" are the primary focus for control and regulatory action, since controlling these eight foods will help control or prevent the most severe allergic reactions *(20)*. Food allergens are all naturally occurring proteins that have proven to be very resilient to heat, proteolysis, and pH *(20)*. While foods contain millions of different proteins, only a few of these proteins are allergens *(7)*.

What defines a true food allergy. A food allergy is an antibody-mediated immune response to a food containing a specific protein or glycoprotein, which could potentially result in life threatening symptoms *(3)*. Humoral immunity, also known as, antibody-mediated immunity, is supplied by antibodies, which are produced by lymphocytes and are present in body fluids. There are two kinds of lymphocytes formed in red bone marrow, including B lymphocytes, and T lymphocytes. B lymphocytes control humoral immunity, and T lymphocytes are non-antibody-producing

lymphocytes. Antibodies can be any group of large glycoproteins that are secreted in the blood serum and that initiate an immune response by binding with specific antigens. The five classes of antibodies or immunoglobulins in humans are IgA, IgD, IgE, IgG, and IgM. An allergic reaction occurs when there is formation of immunoglobulin E (IgE) antibodies. Through a series of biochemical reactions, these antibodies result in the release of histamines into the tissues from the body's mast cells, which are located in the eyes, skin, respiratory system, intestinal tract, and urinary system *(3)*.

An antigen is any substance capable of causing an immune response. Antigens can be grouped into two categories, complete or incomplete. Complete antigens have two important functional properties, immunogenicity and reactivity. Reactivity is the ability to react with the activated lymphocytes and the antibodies, which are released by immunogenic reactions. Complete antigens also have the ability to stimulate proliferation of specific lymphocytes and antibodies. An incomplete antigen, or hapten, has reactivity but not immunogenicity. Some chemicals that act as haptens can be found in poison ivy, animal dander, detergents, cosmetics, and numerous household and industrial products *(14)*.

The first encounter between a lymphocyte and an invading antigen usually takes place in the spleen or lymph node, but can occur in any lymphoid tissue. If the lymphocyte is a B cell, the invading antigen causes the humoral immune response, which results in the production of antibodies *(14)*.

A food allergy is an immune response in which the body's immune system mistakenly attacks harmless food proteins and forms immunoglobulin E-mediated (IgE)

antibodies. Subsequent exposure to a particular food causes the IgE antibodies to detect the protein in that food and alert the cell to pour out chemicals, such as histamines, which may result in allergic symptoms.

Food allergies are the leading cause of anaphylaxis. Anaphylaxis is a severe allergic reaction that is potentially fatal, and can be caused by a food allergy, insect sting, or medication. While symptoms of anaphylaxis will affect the entire body, they may impact each body system differently. Not every allergic reaction will provoke anaphylaxis; however, each allergic episode should be taken very seriously because any episode can result in anaphylaxis. An indication of an allergic reaction affecting the skin can include visible irritation of the skin, including hives, and swelling of the lips, tongue, or face. More severe symptoms involve the respiratory system and can include swelling of the throat, tightness in the chest, wheezing, or coughing. Gastrointestinal tract symptoms may include abdominal pain, nausea, vomiting, or diarrhea. Food allergies affect the circulatory system and may cause a drop in blood pressure, heart failure, loss of consciousness, or even death, if left untreated *(6, 8)*. The proportion of allergic individuals that may be susceptible to anaphylaxis is unknown due to medical professionals reluctance to test the population *(6)*. Historically, when people exhibiting moderate symptoms are re-exposed to a specific allergen anaphylactic shock can occur without warning. Every allergic individual responds differently to his or her offending food. A dose-response phenomenon has been discovered with food allergies. The extent of the response is directly proportional to the amount of allergenic food eaten by the

food allergic individual *(11)*. The response may also be impacted by the individual's overall health status, as people with asthma tend to have more serious reactions *(6)*.

It is also believed that food allergies might result from a breach in oral tolerance to foods while they are being ingested. Oral tolerance can occur when allergenic proteins are digested by infants or children during a presumed time of immunologic immaturity. The gut barrier is a gastrointestinal mucosal barrier that is a complex physical and immunologic structure. If the gut barrier is developmentally immature, this may also provide some explanation for the increased prevalence of food allergies in infants and children, as well as why children may "outgrow" food allergies *(18)*.

People at risk. Approximately 3 to 12 million of American adults are reported to have a food allergy *(3, 4, 8, 18, 20)*. Allergic reactions to shellfish (e.g., shrimp, crab, lobster), and fish are most common in adults *(20)*. Some allergies can provoke more severe allergic reactions, including peanut and tree nut allergies *(8)*.

An estimated 9 to 24 million children in the United States are affected by food allergies *(3, 4, 8, 18, 20)*. In children under 3 years of age, milk and soy allergies are more common. It is not uncommon for children to outgrow milk, soy, or other allergies; however, if the symptoms of a food allergy continue through adolescence into early adulthood, it will most likely be retained for life *(6, 20)*. The foods that are most common for causing an allergic reaction in children over 3 years of age are soy, milk, egg, and wheat. Children are also impacted by peanut and tree nut allergies, which normally persist throughout life *(20)*.

Treatment. Strict avoidance of foods containing allergens is the only way to prevent allergies completely *(22)*. Since trace amounts of a food allergen can elicit an allergic reaction, management of the diet is crucial *(22)*. If an allergic reaction occurs, an early dose of epinephrine is crucial *(22, 23, 27, 28)*. An intramuscular injection of epinephrine or the Epi Pen®, is the common treatment of choice when ingestion of an allergen occurs. Waiting to determine the severity of the reaction is very dangerous and could be fatal *(23, 27)*.

Testing individuals for food allergies is a very important step in identifying which foods to avoid. A simple skin prick test can be performed to determine which foods an individual is allergic to *(3)*. However, a double-blind, placebo-controlled oral food challenge test is considered the gold standard for diagnosing IgE-mediated food allergies. *(3, 5)*. This test method is very expensive and time consuming and the parameters of the test must be fully complied with. When researching food allergies one of the major challenges are diagnostic difficulties *(5)*.

***Food allergies and food intolerances*.** Food allergies are often confused with food intolerances or sensitivities. A true food allergy is an immune response in which the immune system overreacts to protein in food. A food intolerance involves an abnormal reaction to a food, which is usually caused by an enzyme deficiency or other non-immune response *(8, 9, 21, 23)*. Food intolerances are not life threatening, and generally involve less severe symptoms rather than the more serious consequences originating from a true food allergy *(20, 23)*.

About 30% of American adults claim to have a food allergy and alter their eating habits accordingly. Recent epidemiologic estimates suggest that only 1 to 4% of adults have true food allergies with repeatable symptoms resulting from a reaction to food allergens *(15, 16, 19)*.

There are three categories that food intolerances can be divided into including: anaphylactoid reactions, metabolic food disorders, and idiosyncratic illnesses *(23)*. Anaphylactoid reactions result from substances that cause the release of mediators from mast cells without the involvement of IgE *(13)*. That particular substance in the food of concern is thought to destabilize the mast cell membranes which allows the spontaneous release of histamine and other mediators *(23)*. This histamine releasing substance has never been isolated or identified in foods; however, it is well documented with certain drugs *(21, 23)*. An example of an anaphylactoid reaction is strawberry sensitivity *(21, 23)*.

Metabolic food disorders result from inherited defects in the inability to metabolize a component of food or from a genetically determined, enhanced sensitivity to a particular foodborne substance that arises from an altered metabolic pathway *(13)*. Lactose intolerance is an example of an illness that occurs as a result of genetic inability to metabolize a food component *(21)*. Lactose intolerance is caused by a deficiency of intestinal lactase, the enzyme needed for proper digestion of lactose *(21)*. This disease is known to affect a large proportion of certain ethnic populations *(21)*.

Idiosyncratic illnesses cause unfavorable reactions in some individuals, but the mechanism for these symptoms is unknown *(21)*. It has been concluded that a sizeable

amount of different mechanisms could be involved in idiosyncratic reactions *(21)*. The symptoms of idiosyncratic illness range from inconsequential to severe and life threatening *(23)*. The function of particular foods or food ingredients that cause idiosyncratic reactions remains to be determined *(21, 23)*.

Allergen control program. Allergens can be controlled in a processing environment with an allergen control plan, which is part of prerequisite programs. In order for an allergen control program to be effective, a diverse team of people should be organized to take responsibility for developing and implementing such a plan.

An allergen control team should be a diverse group of people including representatives from quality control and regulatory affairs, manufacturing, maintenance, research and development, engineering, and sanitation divisions. Allergen control plans can include: supplier review, allergen-mapping program comprised of traffic patterns through receiving, in the warehouse, and on the production floor, and scheduling of allergen containing products. Other areas that must be addressed include rework, maintenance, labeling, storing of products containing allergens, validation of cleaning methods, and training *(3, 6, 12)*.

It is crucial to make sure that your suppliers have provided you with accurate allergen information about the products that you have purchased. Supplier review should include reviewing the allergen control program of the supplier, identify the range of allergenic products produced by the supplier, and evaluating the allergen cleaning programs performed by the supplier. Allergen training records from the supplier should also be reviewed. Allergenic ingredients that are shipped from the supplier to your plant

should be clearly marked in sealed containers that are not damaged or broken. Effective communication with the supplier is an important component of developing and maintaining an effective allergen control program *(12)*.

An allergen map should be easy to read and understand, and it should clearly depict where the allergens are in the plant and where they are introduced into a production process. Allergen mapping shows the route that the allergen takes once entering the plant and all the way through warehousing, production, and shipping *(12)*.

Prior to receipt the allergen should be marked. Clearly identified staging areas for allergenic products can also be very helpful. It is a good idea to store allergenic products separately from non-allergen products in defined areas and use designated pallets and containers *(12)*.

When possible, production of allergen containing products should be at the end of a production shift and adequate cleaning and sanitation should. The use of long runs of products containing allergens will decrease the number of potentially expensive and time-consuming clean-ups. Non-allergen containing products should be scheduled first followed by allergen containing products. If the product formulation allows, the allergen ingredient should be added as late as possible in the production process. However, this is not always achievable due to last minute product and schedule changes or availability of specific equipment or rework *(6, 12)*.

Dirty equipment is usually defined as food soil, or unwanted matter on food-contact surfaces. Food soil can be visible or invisible, and comes primarily from the food product being produced *(1)*. In the food industry protein-based soils are by far the most

difficult soils to remove *(1)*. Effective cleaning and sanitation procedures must achieve the correct level of cleanliness in food handling or production facilities *(2)*.

Cleaning can be defined as the complete removal of food soil using appropriate detergent chemicals for specific conditions. There are several different types of cleaning methods including mechanical cleaning, clean-out-of-place, and manual cleaning. Mechanical cleaning is usually referred to as clean-in-place which does not require very much disassembly. Clean-out-of-place usually refers to disassembled parts that are cleaned in specialized pressure tanks. Manual cleaning requires total disassembly for cleaning and inspection *(17)*. The suitability of different cleaning methods is dependent upon factors such as size and accessibility of the area to be cleaned and the amount of soil on the equipment *(2)*.

