United States Department of Agriculture

Food Safety and Inspection Service

CLG-ALG1.03

Screening and Confirmation of Allergens by XMAP® Food Allergen Detection Assay

This is the laboratory procedure for screening and confirming 14 allergens as well as gluten in Ready-To-Eat (RTE) products.

Executive Summary

This is a screening and confirmation method for allergens in Ready-To-Eat (RTE) products. The method utilizes the xMAP® Food Allergen Detection Assay Kit (xMAP® FADA) for analysis of 14 allergens (peanut, almond, Brazil nut, cashew, coconut, hazel nut, macadamia nut, pine nut, pistachio nut, walnut, soy, milk, egg, gluten, and crustaceans) as well as gluten in RTE products. Allergen screening levels can be found in Table 16 and are set below the confirmation minimum level of applicability (MLA), the lowest level at which an FSIS method has been validated, to ensure all potential violations are detected. Confirmation MLAs for all allergens are 5 ppm.

Notice of Change

The method was revised with several updates that only impact the work performed by laboratory analysts and does not impact how samples are collected or how results are reported.

The updated version has an improved introduction section that provides additional scientific information describing the xMAP® Food Allergen Detection Assay Kit for food allergen analysis. The method overview has also been enhanced to provide greater clarity to processes involved in the assay.

Other changes improve the method's efficiency for analyzing samples within the FSIS laboratory environment. The extraction and analysis procedure clarifies the preparation of positive controls and includes an additional standard level to improve the screening step and reduce turn around times.

Table of Contents

Safety Precautions

The personnel performing the analysis are to read the Safety Data Sheets for the standards and reagents used in this method. The hazards and recommended safe procedures for use are listed in Table 17. Follow all applicable federal, state, and local regulations regarding the disposal of chemicals listed in this method.

Introduction

Food allergies affect about 2 percent of adults and 4 to 8 percent of children in the United States. Each year in the U.S., it is estimated that anaphylaxis related to food allergens results in 30,000 emergency room visits, 2,000 hospitalizations, and 150 deaths.[1] Under the Federal Meat Inspection Act (FMIA), the Poultry Products Inspection Act (PPIA), and Egg Products Inspection Act (EPIA) under which the Food Safety and Inspection Service (FSIS) operates, all ingredients used to formulate a meat, poultry, or egg product must be declared in the ingredients statement on product labeling.[2]

Ensuring that allergens found in ingredient statements are accurately detected in ready-to-eat (RTE) products is a critical task. Whether it's a peanut or a trace of soy in a hot dog, a single allergen could trigger serious health issues for sensitive consumers. Although several methods have been developed for single allergen detection (i.e., PCR and mass spectrometry methods), the xMAP® Food Allergen Detection Assay Kit is a universally recognized method that is suitable for routine regulatory enforcement.[4,5] The xMAP® FADA takes into consideration several factors such as complex proteins, multiple allergens, cross reactivity, additives, fats, sugars, and other ingredients that may be present in RTE products such as raviolis and hotdogs.

KEY DEFINITIONS

Food Allergy: A food allergy is a specific type of adverse food reaction involving the immune system. The body produces what is called an allergy, or immunoglobulin E (IgE) antibody, to a food (allergen). Once a specific food is ingested and binds with the IgE antibody, an allergic reaction ensues.

Antibody: Immunoglobulin (Ig) protein that is Y-shaped and can specifically bind to a foreign agent such as an allergen.[3]

- Complex proteins such as allergen proteins bind to specific antibodies. During food processing, complex proteins may undergo protein denaturation due to heat, pH changes, or other manufacturing conditions. Protein denaturation is a structural transformation that results in partial folding or total unfolding of a protein. As a result, these changes alter the protein's structure and allergen proteins become less recognizable to bind to antibodies. To handle this possible structural alteration, or denaturation, the xMAP® FADA incorporates two different extraction procedures through different buffers to detect both "non-denatured" (unaltered) and "denatured" proteins.
  - A buffered detergent extraction is used for non-denatured proteins, which are relatively intact and easy for the antibodies to bind.
    - When plant material is not present, Phosphate Buffered Saline and Tween 20 (PBST) is used.
    - When plant material is present, a complex buffer made up of 105 mM Sodium Phosphate/ 75 mM NaCl/ 2.5% nonfat dry milk/ 0.05% Tween-20 (UD) is used.
  - A reduced denatured extraction is used for denatured proteins, which may have been altered by heat, pressure, or other processing conditions. The reduced-denatured extraction uses stronger buffers and denaturing agents to break down the matrix and release allergen proteins from the food, even if they've been structurally altered.
- Processed foods often contain complex mixtures of ingredients such as spices, additives, and proteins that can cause false positives by interacting with the detection antibodies. The xMAP® FADA includes various washing steps to remove unbound substances, while blocking agents in the buffer reduce background interference.