If one product does not contain an allergen but the rest of the products do, a complete wet cleaning becomes an integral part of the process and should be performed before the next product run *(6)*. Published sources agree that verification of the cleaning process between runs should include both a visual inspection that the equipment is clean as well as the use of validated allergen-detection methods when available *(6, 23)*.

Rework of allergen containing products should be addressed by identifying when reworked allergenic products are being produced, where they are stored, products that they are being reworked into, and when these products enter the line. This can be implemented using a “like into like” practice *(12)*.

The processing equipment that the allergen comes in contact with should also be identified. Maintenance personnel should be aware of the seriousness of cross contact

between an allergen and non-allergen equipment or areas. Cross contact can occur from using tools on equipment that has allergen on it, and then using those tools on different equipment that is being used to process non-allergen containing food. Designated tools for allergen and non-allergen equipment can be implemented *(12)*.

Labeling is also an important aspect of allergen management. The label of a package must be correct to make consumers aware of the allergen containing ingredients used in the product. In most cases, the label is the only means of communication to the consumer about what the product contains. Ensure that product formulation changes regarding an allergen are reflected on the label immediately *(6, 12)*.

After production, allergenic products should be stored in designated containers. The area that these products are stored should be clearly marked *(12)*.

Validation of cleaning procedures is a fundamental part of any effective allergen control plan. Cleaning validation refers to the process of assuring that a defined cleaning procedure is able to effectively and reproducibly remove the allergenic food from the specific food processing line or equipment. Also periodic testing of the processing equipment is necessary to ensure that the cleaning procedures are adequately removing the allergen. Training is essential to implement and maintain an allergen control program. All employees should be aware of the health consequences that can occur if equipment is not properly cleaned or if products are not labeled correctly to reflect allergens of concern *(12)*.

Allergen management must be also carried over into retail and foodservice. If allergies are successfully managed through processing, then cross-contaminating

products in a restaurant kitchen or retail deli must not compromise such efforts. All parts of the foodservice chain must do its part to control allergens efficiently *(12)*.

Conclusion. Food allergies can affect the lifestyle of anyone of any age. Food allergies can be serious, and life threatening if not addressed properly. Sensitivity and severity of reactions vary by individual, and by the amount of the allergenic material present. Since there is no cure for food allergies, the only way to effectively manage them is to avoid the food that causes an allergic reaction. People who have food allergies should carry a dose of epinephrine with them at all times to be prepared for a reaction. Allergens can be managed in a processing environment if the right tools are utilized; one of these tools is an effective and validated cleaning program. Training of food personnel is the fundamental step in implementing a successful allergen control program. The allergen control program must not stop at the processor, it must carry over into retail, and possibly into handling of the products at home. Everyone must do their part in managing allergens in order to bring this important food safety issue under control.

CHAPTER III

MATERIALS AND METHODS

Treatments. To develop and validate cleaning practices that could be utilized to remove allergens, two treatments (water wash and scrub/sanitize) were randomized and completed each day, along with a no clean procedure. Each of these treatments were applied to a slicer, grinder, injector, and tumbler. The no clean treatment was used as a control. The equipment was exposed to pork products that contained the selected allergens and tested to determine the initial allergen contamination level on the equipment that would be present and subjected to the water wash and scrub/sanitize treatments.

The water wash treatment consisted of washing the processing equipment and all product contact surfaces with water (34 to 64°C; 15 to 17 liters/min) until visibly clean. The water was warm enough to clean the processing equipment but cool enough to not cook on proteins. The scrub/sanitize treatment included taking the equipment apart, scrubbing with a hand held brush and or white scouring pad (Grainger, San Antonio, TX), followed by sanitizing. Each individual part was scrubbed with a general purpose soap containing (4.06 oz Birko Liquik 10™, Henderson, CO) in 3.8 liters of water (34 to 64°C) and sanitized with a quatenary ammonium compound (Birko Bi-quat™, Henderson, CO) using a hand held polyethylene sprayer until adequately covered (~1.5 liters/min).

Product preparation. To evaluate the treatments, three pork products (hams, ground pork trim, and boneless pork loins) and two different allergens (milk and soy flour) were used. The ready-to-eat ham was coated with parmesan cheese, a milk allergen. Parmesan cheese was also added to the ground pork product. These two products were used because they represented what was currently available in the retail market. However, for the injected product several allergen containing spice blends were initially evaluated, but the allergens were not able to be detected by the test kits; therefore, a marinade containing soy flour was developed.

Nine ready-to-eat hams (1.36 to 1.81 kg) coated with parmesan cheese were purchased from a retail store. The hams were then frozen and stored at -12°C until subsequent use. Prior to each sampling day, one ham was allowed to thaw for 24 h at approximately 4°C. Each ham was cut into 3 sections, weighed, sliced (24 to 30 slices), and assigned to a treatment. A Bizerba Slicer SE-12 (Edison, NJ) was utilized to slice the ham. After the ham was sliced, it was moved around in a clean plastic lug while making sure it had contact with all areas within the lug. The ham was moved around with a gloved hand for 30 s in order to achieve even coverage of allergen inside the lug. In this project, the lug represented a conveyor.

One hundred twenty-two kilograms pork trim containing 70% lean and 30% fat were purchased from a commercial meat processor. The pork trim was then frozen and stored at -12°C until later use. Prior to each sampling day, the pork trim was thawed for 24 h at approximately 4°C. Ten pounds of pork trim were used for each treatment, and all three treatments were completed per day.

A Biro Grinder model 1056 (Canton, OH) was used to grind the pork trim. The pork trim was first ground through a ½ in. plate. Twenty g of shredded parmesan cheese (Churny Company Inc., Weyauwega, WI) were added to the coarse ground pork and mixed with a gloved hand in a lug for 1 min in order to adequately incorporate the cheese into the coarse ground pork. This amount of parmesan cheese was added because it was similar to a recipe formulation from retail. The meat and cheese were then ground through a ¼ in. plate resulting in a fine grind. The allergen containing meat was then moved around in a lug with a gloved hand for 30 s to obtain even coverage of the allergen in the lug.

An allergen containing marinade and boneless pork loins were used to evaluate the injector. Nine boneless pork loins were purchased from a meat processor. The pork loins were frozen and stored and -12° C until later use. Prior to each sampling day, one pork loin was thawed for 24 h at approximately 4°C. The pork loin was cut into thirds, weighed, assigned to a treatment, and injected (≥ 13%) with marinade containing soy flour and then tumbled under a vacuum for 10 min. Tumbling under a vacuum helps to more evenly distribute the marinade that was injected. The marinade consisted of approximately 113 liters of water and 60 g of Bob's Red Mill Soy Flour (Milwaukie, OR).

Equipment preparation. In order to assess the cleaning treatments, four pieces of equipment were used including a slicer, grinder, injector, and tumbler. One piece of equipment was used each day to evaluate all three treatments. For uniform evaluation, equipment was first rinsed with water (34 to 64°C) so that each treatment began with wet

equipment. Each swab (Neogen, Lansing MI) was taken on a different location, no location on the equipment was swabbed twice in a treatment.

Four areas of the slicer including the blade front, metal part of the meat holder, panel next to blade, and base of slicer, as well as the lug were swabbed prior to introduction of the product containing the allergen. Swab samples were taken using a 10 x 10 cm template with a crosshatch technique (10 passes vertically, 10 passes horizontally) to determine if there was any allergen present. The lug was also swabbed as previously described for background allergen at two random areas on the interior side and two areas on the interior bottom.

Four areas of the grinder including the auger, blade, funnel, and channel from the grinder, as well as the lug were swabbed prior to introducing the product containing the allergen using a 10 x 10 cm template with a crosshatch technique to determine the amount of allergen residue present. The lug was swabbed, as previously discussed, at two random areas on the interior side and two areas on the interior bottom.

An Original InjectStar Injector Type: B1-72 (Mountain View, AR) was used to inject the pork loins. Two locations including the area between the chain on the delrin guide, and between the needles of the injector were swabbed prior to each treatment using a 10 x 10 cm template with a crosshatch technique for the amount of soy flour allergen residue. Also two runoff samples were collected from the injector in clean plastic tubes before the allergen was introduced. The lug was swabbed at two random areas on the interior side and two areas on the interior bottom. The vacuum tumbler (VT500, Fort Worth, TX) was swabbed at four locations inside the drum.

No clean (control). The milk allergen was introduced to the slicer by slicing each ham. Immediately following slicing, the blade front, metal part of meat holder, panel next to blade, and base of slicer were swabbed in order to determine the initial level of milk allergen present. The lug containing the sliced ham was swabbed in four random areas, as described above, for the presence of milk allergen.

The milk allergen was introduced to the grinder by grinding the pork trim and parmesan cheese. The auger, blade, funnel, and channel of the grinder were swabbed in order to determine the initial level of milk allergen present. The lug containing the ground pork plus allergen was swabbed in four random areas, as described above, for the presence of milk allergen.

The soy allergen was introduced to the injector through injection as described above. The area between the chain and needles of the injector were swabbed and two runoff samples were taken in order to determine the initial level of soy allergen present. The lug containing the injected pork loin was swabbed in four random areas, as described above, for the presence of soy allergen. The tumbler was also swabbed in four locations after the allergen was introduced.

Water wash treatment. Water in the range of 34 to 64°C was used for the water wash because it was warm enough to rinse out equipment and cool enough not to cook on the proteins. Slicer and lug were sampled as described in equipment preparation. After the ham was sliced the blade front, metal part of meat holder, panel next to blade, and base of slicer were swabbed to determine the initial amount of allergen present. Each part was then water washed separately (34 to 64°C; flow rate 15 to 17 liters/min) until

visibly clean approximately 15 to 20 s. The water washed parts were then placed in a clean lug and swabbed as described above for presence of remaining milk allergen.

Grinder and lug were sampled as described in equipment preparation. The milk allergen was introduced to the processing equipment as previously described. The face on the front of the grinder was opened and the grinding plate, blade, and auger were removed, swabbed, and placed in a plastic lug. Each part was then water washed separately (34 to 64°C; 15 to 17 liters/min) until visibly clean approximately 35 to 45 s. The water washed parts were then placed in a clean lug and swabbed as described above for presence of milk allergen remaining. The grinder including the funnel and channel were washed (34 to 64°C; 15 to 17 liters/min) until visibly cleaned. After the water wash treatment areas of the grinder and lug were then randomly swabbed to determine the amount of allergen remaining.

Injector, lug, and tumbler were sampled as described in equipment preparation. After the pork loin was injected the area between the chain and needles of the injector were swabbed, and runoff samples were collected to determine the initial amount of soy present. The injector was water washed with approximately 189 liters of water that was pumped through the injector for the water wash. After the pork loin was tumbled for 10 min under a vacuum two swabs were taken from the inside the drum and each side inside the drum. The tumbler was then water washed for approximately 15 to 20 s (34 to 64°C; 15 to 17 liters/min) until visibly clean. The water washed parts were then swabbed, and runoff samples were taken as described above for presence of soy allergen remaining.

Scrub/sanitize treatment. Slicer and lug were prepared as noted in equipment preparation and swabbed for background allergens. After the allergen had been introduced, the slicer and lug were swabbed to obtain an initial level of milk allergen present. The slicer was then rinsed with water 34 to 64°C. Each individual part was then scrubbed with a general purpose soap containing (4.06 oz Birko Liquik 10™, Henderson, CO) in 3.8 liters of water (34 to 64°C). The blade was turned clockwise three times while scrubbing horizontally making sure to scrub the blade completely. To scrub the base, 15 to 20 brush strokes were used in a clockwise circular motion to ensure that the entire base was scrubbed. In order to wash the panel to the left of the blade, 12 to 15 brush strokes were used with a left to right motion while adequately cleaning the entire panel. The entire meat holder was cleaned using 12 to 15 vertical brush strokes. The entire lug was cleaned with 8 to 14 horizontal brush strokes on the short side and 12 to 18 brush strokes on the long side. The bottom of the lug was cleaned with 15 to 17 circular brush strokes. The lug, slicer, and all parts were sanitized with a quatenary ammonium compound (Birko Bi-quat™, Henderson, CO) using a hand held polyethylene sprayer until adequately covered (~1.5 liters/min). After the scrub/sanitize treatment was applied the slicer and lug were swabbed to determine the amount of allergen removed.

Grinder and lug were prepared as noted in equipment preparation and swabbed for residual milk allergens. After the allergen had been introduced, the grinder and lug were swabbed to obtain an initial level of milk allergen present. The grinder was then rinsed with water 34 to 64°C. The grinder was then taken apart and the scrub/sanitize

treatment was applied to the grinder and lug. Each individual part was scrubbed with a general purpose soap containing (.12 liter Birko Liquik 10™, Henderson, CO) in 3.8 liters of water (34 to 64°C).

In order to scrub the auger, 5 to 6 brush strokes were used per groove yielding approximately 30 to 36 brush strokes in order to clean the entire auger. Each side of the blade was scrubbed using 12 to 15 circular brush strokes covering the entire blade. In order to clean the entire funnel 14 to 16 circular brush strokes were used. The complete channel was cleaned using 18 to 20 vertical brush strokes. The grinder and all parts were rinsed until no soap remained (45 to 55 s). The entire lug was cleaned with 8 to 14 horizontal brush strokes on the short side and 12 to 18 brush strokes on the long side. The bottom of the lug was cleaned with 15 to 17 circular brush strokes. The lug, grinder, and all parts were sanitized with a quatenary ammonium compound (Birko Bi-quat™, Henderson, CO) using a hand held polyethylene sprayer until adequately covered (~1.5 liters/min). After the scrub/sanitize treatment, the grinder and lug were then swabbed to determine the amount of allergen removed.

Injector, lug, and tumbler were prepared as noted in equipment preparation and swabbed for naturally occurring soy allergens. After the allergen had been introduced, the injector and lug were swabbed and runoff samples were taken to obtain an initial level of soy allergen present. Next approximately 113 liters of water containing .07 liter of Birko Liquik 10™ soap (Henderson, CO) were pumped through the injector, then rinsed with approximately 189 liters of water. Each delrin guide was scrubbed with 10 vertical brush strokes on each side. The lug was cleaned with 8 to 14 horizontal brush

strokes on the short side and 12 to 18 brush strokes on the long side. The bottom of the lug was cleaned with 15 to 17 circular brush strokes. After the pork loin was tumbled under a vacuum, four swabs were taken from the inside the drum. The tumbler was washed using 15 to 20 horizontal brush strokes on the inside of the drum. The injector, tumbler, and all parts were sanitized with a quatenary ammonium compound (Birko Bi-quat™, Henderson, CO) using a hand held polyethylene sprayer until adequately covered (~1.5 liters/min). The injector, lug, and tumbler were then swabbed, and runoff samples were collected as described above for the presence of soy allergen remaining.

Each piece of equipment was cleaned using the scrub/sanitize treatment method between each treatment. The processing equipment was cleaned between each treatment to remove any remaining allergen, and to ensure that each treatment was started with adequately clean equipment.

Packaging and personal protective equipment. The sliced allergen containing product was packaged, using a vacuum packaging machine (Boss B-14, Willawong Queensland, Australia). The packaging equipment was also swabbed for the presence of milk allergen. The entire heat seal bar and vacuum channel of the packaging machine were swabbed in one location prior to and after packaging the allergen containing product. No location was swabbed twice during a day's treatment.

The ground pork trim containing the milk allergen was packaged, using a BIVAC vacuum packaging machine (Saddlebrook, NJ) the packaging equipment was also swabbed for the presence of milk allergen. The conveyor chain and metal guides were

swabbed in one location prior to and after packaging the allergen containing product. Each swab was used on a different location, no location was swabbed twice.

The pork loin injected with soy flour was packaged, using a vacuum packaging machine (Ultravac Koch Packaging Machine, Kansas City, MO). The packaging equipment was also swabbed for the presence of soy allergen. The entire heat seal bar and vacuum channel of the packaging machine were swabbed in one location prior to and after packaging the allergen containing product. No location was swabbed twice.

Personal protective equipment, including gloves (VWR, Suwanee, GA) and disposable aprons (VWR) were also swabbed in one location before and after each treatment. This was to validate the appropriate need for employee change out between allergen and non-allergen product runs. At the completion of each treatment, the personal protective equipment was discarded and replaced with clean gloves and aprons.

Testing. The swabs were transferred to the Food Microbiology Laboratory (Texas A&M University, College Station, TX) for analysis using Neogen Veratox Total Milk Allergen Quantitative Test and Neogen Alert Total Milk Allergen Screening Test. The swabs were tested according to the manufacturer's instructions. For this project, a threshold of 5 ppm was set as verbally recommended by the kit manufacturer. If a sample contained greater than 5 ppm it was considered positive, if it contained less than 5 ppm the sample was considered negative. If necessary, due to time constraints, the swabs were stored for no longer than 24 h at 4°C before analysis. An overview of each kit procedure is listed below.

For the Veratox kit, extraction additive was added to each swab and vortexed. Five controls included with the kit and the extracted samples were added to separate transfer microtiter wells. The samples were then transferred to antibody-coated microtiter wells and allowed to incubate on the benchtop (~25ºC) for 10 min. The wells were then washed and the conjugate was added and allowed to incubate (~25ºC) for 10 additional min. The wells were washed once again and the substrate was added and allowed to incubate (~25ºC) for 10 min. The stop solution was added and the results were read using the microwell strip reader with a 650 nm filter (Neogen, Lansing, MI). For complete kit instructions see Appendices A and B.

In order to begin analysis using the Alert kit, extraction additive was added to each swab and vortexed. The control was added to the appropriate antibody-coated microtiter well followed by the samples and allowed to incubate (~25ºC) for 10 min. The wells were then washed with the wash buffer provided. Next the conjugate was added to all of the wells and allowed to incubate (~25ºC) for an additional 10 min. The wells were washed again with the wash buffer. The substrate was added to the wells and allowed to incubate (~25ºC) for a remaining 10 min. Results were interpreted by comparing the intensity of color of each sample to the control provided. If the sample was darker in color than the control, the sample was positive; however, if the sample was lighter in color compared to the control, the sample was negative. For complete kit instructions see Appendices C and D.

Data were analyzed by Analysis of Variance using the PROC GLM procedure of SAS (v. 9.1, Cary, NC). Processing day was defined as a block and the cleaning

treatments, location and their interaction were the main effects. Least squares were calculated and if differences were found in the Analysis of Variance table ($P < 0.05$), least squares means were separated using the P DIFF function.

CHAPTER IV

RESULTS AND DISCUSSION

Food allergens are a potential food safety concern that the food industry must address to ensure that there is no cross contact between allergen and non-allergen products. The results of this research demonstrated effective validation of cleaning procedures for different processing equipment.

Although there was a numerical difference, there was no statistical difference between the water wash and scrub/sanitize treatments on any equipment, which is an important aspect to consider. The food production industry is a business that strives to be as efficient as possible. As a result, product changeovers between allergen and non-allergen containing products requires maintaining an allergen control program which costs the company time and money. After the water wash and scrub/sanitize treatments, allergen levels were decreased below 5 ppm, which is the threshold level. Although the scrub/sanitize treatment had a lower numerical value, this research validated cleaning procedures and showed that the water wash treatment is very effective in removing allergens. These findings are consistent with results from a study done by the National Food Center for Food Safety and Technology Allergen Task Force (NCFST). The data reviewed by Jackson *(12)* stated that NCFST researchers showed that water alone at 62.8 and 73.8°C was effective at removing cold milk solids.

Table 1 illustrates the effects of treatments on the amount of allergen remaining on the equipment. The amount of residual allergen on the grinder and lug before the

TABLE 1. *Effect of treatments on the amount (ppm) of allergen remaining on the grinder and lug.*

	Veratox		
Equipment/Treatment	Before Allergen[c]	After Allergen[d]	After Treatment[e]
Grinder (n=306)			
Positive Control	0.27[a]	34.59[a]	
Water Wash	0.15[a]	25.39[a]	1.14[b]
Scrub/sanitize	0.19[a]	26.74[a]	0.28[b]
P value	0.35	0.42	< 0.0001
RMSE[f]	0.59	22.37	18.17
Lug (n=306)			
Positive Control	0.31[a]	27.12[a]	
Water Wash	0.17[a]	22.76[a]	0.961[b]
Scrub/sanitize	0.34[a]	26.53[a]	0.156[b]
P value	0.39	0.83	< 0.0001
RMSE[f]	0.41	23.21	15.47

Least squares means within a column and piece of equipment with different letters (*a*-*b*) differ ($P < 0.05$).

[c]Prior to allergen exposure.

[d]After allergen exposure, but before treatment.

[e]Allergen remaining after treatment.

[f]RMSE=Root mean square error from Analysis of Variance Table.

allergen-containing product was introduced to the equipment was not different ($P \geq 0.05$) between treatments, and levels were very low. There were no differences ($P \geq 0.05$) in the amount of allergen on the grinder and lug following exposure to the allergen-containing product. This indicates that after exposure to the allergen containing product allergens remained on the equipment and levels were similar across treatments. The control levels of allergen for both the grinder and lug were higher than the treated equipment.

The effects of treatments on the amount of allergen remaining on the slicer and lug are found in Table 2. The amount of allergen on the slicer and lug before introducing the allergen was not different ($P \geq 0.05$) between treatments. The amount of allergen on the slicer following exposure to the allergen-containing product was not different ($P \geq 0.05$) between treatments as would be expected. However the amount of allergen on the lug differed between the control and water wash. While the lug was exposed to the allergen using a standardized protocol, the water wash lug had higher levels of allergens remaining. These results are most likely due to product with higher levels of allergen being placed in the water wash lug prior to treatment. The amount of allergen remaining after the slicer and lug were treated was not different ($P \geq 0.05$) between the water wash and scrub/sanitize, but these two treatments were different from the control.

The effects of treatments on the amount of allergen remaining on the injector, tumbler, and lug are presented in Table 3. The amount of residual allergen on the injector and lug were not different ($P \geq 0.05$) between treatments. After introducing the allergen-containing product to the injector and lug the amount found on the equipment

was not different ($P \geq 0.05$) between treatments. The amount of allergen on the injector and lug after treatment was not different for the water wash or scrub/sanitize treatments; however, these two treatments were different from the control.