[1] https://www.fsis.usda.gov/food-safety/safe-food-handling-and-preparation/food-safety-basics/food-allergies
[2] https://www.fsis.usda.gov/policy/food-safety-acts/federal-meat-inspection-act
[3] Hage DS, Carr JD, *Analytical Chemistry and Quantitative Analysis.* Pearson, 2010
[4] Cho, CY, Nowatzke, W, Oliver, K, Garber, EAE. (2015) Multiplex detection of food allergens and gluten. Anal Bioanal Chem 407:4195
[5] Garber, EAE, Cho, CY, Rallabhandi R, Nowatzke, WL, Oliver, KG, Venkateswaran, KV, Venkateswaran N. (2020) Multi-laboratory validation of the xMAP–Food Allergen Detection Assay: A multiplex, antibody-based assay for the simultaneous detection of food allergens. PLOS One https://doi.org/10.1371/journal.pone.0234899
[6] Lepock, JR. (2005) Measurement of protein stability and protein denaturation in cells using scanning calorimetry. Methods 35:117

- For redundancy of most allergens, a dual-bead approach is incorporated into the analysis where each allergen is detected using two separate bead sets. Only if both beads show a consistent signal can the allergen be confirmed, helping to reduce false positives due to cross-reactivity. An exception is crustacean, where only a single allergen bead set is used.
- To ensure accuracy of analyzing allergens, the xMAP® FADA includes the use of calibration standards as well as negative and positive controls. These quality control samples allow the system to distinguish between actual allergen signals and any noise that might be present in the sample matrix. If the allergen concentration is close to the detection limit, the dual-bead method, along with the calibration data and quality control checks ensure that true positives are accurately identified.

Method Overview

The following method utilizes the xMAP® Food Allergen Detection Assay Kit to analyze allergens in Ready-to-Eat (RTE) products. The method is applicable for the 14 most common food allergens (peanut, soy, milk, egg, crustaceans, and various tree nuts) as well as gluten in RTE products. This procedure provides a powerful tool for both screening and confirming allergen presence, with a detection sensitivity of 5 parts per million (ppm).

In brief, the RTE product is carefully processed into a homogeneous mixture, which helps to guarantee that the allergens, if present, are evenly distributed and ready for extraction. Then the allergens are extracted from the food matrix using different extraction processes depending on sample components. A buffered extraction with either the PBST or UD buffers is used if plant material is present in the sample while a reduced denatured extraction is used for samples with denatured proteins.

The extracted sample is then loaded onto a 96-well plate. A special set of color-coded magnetic beads, each coated with covalently linked antibodies that are tailored to selectively capture a different allergen, are added to the mix (Capture Antibody). The allergens (e.g., peanut, soy, or gluten) in the sample bind to these beads, forming an allergen-antibody complex, as shown in Box 1 of Figure 1.

Next, a secondary antibody with a fluorescent receptor (Detection Antibody) is added. This antibody attaches itself to the allergen proteins bound to the beads, completing a sandwich like structure forming the Detection Complex, as shown in Box 2 of Figure 1.

- For gluten, there are two detection antibodies used to target gluten in its native (non-denatured) and denatured state. These antibodies bind to gluten proteins, primarily epitopes within gliadin proteins, which are a major component of gluten.

A fluorescence dye (Detection Dye) is added and the structure that forms on the beads generates an allergen specific fluorescence signal, as shown in Box 3 of Figure 1.