According to the kit manufacturer, industry personnel typically use the Veratox (quantitative) kit to initiate an allergen control program. After the allergen is brought under control, the Alert (qualitative) kit is used to monitor the allergen of concern.

The Veratox and Alert kits were both straightforward and easy to use which made training operators uncomplicated. However, when reading the results of the Alert kit bias may have been introduced due to the subjectivity of reading the test results. Two trained operators interpreted each Alert test, but a small number of results varied between interpreters. There were several factors that could influence the intensity of color developed in the test kit including room temperature, conditions and time that the kits were stored at, and conditions that the reagents were stored at during use.

TABLE 2. *Effect of treatments on the amount (ppm) of allergen remaining on the slicer and lug.*

	Veratox		
Equipment/Treatment	Before Allergen[c]	After Allergen[d]	After Treatment[e]
Slicer (n=306)			
Positive Control	1.83[a]	58.42[a]	
Water Wash	1.13 [a]	66.67[a]	2.94[b]
Scrub/sanitize	0.94[a]	63.16[a]	0.58[b]
P value	0.53	0.90	< 0.0001
RMSE[f]	2.48	52.81	34.17
Lug (n=306)			
Positive Control	0.82[a]	41.94[b]	
Water Wash	0.28[a]	64.41[a]	1.24[b]
Scrub/sanitize	0.58[a]	48.86[ab]	0.43[b]
P value	0.22	0.02	< 0.0001
RMSE[f]	0.91	22.8	14.96

Least squares means within a column and piece of equipment with different letters (*a-b*) differ ($P < 0.05$).

[c]Prior to allergen exposure.

[d]After allergen exposure, but before treatment.

[e]Allergen remaining after treatment.

[f]RMSE=Root mean square error from Analysis of Variance Table.

TABLE 3. *Effect of treatments on the amount (ppm) of allergen remaining on the injector, tumbler, and lug.*

	Veratox		
Equipment/Treatment	Before Allergen[c]	After Allergen[d]	After Treatment[e]
Injector (n=324)			
Positive Control	0.44[a]	173.76[a]	
Water Wash	0.38[a]	145.83[a]	0.54[b]
Scrub/sanitize	0.46[a]	179.78[a]	0.71[b]
P value	0.92	0.15	< 0.0001
RMSE[f]	0.59	54.87	57.58
Tumbler (n=228)			
Positive Control	0.50[a]	44.12[b]	
Water Wash	0.27[a]	71.05[a]	0.66[b]
Scrub/sanitize	0.26[a]	46.18[ab]	0.15[b]
P value	0.05	0.40	< 0.0001
RMSE[f]	0.23	23.71	13.77
Lug (n=324)			
Positive Control	0.79[a]	79.08[a]	
Water Wash	4.13[a]	87.72[a]	0.94[b]
Scrub/sanitize	0.56[a]	111.59[a]	0.46[b]
P value	0.46	0.29	< 0.0001
RMSE[f]	9.58	63.31	18.15

Least squares means within a column and piece of equipment with different letters (*a-b*) differ ($P < 0.05$).

[c]Prior to allergen exposure.

[d]After allergen exposure, but before treatment.

[e]Allergen remaining after treatment.

[f]RMSE=Root mean square error from Analysis of Variance Table.

Tables 4, 5, and 6 demonstrate the percentages of positive and negative samples for each piece of equipment and treatments using the Alert (qualitative) kit. The amount of residual allergen detected was found to be similar for the grinder, slicer, injector, tumbler, and lugs in Tables 4, 5, and 6. A similar trend was found in the amount of allergen detected after the allergen-containing product was introduced to the processing equipment. The amount of allergen remaining after the treatments were similar for the water wash and scrub/sanitize. The amount of allergen detected in packaging before and after allergen was introduced followed a similar pattern through out packaging and packaging equipment. The amount of allergen found on the personal protective equipment had similar tendencies across all equipment and treatments.

TABLE 4. *Percentages of positive and negative samples for grinder and lug and each treatment.*

Equipment/ treatment	Alert					
	Before allergen		After Allergen		After Treatment	
	negative	positive	negative	positive	negative	positive
Grinder						
Positive control	100.00%		11.10%	88.90%		
Water Wash	100.00%		22.20%	77.80%	88.90%	11.10%
Scrub/ sanitize	100.00%		22.20%	25.93%	94.40%	5.60%
Lug						
Positive control	100.00%			100.00%		
Water Wash	100.00%		11.10%	88.90%	100.00%	
Scrub/ sanitize	100.00%		11.10%	88.90%	100.00%	
Packaging	100.00%		92.85%	7.14%		
Packaging Equipment	100.00%		100.00%			
Contaminated PPE			30.76%	69.23%	96.15%	3.85%

PPE= Personal Protective Equipment

TABLE 5. *Percentages of positive and negative samples for injector, tumbler, and lug for each treatment.*

Equipment/ treatment	Alert					
	Before allergen		After Allergen		After Treatment	
	negative	positive	negative	positive	negative	positive
Injector						
Postive control	90.80%	9.20%		100.00%		
Water wash	100.00%		9.20%	90.80%	94.40%	5.60%
Scrub/ sanitize	94.44%	5.55%		100.00%	100.00%	
Lug						
Positive control	100.00%			100.00%		
Water Wash	100.00%		5.60%	94.44%	94.40%	5.60%
Scrub/ sanitize	100.00%			100.00%	100.00%	
Tumbler						
Positive control	100.00%			61.10%		
Water Wash	100.00%			38.90%	61.10%	
Scrub/ sanitize	100.00%			38.90%	61.10%	
Packaging	100.00%		88.24%	11.76%		
Packaging Equipment	94.12%	5.88%	100.00%			
Contaminated PPE			23.53%	76.47%	83.35%	17.65%

PPE=Personal Protective Equipment

TABLE 6. *Percentages of positive and negative samples for slicer and lug and each treatment.*

Equipment/ treatment	Alert					
	Before allergen		After Allergen		After Treatment	
	negative	positive	negative	positive	negative	positive
Slicer						
Positive control	100.00%			100.00%		
Water Wash	100.00%			100.00%	94.40%	5.60%
Scrub/ sanitize	100.00%		11.10%	88.90%	100.00%	
Lug						
Positive control	100.00%		100.00%			
Water Wash	100.00%		100.00%		100.00%	
Scrub/sanitize	100.00%		100.00%		94.40%	5.60%
Packaging	94.44%	5.56%	22.30%	77.70%		
Packaging Equipment	100.00%		94.40%	5.60%		
Contaminated PPE			22.30%	77.70%	88.90%	11.10%

PPE=Personal Protective Equipment

CHAPTER V

CONCLUSIONS

Food allergens are a serious food safety issue that affect children and adults and that can be life threatening. There are several options that the food industry can implement to control food allergens. This research demonstrates that validated cleaning procedures are effective in removing food allergens from processing equipment of varying complexity. It is imperative that food processors evaluate the efficiency of their cleaning procedures for each type of allergen of concern and all processing equipment involved. All parts of the food service chain must do their part in order for allergen management to be successful and worth the effort.

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APPENDIX A

Veratox®

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Service can be reached between 8 a.m. and 6 p.m. Eastern time by calling 800/234-5333 or 517/372-9200 and asking for a Neogen sales representative or Technical Services. Assistance is available on a 24-hour basis by calling 517/334-0460. Training on this product, and all Neogen test kits, is available.

MSDS INFORMATION AVAILABLE
Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's Web site at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY
Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

TESTING KITS AVAILABLE FROM NEOGEN

Natural Toxins
- Aflatoxin, DON, Ochratoxin, Zearalenone, T-2 Toxin, Fumonisin, Histamine

Foodborne Bacteria
- *E. coli* O157:H7, *Salmonella, Listeria, Listeria monocytogenes, Campylobacter, Staphylococcus aureus, Vibrio parahaemolyticus*

Sanitation
- ATP, Yeast and Mold, Total Plate Count, Generic *E. coli* and Total Coliforms, Protein Residues

Food Allergens
- Peanuts, Milk, Eggs, Almonds, Gliadin, Soy Flour, Hazelnut

Genetic Modification
- CP4 (Roundup Ready®)

Ruminant By-products
- Meat and Bone Meal, Feed

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16021D V-SoyFlour-1107

Product #8490

Read instructions carefully before starting test

Veratox®

Quantitative Soy Flour Allergen Test

REFRIGERATE at 2-8°C (35-46°F) — DO NOT FREEZE

SOY FLOUR ALLERGEN
Food allergens are proteins in food that can create an immune response in sensitive individuals. Once ingested, food allergens can cause a number of reactions, ranging in severity from hives and itching to anaphylaxis. Anaphylaxis is a severe allergic reaction, involving vomiting, diarrhea, difficulty breathing, swelling of the mouth and tongue, and a rapid drop in blood pressure.

An estimated 3.5 to 4 percent of adults, and 6 to 8 percent of children, are sensitive in some degree to food allergens. More than 12 million people in the United States alone are known to have a food allergy.

Food manufacturers protect those with food allergies by clearly labeling their products with a list of ingredients. Testing for the presence of soy flour ensures food manufacturers that an unlabeled—and potentially dangerous—ingredient did not make its way into a food product.

INTENDED USE
Veratox for Soy Flour Allergen is intended for the quantitative analysis of soy flour protein residue in food products such as cookies, crackers, chocolate bars, ice cream and cereals.

INTENDED USER
This test kit is designed for use by quality control personnel and others familiar with foods possibly contaminated by soy flour or soy flour products. Since technique is very important, operators should be trained by a Neogen representative or someone who has completed the Neogen training.

ASSAY PRINCIPLES
The Veratox Soy Flour Allergen test is a sandwich enzyme-linked immunosorbent assay (S-ELISA). Soy flour protein residue is extracted from samples with a buffered salt solution (PBS) by shaking in a heated water bath, followed by centrifugation or filtration. Extracted soy flour protein is sampled and added to antibody-coated wells (capture antibody) where it binds to the antibody during an incubation. Any unbound soy flour protein is washed away and a second antibody (detector antibody), which is enzyme labeled, is added. The detector antibody binds to the already bound soy flour protein. After a second wash, substrate is added. Color develops as a result of the presence of bound detector antibody. Red Stop reagent is added and the color of the resulting solution is observed. The test is read in a microwell reader to yield optical densities. The optical densities of the controls form a standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of soy flour.

STORAGE REQUIREMENTS
The kit can be used until the expiration date on the label when stored refrigerated at 2-8°C (35-46°F).