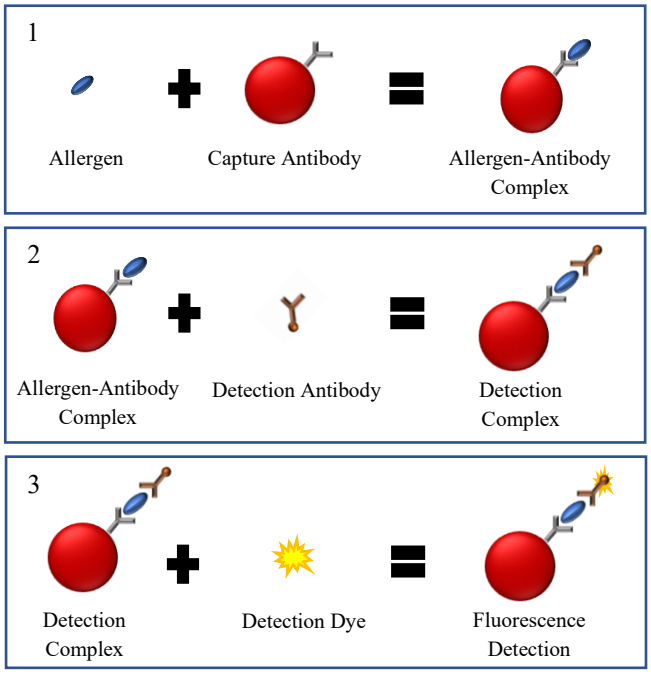

Figure 1: Overview of xMAP® Food Allergen Detection Assay Kit Photos and instrument analysis figure courtesy of Marie Kelleher, USDA-FSIS

The fluorescence response is the key to understanding whether allergens are present and how much of them are in the sample. The system uses a red laser to identify the bead type and tells the system which allergen is present. Then, a green laser measures the intensity of the fluorescence, providing a quantitative reading of how much allergen is bound to the bead.

KEY DEFINITIONS

Fluorescence: The emission of light by a sample after this sample has become electronically excited by the absorption of a photon.