MATERIALS PROVIDED
1. 48 antibody-coated microwells
2. 48 red-marked transfer wells
3. 5 yellow-labeled bottles of 0, 2.5, 5, 10, 25 ppm soy flour controls
4. 2 blue-labeled bottles of enzyme-labeled antibody conjugate
5. 1 green-labeled bottle of K-Blue® Substrate
6. 1 red-labeled bottle of Red Stop solution
7. Foil pouch of 10 mM PBS dry powder extraction solvent. Each pouch is enough to prepare 1 L in distilled or deionized water (pH 7.4)
8. 40 mL of 10 mM PBS-Tween washing reagent in a wide mouth bottle. Each bottle is enough to prepare 1 L in distilled or deionized water (pH 7.4)
9. 50 grams of extraction additive in a specimen cup
10. Plastic scoop to measure extraction additive

MATERIALS RECOMMENDED BUT NOT PROVIDED
1. Allergen Extraction Kit (Neogen item #8429)
   a. 20 disposable plastic extraction bottles
   b. 20 sample collection tubes (12x75 mm) with caps
2. Shaker water bath capable of maintaining 60°C ± 1° with clamps to hold 250 mL extraction bottles
3. Whatman #4 filters or equivalent
4. Centrifuge (optional)
5. 50-200 µL adjustable pipettor (Neogen item #9276)
6. 12-channel pipettor (Neogen item #9273)
7. Pipette tips (Neogen item #9410)
8. Timer (Neogen item #9426)
9. Microwell reader with a 650 nm filter (Neogen item #9301/9302)
10. 1 L bottle to prepare washing solution (Neogen item #9472)
11. 1 L heat safe bottle to prepare extract solution (Neogen item #9472)
12. Paper towels or equivalent absorbent material
13. Microwell holder (Neogen item #9402)
14. Waterproof marker

15. Wash bottle (Neogen item #9400)
16. Distilled or deionized water
17. 3 reagent boats for 12-channel pipettor (Neogen item #9435)
18. Graduated cylinder capable of measuring 125 mL (Neogen item #9368)
19. Scale capable of weighing 5 ± 0.1 g (Neogen item #9427)

PRECAUTIONS

1. Components of Veratox for Soy Flour Allergen, such as controls and extraction reagents, may contain one or more of the following potentially allergic materials: casein; egg protein; Soy Flour protein; soy protein; tree nut protein. If allergic to any of these compounds, please use caution when using this product.
2. Concentrated food additives, colors and flavors may cause interferences on ELISA test methods. Contact Neogen's technical services for validation information.
3. Hydrolyzed and fermented proteins may not be detected using ELISA methods for allergen testing. Due to the breakdown of the proteins to small peptides or amino acids, they may be come undetectable by this assay, but still could be allergenic and cause an allergic reaction.
4. Store test kit between 2-8°C (35-46°F) when not in use, do not freeze.
5. Bring kits to room temperature (18-30°C, 64-86°F) prior to use.
6. Avoid prolonged storage of kits at ambient temperatures.
7. Do not use kit components beyond expiration date.
8. Do not mix reagents from one kit serial with reagents from a different kit serial.
9. Do not run more than 24 wells per test.
10. Follow proper pipetting techniques (e.g., prime tips and use clean tips).
11. Use only incubation times specified. Others may give inaccurate results.
13. Use clean pipette tips and glassware for each sample to avoid cross-contamination. Thoroughly wash all glassware between samples.

PROCEDURAL NOTES

1. **Substrate.** K-Blue Substrate is ready for use. The substrate should be clear to light blue — discard if it has turned dark blue. Only pour the needed volume of substrate into a reagent boat. **Do not return unused substrate to the bottle.** Cover the reagent boat to keep the substrate protected from light until needed.
2. **Conjugate.** The conjugate supplied with this kit is ready to use. One bottle is enough for 24 wells. Cover the reagent boat to keep the conjugate protected from direct light and contaminants.
3. **Extraction solution.** Prepare extraction solution by adding a foil pouch of extraction solvent, 10mM PBS, to 1 L distilled or deionized water. Swirl to mix thoroughly. Cover and store any unused portions refrigerated at 2-8°C (35-46°F).
4. **Wash buffer.** Prepare the wash buffer solution by pouring all the wash buffer concentrate into an empty 1 L container. Rinse the wash buffer concentrate bottle with distilled or deionized water and pour into the 1 L container to ensure all the concentrate is used. Fill the 1 L container with additional distilled or deionized water, and swirl to assure thorough mixing. Cover and store any unused portions refrigerated at 2-8°C (35-46°F).

NOTE: Discard unused portions of extraction solution and wash buffer when the test kit has been used completely.

5. **Antibody wells.** Keep wells sealed in the foil pouch until needed. Remove wells from the foil pouch only after samples are extracted, and the test procedure is set to begin.

SAMPLE PREPARATION AND EXTRACTION

The sample to be tested should be collected according to accepted sampling techniques (see Neogen's Food Allergen Handbook). The sample should be ground and thoroughly mixed prior to proceeding with the extraction procedure.

1. Prepare the extraction solution as described in the procedural notes.
2. Preheat extraction solution to 60°C (140°F) by immersing the bottle containing the solution into the water bath and allowing it to reach 60°C.
3. Using your sampling and collection procedure, obtain a representative sample and grind it to a very fine particle size.
4. Transfer 5 grams of sample or 5 mL of liquid sample into a 250 mL disposable extraction bottle.
5. Add one level scoop of the extraction additive to the sample bottle.
6. Pour 125 mL of the 60°C (140°F) extraction solution to the sample bottle.
7. Cap the sample bottle to prevent contents from splashing during the extraction.
8. Extract by shaking (150 rpm) in a water bath at 60°C (140°F) for 15 minutes. Remove the bottle from the bath.
9. Let material settle for 5 minutes to enable some of the sample to settle before proceeding to the next step.
10. Filter the extract by pouring at least 5 mL through a Whatman #4 filter and collecting the filtrate as a sample. ALTERNATIVE: Centrifuge at 14,000 rpm for 5 minutes (20 minutes for lower speeds). Use the clear supernatant as a sample.
11. Allow extracts to cool to room temperature before beginning analysis.
12. Discard extracts after completion of analysis.

TEST PROCEDURE

Allow the test kit and all reagents to warm to room temperature (18-30°C, 64-86°F) before using.

1. Remove 1 red-marked mixing well for each sample to be tested plus 5 red-marked wells for controls, and place in the well holder.
2. Remove an equal number of antibody-coated wells. Return antibody wells which will not be used immediately to the foil pack with desiccant. Reseal the foil pack to protect the antibody. Mark one end of the strip with a "1", and place strip in the well holder with the marked end on the left.
3. Mix each reagent by swirling the reagent bottle prior to use.
4. Using a new pipette tip for each, transfer 150 µL of controls and sample extracts to the red-marked transfer wells as shown in the template below. Only run up to two 12-well strips at a time.

0	2.5	5	10	25	S1	S2	S3	S4	S5	S6	S7
S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19

5. Place tips on the 12-channel pipettor and transfer 100 µL of the controls and sample extracts to the antibody-coated wells. Mix for 20 seconds by sliding the well holder back and forth on a flat surface.
6. Incubate microwells **10 minutes** at room temperature (18-30°C, 64-86°F). Discard the red-marked transfer wells.
7. Empty the contents of the wells into a sink. With a wash bottle fill each antibody well with the wash buffer solution and dump out. Repeat the washing 5 times, then turn the wells upside down and tap out on a paper towel until the remaining washing solution is removed.
8. Pour the needed volume of conjugate from the blue-labeled bottle into a clean reagent boat.
9. Using the 12-channel pipettor and new tips, transfer 100 µL of the conjugate into all the wells and mix for 20 seconds by sliding the well holder back and forth on a flat surface.
10. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
11. Wash all wells with the wash buffer solution as described in step 7.
12. Pour the needed volume of substrate solution from the green-labeled bottle into a clean reagent boat.
13. Place new tips on the 12-channel pipettor and transfer 100 µL of substrate into each well and mix for 20 seconds. Do not eject tips.
14. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
15. Pour the needed volume of Red Stop solution from the red-labeled bottle into a clean reagent boat.
16. With the same tips used to dispense the substrate, transfer 100 µL of Red Stop into each well and mix for 20 seconds.
17. Wipe the bottom of the microwells and read in a microwell reader with a 650 nm filter.
18. Interpret the test's results using Neogen's Stat Fax microwell reader, or an equivalent strip reader. If using a strip reader, calculate the results using Neogen's Log/Logit software.

PERFORMANCE CHARACTERISTICS

Limit of quantitation: 2.5 ppm (Described as the lowest concentration point on the calibration curve that this test can reliably detect soy flour allergen.)

Range of quantitation: 2.5 – 25 ppm (For quantitating samples above 25 ppm, contact a Neogen representative for dilution instructions.)

Allergen detection: This test detects soy flour protein and the results are expressed as ppm of soy flour.

NOTE: Due to variations in food additives and commodity compositions, levels below 10 ppm may be considered suitable for research purposes only.

APPENDIX B

Veratox®

Product #8470

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Service can be reached between 8 a.m. and 6 p.m. Eastern time by calling 800/234-5333 or 517/372-9200 and asking for a Neogen sales representative or Technical Services. Assistance is available on a 24-hour basis by calling 800/867-0308. Training on this product, and all Neogen test kits, is available.

MSDS INFORMATION AVAILABLE
Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's Web site at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY
Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

TESTING KITS AVAILABLE FROM NEOGEN

Natural Toxins
- Aflatoxin, DON, Ochratoxin, Zearalenone, T-2 Toxin, Fumonisin, Histamine

Foodborne Bacteria
- *E. coli* O157:H7, *Salmonella, Listeria, Listeria monocytogenes, Campylobacter, Staphylococcus aureus, Vibrio parahaemolyticus*

Sanitation
- ATP, Yeast and Mold, Total Plate Count, Generic *E. coli* and Total Coliforms, Protein Residues

Food Allergens
- Peanuts, Milk, Eggs, Almonds, Gliadin, Soy Flour, Hazelnut

Genetic Modification
- CP4 (Roundup Ready®)

Ruminant By-products
- Meat and Bone Meal, Feed

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16231E V-TotMilkAll-0708

Read instructions carefully before starting test

Veratox®

Total Milk Allergen Quantitative Test

REFRIGERATE at 2-8°C (35-46°F) — DO NOT FREEZE

MILK ALLERGEN
Food allergens are proteins in food that can create an immune response in sensitive individuals. Once ingested, food allergens can cause a number of reactions, ranging in severity from hives and itching to anaphylaxis. Anaphylaxis is a severe allergic reaction, involving vomiting, diarrhea, difficulty breathing, swelling of the mouth and tongue, and a rapid drop in blood pressure.

An estimated 3.5 to 4 percent of adults, and 6 to 8 percent of children, are sensitive in some degree to food allergens. More than 12 million people in the United States alone are known to have a food allergy.

Food manufacturers protect those with food allergies by clearly labeling their products with a list of ingredients. Testing for the presence of milk components ensures food manufacturers that an unlabeled — and potentially dangerous — ingredient did not make its way into a food product.

INTENDED USE
Veratox for Total Milk Allergen is intended for the quantitative analysis of food products, such as juices, cake mixes, cookies, sauces and sorbets, for the presence of milk proteins casein and whey.

INTENDED USER
The Veratox for Total Milk Allergen test kit is designed for use by quality control personnel and others familiar with foods possibly contaminated by casein or whey. Since technique is very important, operators should be trained by a Neogen representative or someone who has successfully completed the Neogen training.

ASSAY PRINCIPLES
Veratox for Total Milk Allergen is a sandwich enzyme-linked immunosorbent assay (S-ELISA). Milk protein residue is extracted from samples with a buffered salt solution (PBS) by shaking in a heated water bath. Extracted milk protein residue is sampled and added to antibody-coated wells (capture antibody) where it binds to the antibody during an incubation. Any unbound protein residue is washed away and a second antibody (detector antibody), which is enzyme labeled, is added. The detector antibody binds to the already bound milk protein residue. After a second wash, the substrate is added. Color develops as a result of the presence of bound detector antibody. Red Stop reagent is added and the color of the resulting solution is observed. The test is read in a microwell reader to yield optical densities. The optical densities of the controls form a standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of allergen.

STORAGE REQUIREMENTS
The kit can be used until the expiration date on the label when stored refrigerated at 2-8°C (35-46°F).

MATERIALS PROVIDED
1. 48 antibody-coated microwells (24 per pouch)
2. 48 red-marked transfer wells (24 per pouch)
3. 5 yellow-labeled bottles of 0, 2.5, 5, 10 and 25 ppm milk protein controls
4. 4 blue-labeled bottles of enzyme-labeled antibody conjugate
5. 1 green-labeled bottle of K-Blue® Substrate
6. 1 red-labeled bottle of Red Stop solution
7. 5 foil pouches of 10 mM PBS dry powder extraction solvent; each pouch contains enough powder to prepare 1 L of extraction solvent
8. 2 wide-mouth bottles of 40 mL PBS-Tween wash buffer concentrate; each bottle contains enough concentrate to prepare 1 L of wash buffer
9. 50 g of extraction additive in a specimen cup
10. Plastic scoop to measure extraction additive

MATERIALS RECOMMENDED BUT NOT PROVIDED
1. Allergen Extraction Kit (Neogen item #8429)
   a. 20 disposable plastic extraction bottles
   b. 20 disposable transfer pipettes
2. Shaker water bath adjusted to 60°C ± 1° (140°F) with clamps to hold extraction bottles
3. Pipettor, adjustable 50-200 µL (Neogen item #9276)
4. Pipettor, 1 mL (Neogen item #9418)
5. Pipette tips (Neogen item #9410)
6. Pipettor, 12-channel (Neogen item #9273)
7. Timer (Neogen item #9426)
8. Microwell strip reader with a 650 nm filter (Neogen item #9302)
9. 1 L bottle to prepare washing solution (Neogen item #9472)
10. 1 L heat safe bottle to prepare extract solution (Neogen item #9472)
11. Paper towels or equivalent absorbent material
12. Microwell holder (Neogen item #9402)
13. Waterproof marker
14. Wash bottle (Neogen item #9400)

15. Distilled or deionized water
16. 3 reagent multichannel pipettor boats (Neogen item #9435)
17. Graduated cylinder capable of measuring 125 mL
18. Scale capable of weighing 5 g (Neogen item #9435)

PRECAUTIONS

1. Samples intended to be tested for **milk must be extracted separately** from samples intended to be tested for other food allergens, such as peanut and egg residues. The extraction additives for each type of test are designed specifically for the target food allergen.
2. The controls and extraction reagents of the Veratox for Total Milk Allergen test kit may contain one or more of the following potentially allergic materials: milk, egg protein, peanut protein, soy protein, or tree nut protein. If allergic to any of these compounds, please use caution when using this product.
3. Concentrated food additives, colors and flavors may cause interferences on ELISA test methods. Contact Neogen's technical services for validation information.
4. Hydrolyzed and fermented proteins may not be detected using ELISA methods for allergen testing. Due to the nature of the proteins it may be undetectable in the assay, but there could still be active allergenic protein residue present.
5. Infant formula – Contact Neogen for additional information concerning the testing of infant formulas.
6. Disposable extraction bottles and tubes must be used to extract milk samples to avoid cross-contamination.
7. The testing area must be totally free of milk products. A minute amount of milk protein in the environment can affect test results.
8. Store test kit between 2-8°C (35-46°F) when not in use. Do not freeze test kits.
9. Do not use kit components beyond expiration date.
10. Sample extracts must be cooled to room temperature (18-30°C, 64-86°F) prior to use.
11. Bring kits to room temperature (18-30°C, 64-86°F) prior to use.
12. Do not mix reagents from kit serial with reagents from a different kit serial.
13. Do not run more than 24 wells per test.
14. Follow proper pipetting techniques (e.g. prime tips and use clean tips).
15. Use only the incubation times specified. Others may give inaccurate results.
16. Avoid prolonged storage of kits at ambient temperatures.

PROCEDURAL NOTES

1. **Substrate**: K-Blue Substrate is ready for use. The substrate should be clear to light blue — discard if it has turned dark blue. Only pour the needed volume of substrate into a reagent boat. **Do not return unused substrate to the bottle.** Cover the reagent boat to keep the substrate protected from light until it is needed.
2. **Extraction solution.** Prepare extraction solution by adding a foil pouch of extraction solvent, 10mM PBS, to 1 L distilled or deionized water at pH 7.4. Cover and store unused portion, refrigerated at (2-8°C, 35-46°F).

3. **Wash buffer.** Prepare wash buffer solution by mixing 40 mL of wash buffer concentrate in wide mouth bottle into 960 mL of distilled or deionized water at pH 7.4. Cover and store unused portion refrigerated (2-8°C, 35-46°F).
   NOTE: Discard unused portions of extraction solution and wash buffer when test kit has been used completely.
4. **Antibody wells.** Keep wells sealed in the foil pouch until needed. Remove wells from the foil pouch only after samples are extracted, and the test procedure is set to begin.

SAMPLE PREPARATION AND EXTRACTION

The sample to be tested should be collected according to accepted sampling techniques (see Neogen's Food Allergen Handbook). The sample should be ground and thoroughly mixed prior to proceeding with the extraction procedure.

NOTE: *Glassware and material used for peanut and egg allergen testing cannot be used for milk residue testing due to the potential of cross-contamination. For this reason it is also highly recommended that any labware, such as wash bottles, graduated cylinders, and 1 L bottles, be solely dedicated for use with the milk allergen kit.*

1. Prepare the extraction solution (PBS, pH 7.4) as described in the procedural notes.
2. Preheat extraction solution to 60°C (140°F) by immersing the bottle containing the solution into the water bath and allowing it to reach 60°C.
3. Using your sampling and collection procedure, obtain a representative sample and grind it to a very fine particle size.
4. Transfer 5 grams of sample, or 5 mL of liquid sample, into a 250 mL disposable plastic extraction bottle.
5. Add one level scoop of the extraction additive into the sample bottle. (Do not use the extraction additive from another allergen test kit.)
6. Pour 125 mL of the 60°C (140°F) extraction solution into the sample bottle.
7. Cap the sample bottle to prevent contents from splashing during the extraction.
8. Extract by shaking (150 rpm) in a 60°C water bath for 15 minutes. Remove the bottle from the bath.
9. Let the material settle for 5 minutes before proceeding to the next step.
10. Use the supernatant (the top liquid portion of the extract) as your sample. **Do not filter**. Begin the test procedure once the sample has cooled to room temperature (at least 15 minutes).

TEST PROCEDURE

Allow the test kit and all reagents to warm to room temperature (18-30°C, 64-86°F) before using.

1. Mix each reagent by swirling the reagent bottle prior to use.
2. Using a new pipette tip for each, transfer 150 µL of controls and sample extracts to the red-marked transfer wells as shown in the template below. Run only two 12-well strips at a time.

0	2.5	5	10	25	S1	S2	S3	S4	S5	S6	S7
S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19

3. Place tips on the 12-channel pipettor and transfer 100 µL of the controls and sample extracts to the antibody-coated wells. Mix for 10 seconds by sliding the microwell holder back and forth on a flat surface.
4. Incubate microwells **10 minutes** at room temperature (18-30°C , 64-86°F). Discard the red-marked transfer wells.
5. Empty the contents of the wells into a sink. With a wash bottle, fill each antibody well with the wash buffer solution and dump out. Repeat the **washing 10 times**, then turn the wells upside down and tap out on a paper towel until the remaining washing solution is removed.
6. Pour the needed volume of conjugate from the blue-labeled bottle into a clean reagent boat.
7. Using the 12-channel pipettor transfer 100 µL of the conjugate into all the wells and mix for 10 seconds by sliding the microwell holder back and forth on a flat surface.
8. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
9. Wash all wells with the wash buffer solution as described in step 5.
10. Pour the needed volume of substrate solution from the green-labeled bottle into a clean reagent boat.
11. Place new tips on the 12-channel pipettor and transfer 100 µL of substrate into each well and mix for 10 seconds by sliding the microwell holder back and forth on a flat surface.
12. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
13. Pour the needed volume of Red Stop solution from the red-labeled bottle into a clean reagent boat.
14. Place new tips on the 12-channel pipettor and transfer 100 µL of Red Stop into each well and mix for 10 seconds by sliding the microwell holder back and forth on a flat surface.
15. Wipe the bottom of the microwells with a dry cloth or towel and read in a microwell reader with a 650 nm filter. Air bubbles should be eliminated, as they could affect analytical results. Results should be read within 20 minutes after the addition of Red Stop.
16. Read and calculate the test's results using Neogen's Stat Fax microwell reader, or an equivalent strip reader. If using a strip reader, calculate the results using Neogen's Log/Logit software.

PERFORMANCE CHARACTERISTICS

Limit of detection: 1 ppm (described as the lowest concentration point on the calibration curve that this test can reliably detect milk protein.)
Range of quantitation: 2.5 – 25 ppm (for quantitating samples above 25 ppm, contact a Neogen representative for dilution instructions.)
Allergen detection: This test detects casein and whey proteins from cow, goat and sheep and the results are expressed as ppm of non fat dried milk (NFDM).

NOTE: Due to variations in food additives and commodity compositions, levels below 10 ppm may be considered suitable for research purposes only.

APPENDIX C

ALERT®

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Service can be reached between 8 a.m. and 6 p.m. Eastern Time by calling 800/234-5333 or 517/372-9200 and asking for a Neogen sales representative or Technical Services. Assistance is available on a 24-hour basis by calling 800/867-0308. Training on equipment use for all Neogen test kits is available.

MSDS INFORMATION AVAILABLE
Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's Web site at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY
Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

TESTING KITS AVAILABLE FROM NEOGEN

Natural Toxins
- Aflatoxin, DON, Ochratoxin, Zearalenone, T-2 Toxin, Fumonisin, Histamine

Foodborne Bacteria
- *E. coli* O157:H7, *Salmonella, Salmonella enteritidis, Listeria, Listeria monocytogenes, Campylobacter, Staphylococcus aureus, Vibrio parahaemolyticus*

Sanitation
- ATP, Yeast and Mold, Total Plate Count, Generic *E. coli* and Total Coliforms, Protein Residues

Food Allergens
- Peanuts, Total Milk, Eggs, Almonds, Gliadin, Soy Flour, Hazelnut

Genetic Modification
- CP4 (Roundup Ready®)

Ruminant By-products
- Meat and Bone Meal, Feed

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16020B A-Soy Flour-0508

Product #8491

Read instructions carefully before starting test

ALERT®

Soy Flour Allergen Screening Test

REFRIGERATE at 2-8°C (35-46°F) — DO NOT FREEZE

SOY FLOUR
Food allergens are proteins in food that can create an immune response in sensitive individuals. Once ingested, food allergens can cause a number of reactions, ranging in severity from hives and itching to anaphylaxis. Anaphylaxis is a severe allergic reaction, involving vomiting, diarrhea, difficulty breathing, swelling of the mouth and tongue, and a rapid drop in blood pressure.

An estimated 3.5 to 4 percent of adults, and 6 to 8 percent of children, are sensitive in some degree to food allergens. More than 12 million people in the United States alone are known to have a food allergy.

Food manufacturers protect those with food allergies by clearly labeling their products with a list of ingredients. Testing for the presence of unprocessed soy flour ensures food manufacturers that an unlabeled — and potentially dangerous — ingredient did not make its way into a food product.

INTENDED USE
Alert for Soy Flour is intended for the qualitative analysis of soy flour protein residue in food products such as cookies, crackers, meat, and cereals and on environmental surfaces.

INTENDED USER
This test kit is designed for use by quality control personnel and others familiar with foods possibly contaminated by soy flour or soy flour products. Since technique is very important, operators should be trained by a Neogen representative or someone who has successfully completed the Neogen training.

ASSAY PRINCIPLES
Alert for Soy Flour is a sandwich enzyme-linked immunosorbent assay (S-ELISA). Soy flour protein residue is extracted from samples by blending with a hot buffered salt solution (PBS). Extracted soy flour protein is sampled and added to antibody-coated wells (capture antibody) where it binds to the antibody during an incubation. Any unbound soy flour protein is washed away and a second antibody (detector antibody), which is enzyme labeled, is added. The detector antibody binds to the already bound soy flour protein. After a second wash, the substrate is added. Color develops as a result of the presence of bound detector antibody. Red Stop reagent is added and the color of the resulting solution is observed.

STORAGE REQUIREMENTS
The kit can be used until the expiration date on the label when stored refrigerated at 2-8°C (35-46°F).

MATERIALS PROVIDED
1. 24 antibody-coated microwells
2. 2 yellow-labeled dropper bottles of controls; one bottle at a concentration of 5 ppm and one at 10 ppm soy flour.
3. 1 blue-labeled dropper bottle of enzyme-labeled antibody conjugate
4. 1 green-labeled dropper bottle of K-Blue® Substrate solution
5. 1 red-labeled dropper bottle of Red Stop solution
6. 20 unlabeled empty sample droppers with tips
7. 3 foil pouches of 10 mM PBS dry powder extraction solvent; each pouch contains enough powder to prepare 1 L of extraction solution
8. 1 wide-mouth bottle of 10 mM PBS-Tween wash buffer concentrate; a bottle contains enough concentrate to prepare 1 L of wash buffer
9. Extraction additive in a specimen cup
10. Plastic 1 gram scoop to measure extraction additive

MATERIALS RECOMMENDED BUT NOT PROVIDED
1. High-speed blender with a 250 mL jar
2. Scale capable of weighing 5 ± 0.1 grams (Neogen item #9427)
3. Shaker water bath, hot plate or equivalent heat source capable of maintaining 60°C ± 1°
4. Thermometer
5. Whatman #4 filter paper or equivalent

6. Two 1 L bottles to prepare washing and extraction solutions (Neogen item #9472)
7. Paper towels or equivalent absorbent material
8. Microwell holder (Neogen item #9402)
9. Waterproof marker
10. Wash bottle (Neogen item #9400)
11. Distilled or deionized water
12. Timer (Neogen item #9426)
13. Graduated cylinder capable of measuring 125 mL (Neogen item #9368)
14. Allergen Environmental Swabbing kit (Neogen #8432)

PRECAUTIONS

1. Samples intended to be tested for **soy must be extracted separately** from samples intended to be tested for other food allergens, such as peanut, egg and milk residues. The extraction additives for each type of test are designed specifically for the target food allergen.
2. Components of the Alert for Soy Flour test kit, such as controls and extraction reagents, may contain one or more of the following potentially allergic materials: milk, egg protein, peanut protein or soy protein. If allergic to any of these compounds, please use caution when using this product.
3. Concentrated food additives, colors and flavors may cause interferences on ELISA test methods. Contact Neogen's technical services for validation information.
4. Hydrolyzed and fermented proteins may not be detected using ELISA methods for allergen testing. Due to the nature of the proteins it may be undetectable in the assay, but there could still be active allergenic protein residue present.
5. The testing environment should be clean and dust-free.
6. Store test kit between 2-8°C (35-46°F) when not in use, do not freeze.
7. Bring kits to room temperature (18-30°C, 64-86°F) prior to use.
8. Avoid prolonged storage of kits at ambient temperatures.
9. Do not use kit components beyond expiration date.
10. Do not mix reagents from kit serial with those from a different serial.
11. Do not run more than 6 wells per test.
12. Use only incubation times specified. Others may give inaccurate results.

PROCEDURAL NOTES

1. **Extraction solution.** Prepare extraction solution by adding a foil pouch of extraction solvent, 10mM PBS, to 1 L distilled or deionized water. Swirl to mix thoroughly. Cover and store any unused portions refrigerated at 2-8°C (35-46°F).
2. **Wash buffer.** Prepare the wash buffer solution by pouring all the wash buffer concentrate into an empty 1 L bottle. Rinse the wash buffer concentrate container with distilled or deionized water and pour into the 1 L bottle to ensure all the concentrate is used. Fill the 1 L bottle with additional distilled or deionized water, and swirl to assure thorough mixing. Cover and store any unused portions refrigerated at 2-8°C (35-46°F).
   NOTE: Discard unused portions of extraction solution and wash buffer when the test kit has been used completely.

3. **Antibody wells.** Keep wells sealed in the foil pouch until needed. Remove wells from the foil pouch only after samples are extracted, and the test procedure is set to begin.

SAMPLE PREPARATION AND EXTRACTION

The sample to be tested should be collected according to accepted sampling techniques (see Neogen's Food Allergen Monitoring Handbook). The sample should be ground and thoroughly mixed prior to proceeding with the extraction procedure. **NOTE:** For environmental sampling, refer to the Allergen Environmental Swabbing Kit, available from Neogen.

1. Prepare the extraction solution as described in the procedural notes.
2. Preheat the extraction solution to 60°C (140°F).
3. Obtain a representative sample. If the sample is of a larger particle size, grind it to a very fine particle size.
4. Transfer 5 grams of sample, or 5 mL of liquid sample, to a 250 mL plastic bottle or blender jar.
5. Add one level scoop of soy extraction additive to the plastic bottle or blender jar. (Do not use the extraction additive from another allergen test kit.)
6. Pour 125 mL of the 60°C (140°F) extraction solution into the plastic bottle or blender jar and cap.
7. Extract by shaking (150 rpm) in a waterbath at 60°C (140°F) for 15 minutes. Remove bottle from bath. **Alternative:** Blend at high speed for 2 minutes.
8. Let the material settle for 5 minutes. *Note: In rare cases, filtering may be necessary to achieve a supernatant free of suspended material. Filter the extract by pouring at least 5 mL through a Whatman #4 filter and collecting the filtrate as a sample, or centrifuging at 14,000 rpm for 5 minutes.*
9. Using a disposable pipette, transfer approximately 1 mL of the supernatant (the top liquid portion of the extract) to a sample dropper bottle. Then, label and place a dropper tip on the bottle.
10. Begin the test procedure once the sample has cooled to room temperature (at least 15 minutes).

TEST PROCEDURE

Allow the test kit and all reagents to warm to room temperature (18-30°C, 64-86°F) before using.

1. Remove 1 well for each sample to be tested plus 1 well for the control, and place into the well holder.
2. Mix each reagent by swirling its dropper bottle prior to use.
3. Add 3 drops from the yellow-labeled control dropper bottle to the first well. Add three drops from each sample extract to a respective well as indicated in the template below. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface.

   Control S1 S2 S3 S4 S5
4. Incubate microwells **10 minutes** at room temperature (18-30°C, 64-86°F).

5. Shake out the contents of the wells. Using a wash bottle filled with wash buffer, fill each well and shake out. Repeat 10 times. Remove excess wash buffer by turning wells upside down and vigorously tapping wells on absorbent towel.
6. Add 3 drops from the blue-labeled conjugate dropper bottle to each well. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface.
7. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
8. Shake out the contents of the wells. Using a wash bottle filled with wash buffer, fill each well and shake out. Repeat 10 times. Remove excess wash buffer by turning wells upside down and vigorously tapping wells on absorbent towel.
9. Add 3 drops from the green-labeled substrate dropper bottle to each well. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface.
10. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
11. Add 3 drops from the red-labeled Red Stop dropper bottle to each well. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface. The results are now ready to be interpreted.

INTERPRETATION OF RESULTS

Visually compare the color of a sample well to the color of the control well. If the sample well has **more blue color** than the control well, the sample tests positive for soy flour contamination of **more than 5 ppm**. If the sample well has **less blue color, or more red color,** than the control well, the sample contains **less than 5 ppm** of soy flour contamination.

Alternative: Read wells (wipe bottom of wells first) in a microwell reader with a 650 nm filter. If the sample well has an optical density (OD) higher than the control well, the sample is positive for soy flour contamination of more than 5 ppm. If the sample well has an OD lower than the control well, the sample contains less than 5 ppm of soy flour contamination.

PERFORMANCE CHARACTERISTICS

Allergen detection: This test kit detects soy flour protein and the results are expressed in terms of the detection of soy flour.

APPENDIX D

ALERT®

Product #8471

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Service can be reached between 8 a.m. and 6 p.m. Eastern Time by calling 800/234-5333 or 517/372-9200 and asking for a Neogen sales representative or Technical Services. Assistance is available on a 24-hour basis by calling 800/867-0308. Training on this product and all Neogen test kits is available.

MSDS INFORMATION AVAILABLE
Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's Web site at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY
Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

TESTING KITS AVAILABLE FROM NEOGEN

Natural Toxins
- Aflatoxin, DON, Ochratoxin, Zearalenone, T-2 Toxin, Fumonisin, Histamine

Foodborne Bacteria
- *E. coli* O157:H7, *Salmonella, Salmonella enteritidis, Listeria, Listeria monocytogenes, Campylobacter, Staphylococcus aureus, Vibrio parahaemolyticus*

Sanitation
- ATP, Yeast and Mold, Total Plate Count, Generic *E. coli* and Total Coliforms, Protein Residues

Food Allergens
- Peanuts, Milk, Eggs, Almond, Gliadin, Soy Flour, Hazelnut

Genetic Modification
- CP4 (Roundup Ready®)

Ruminant By-products
- Meat and Bone Meal, Feed

NEOGEN CORPORATION
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e-mail: foodsafety@neogen.com • www.neogen.com

16230C A-TotMilkAll-0708

Read instructions carefully before starting test

ALERT®

Total Milk Allergen Screening Test

*REFRIGERATE at 2-8°C (35-46°F) — **DO NOT FREEZE***

MILK ALLERGEN
Food allergens are proteins in food that can create an immune response in sensitive individuals. Once ingested, food allergens can cause a number of reactions, ranging in severity from hives and itching to anaphylaxis. Anaphylaxis is a severe allergic reaction, involving vomiting, diarrhea, difficulty breathing, swelling of the mouth and tongue, and a rapid drop in blood pressure.

An estimated 3.5 to 4 percent of adults, and 6 to 8 percent of children, are sensitive in some degree to food allergens. More than 12 million people in the United States alone are known to have a food allergy.

Food manufacturers protect those with food allergies by clearly labeling their products with a list of ingredients. Testing for the presence of milk residue ensures food manufacturers that an unlabeled—and potentially dangerous—ingredient did not make its way into a food product.

INTENDED USE
Alert for Total Milk Allergen is intended to be used to screen food, such as juices, sauces and sorbets, or environmental surfaces for the presence of the milk components, casein and whey.

INTENDED USER
The test kit is designed for use by quality control personnel and others familiar with foods possibly contaminated by casein or whey. Since technique is very important, operators should be trained by a Neogen representative or someone who has successfully completed the Neogen training.

ASSAY PRINCIPLES
Alert for Total Milk Allergen is a sandwich enzyme-linked immunosorbent assay (S-ELISA). Milk protein residue is extracted from samples by blending with a hot buffered salt solution (PBS). Extracted milk protein is sampled and added to antibody-coated wells (capture antibody) where it binds to the antibody during an incubation. Any unbound milk protein is washed away and a second antibody (detector antibody), which is enzyme labeled, is added. The detector antibody binds to the already bound milk protein. After a second wash, substrate is added. Color develops as a result of the presence of bound detector antibody. Red Stop reagent is added and the color of the resulting solution is observed.

STORAGE REQUIREMENTS
The kit can be used until the expiration date on the label when stored refrigerated at 2-8°C (35-46°F).

MATERIALS PROVIDED
1. 24 antibody-coated microwells
2. 2 yellow-labeled bottles of controls made from nonfat dried milk; 1 bottle at a concentration of 5 ppm and 1 bottle at 10 ppm *(see procedural note No. 1)*
3. 1 blue-labeled bottle of enzyme-labeled antibody conjugate
4. 1 green-labeled dropper bottle of K-Blue® Substrate solution
5. 1 red-labeled dropper bottle of Red Stop solution
6. 20 unlabeled empty sample dropper bottles with tips
7. 3 foil pouches of 10 mM PBS dry powder extraction solvent; each pouch contains enough powder to prepare 1 L of extraction solution
8. 1 wide-mouth bottle of 40 mL PBS-Tween wash buffer concentrate; a bottle contains enough concentrate to prepare 1 L of wash buffer
9. 50 grams of extraction additive in a specimen cup
10. Plastic 1 gram scoop to measure extraction additive

MATERIALS RECOMMENDED BUT NOT PROVIDED
1. Allergen Extraction Kit (Neogen item #8429)
   a. 20 disposable extraction bottles
   b. 20 disposable transfer pipettes
2. Scale capable of weighing 5 ± 0.1 grams (Neogen item #9427)
3. Shaker water bath, hot plate or equivalent heat source capable of maintaining 60°C ± 1°
4. Thermometer
5. Two 1 L bottles to prepare washing and extraction solutions (Neogen item #9472)
6. Paper towels or equivalent absorbent material
7. Microwell holder (Neogen item #9402)
8. Waterproof marker
9. Wash bottle (Neogen item #9400)
10. Distilled or deionized water
11. Timer (Neogen item #9426)
12. Graduated cylinder capable of measuring 125 mL
13. Pipettor, 100 µL (Neogen item #9272, 9278 or 9276)
14. Pipette tips (Neogen item #9410)
15. Vortex
16. 50 mL sample tubes

PRECAUTIONS

1. Samples intended to be tested for **milk must be extracted separately** from samples intended to be tested for other food allergens, such as peanut and egg residues. The extraction additives for each type of test are designed specifically for the target food allergen.
2. Components of Alert for Total Milk Allergen, such as controls and extraction reagents, may contain one or more of the following potentially allergic materials: milk, egg protein, peanut protein or soy protein. If allergic to any of these compounds, please use caution when using this product.
3. Concentrated food additives, colors and flavors may cause interferences on ELISA test methods. Contact Neogen's technical services for validation information.
4. Hydrolyzed and fermented proteins may not be detected using ELISA methods for allergen testing. Due to the nature of the proteins it may be undetectable in the assay, but there could still be active allergenic protein residue present.
5. Infant formula – Contact Neogen for additional information concerning the testing of infant formulas.
6. Disposable extraction bottles and transfer pipettes must be used to extract milk samples to avoid cross-contamination.
7. The testing environment should be clean and dust-free.
8. Store test kit between 2-8°C (35-46°F) when not in use. Do not freeze
9. Do not use kit components beyond expiration date.
10. Do not mix reagents from kit serial with those from a different serial.
11. Do not run more than 6 wells per test.
12. Use only the incubation times specified. Others may give inaccurate results.
13. Bring kits to room temperature (18-30°C, 64-86°F) prior to use.
14. Avoid prolonged storage of kits at ambient temperatures.

PROCEDURAL NOTES

1. **Controls.** To increase the flexibility of this test kit, two test controls are supplied—5 ppm and 10 ppm. **Choose one control** level to screen samples against for each test performed.
2. **Extraction solution.** Prepare extraction solution by adding a foil pouch of extraction solvent, 10mM PBS, to 1 L distilled or deionized water at pH 7.4. Cover and store unused portion refrigerated at 2-8°C (35-46°F).
3. **Wash buffer.** Prepare the wash buffer solution by pouring all the wash buffer concentrate into an empty 1 L bottle. Rinse the wash buffer concentrate container with distilled or deionized water and pour into the 1 L bottle to ensure all the concentrate is used. Fill the 1 L bottle with additional distilled or deionized water, and swirl to assure thorough mixing. Cover and store any unused portions refrigerated at 2-8°C (35-46°F).
   NOTE: Discard unused portions of extraction solution and wash buffer when the test kit has been used completely.
4. **Antibody wells.** Keep wells sealed in the foil pouch until needed. Remove wells from the foil pouch only after samples are extracted, and the test procedure is set to begin.

SAMPLE PREPARATION AND EXTRACTION

The sample to be tested should be collected according to accepted sampling techniques (see Neogen's Food Allergen Monitoring Handbook). For environmental sampling, refer to the Allergen Environmental Swabbing Kit, available from Neogen.

NOTE: *Glassware and material used for other allergen testing cannot be used for milk residue testing due to the potential of cross-contamination. For this reason, it is highly recommended that any labware, such as wash bottles, graduated cylinders or 1 L bottles, are dedicated solely for use with the milk allergen kit.*

1. Prepare the extraction solution as described in the procedural notes.
2. Preheat extraction solution to 60°C (140°F).
3. Using your sampling and collection procedure, obtain a representative sample and grind it to a very fine particle size.
4. Transfer 5 grams of sample, or 5 mL of liquid sample, into a 250 mL disposable plastic extraction bottle.
5. Add one level scoop of the milk extraction additive into the sample bottle. (Do not use the extraction additive from another allergen test kit.)
6. Pour 125 mL of the 60°C (140°F) extraction solution into the sample bottle.
7. Cap the sample bottle to prevent contents from splashing during the extraction.
8. Extract by shaking (150 rpm) in a 60°C water bath for 15 minutes.
9. Remove the bottle from the bath and let the material settle for 5 minutes.
10. Using a disposable pipette, transfer approximately 1 mL of the supernatant (the top liquid portion of the extract) to a sample dropper bottle. Then, label and place a dropper tip on the bottle.
11. Begin the test procedure once the sample has cooled to room temperature (at least 15 minutes).

ALTERNATE EXTRACTION

1. Prepare the extraction solution as described in the procedural notes.
2. Prepare a working solution by adding 125 mL of extraction solution into container and adding one scoop of extraction additive and mix.
3. Preheat the working solution to 60°C (140°F) by using a hot plate, incubator, microwave, water bath or equivalent. Use a thermometer to assure temperature is correct.
4. Transfer 1 gram of sample, or 1 mL of liquid sample, into a 50 mL tube.
5. Pour 25 mL of the 60°C (140°F) working extraction solution into the sample tube and cap.
6. Extract by vortexing for 3 minutes. Use caution the liquid is hot.
7. Let the material settle for 5 minutes.
8. Using a disposable pipette, transfer approximately 1 mL of the supernatant (the top liquid portion of the extract) to a sample dropper bottle. Then label and place a dropper tip on the bottle.
9. Begin the test procedure once the sample has cooled to room temperature (at least 15 minutes).

TEST PROCEDURE

Allow the test kit and all reagents to warm to room temperature (18-30°C, 64-86°F) before using.

1. Remove 1 well from the foil pouch for each sample to be tested plus 1 well for the control, and place into the well holder.
2. Mix each reagent by swirling the reagent bottle prior to use.
3. Pipette 100 µL from a yellow-labeled control bottle *(choose the 5 ppm or 10 ppm control supplied—see procedural note No.1)* to the first well. Add three drops, or pipette 100 µL from each sample extract to a respective well as indicated in the template below. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface.

   Control S1 S2 S3 S4 S5

4. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
5. Shake out the contents of the wells. Using a wash bottle filled with wash buffer, fill each well and shake out. **Repeat 10 times.** Remove excess wash buffer by turning wells upside down and vigorously tapping wells on an absorbent towel.
6. Pipette 100 µL from the blue-labeled conjugate bottle to each well. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface.
7. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
8. Shake out the contents of the wells. Using a wash bottle filled with wash buffer, fill each well and shake out. **Repeat 10 times.** Remove excess wash buffer by turning wells upside down and vigorously tapping wells on an absorbent towel.
9. Add 3 drops from the green-labeled substrate dropper bottle to each well. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface.
10. Incubate for **10 minutes** at room temperature (18-30°C, 64-86°F).
11. Add 3 drops from the red-labeled Red Stop dropper bottle to each well. Mix for 20 seconds by sliding the microwell holder back and forth on a flat surface. The results are now ready to be interpreted.

INTERPRETATION OF RESULTS

Visually compare the color of a sample well to the color of the control well. If the sample well has **more blue color** than the control well, the sample tests positive for milk protein contamination of **more than 5 ppm (or 10 ppm, depending on the control used)**. If the sample well has **less blue color, or more red color,** than the control well, the sample contains **less than 5 ppm (10 ppm)** of milk protein contamination.

Alternative: Read wells (wipe bottom of wells first) in a microwell reader with a 650 nm filter. If the sample well has an optical density (OD) higher than the control well, the sample is positive for milk contamination of more than 5 ppm (10 ppm). If the sample well has an OD lower than the control well, the sample contains less than 5 ppm (10 ppm) of milk protein contamination.

PERFORMANCE CHARACTERISTICS

Allergen detection: This test detects casein and whey proteins from cow, goat and sheep. The results are expressed in terms of detection of non-fat dried milk (NFDM).

VITA

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