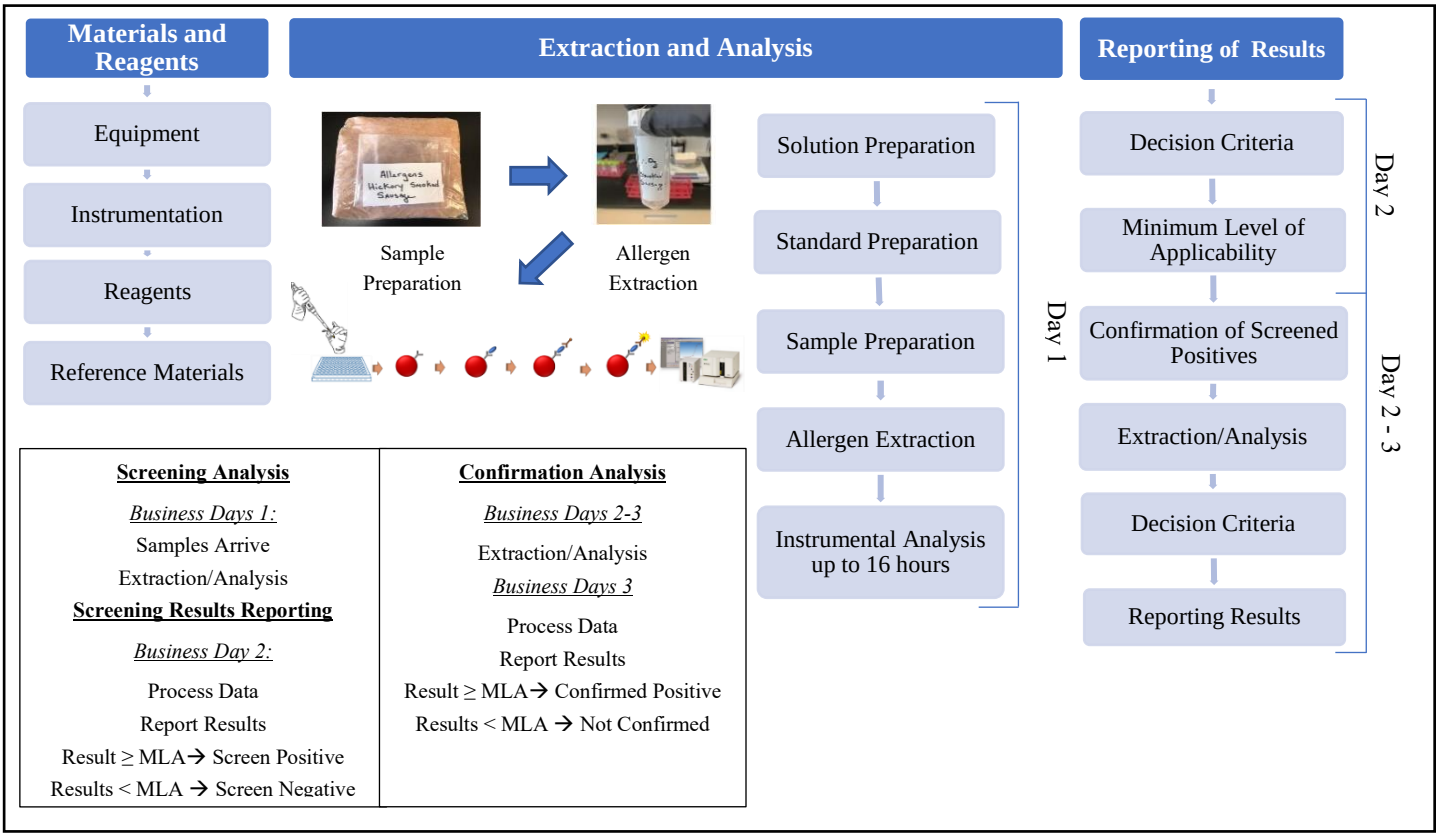

Figure 2: Overview and timeframe of the allergen analysis of Ready-to-Eat samples. Samples arrive at the laboratory on Business Day 1 and are prepared into a homogenized mixture and extracted with a buffer solution. After extraction, allergens are then analyzed the xMAP® Food Allergen Detection Assay Kit and a fluorescence plate reader. Results are reported on Business Day 2. If needed, confirmation would be conducted from Business Days 2-3. This chart represents the best-case scenario, but analyses may take longer due to analytical testing circumstances. Photos and instrument analysis figure courtesy of Marie Kelleher, USDA-FSIS

Decision Criteria

A sample is considered negative if the results are less the than the minimum level of applicability (MLA). A sample is considered positive for allergens if the results are greater than or equal to the screening MLA. The screening MLA is set at a level to ensure that all potential violations are detected. Screened positive results will undergo a second extraction through use of the UD buffer and/or reduced denatured buffer to confirm the allergen at the regulatory action level or MLA of 5 ppm.

KEY DEFINITIONS

MLA: Lowest level at which an FSIS method has been successfully validated for a residue in each matrix. Full definition is on the CLG website.

Disclosure statement

FSIS does not specifically endorse any test products listed in this method. FSIS acknowledges that equivalent equipment, reagents, or solutions may be suitable for laboratory use. The FSIS laboratory system uses method performance requirements when evaluating the equivalence of an alternative equipment, reagent, or solution for a given analyte and sample matrix pair. Significant equivalence changes would require FSIS laboratory leadership approval.

Materials and Reagents

Equipment

Table 1: Equipment

Equipment	Manufacturer and Part Number	Purpose
Forma Orbital Shaker	Thermo Electron Corp., Model 4520	Facilitates extraction of allergens from the sample.
Microcentrifuge capable of 14000 RCF	General Lab Supplier	Separates the solid sample material from the extraction solution.
Ecotherm Orbital Mixing Dry Bath	Torrey Pines Scientific Inc., Model SC25 with round bottom smartblock for 96-well plates	Facilitates extraction of allergens from the sample.
Plate Shaker	General Lab Supplier	Facilitates extraction of allergens from the sample.
96-Well Magnetic Plate	Ambion Inc., 10050	Magnet used to attract magnetic beads.
Vortex Genie 1	Scientific Industries Inc., Model SI-0136	Facilitates extraction of allergens from the sample.
Analytical Balance	General lab supplier	Record weight of standard reagent. Minimum readability ±0.0001 g.
Top Loading Balance	General Lab Supplier	Record weight of quality controls and samples. Minimum readability ±0.01 g.
50 mL Centrifuge tubes	Protein LoBind®, Eppendorf 0030122240	Extraction vessel.
1.5 mL Microcentrifuge Tubes	Thermo Scientific, 90410, Low-protein binding microcentrifuge tubes	Contain standards and sample extracts.
Round Bottom Plates	Fisher Scientific, 07-200-336, 96-well Polystyrene	Container to mix extract with capture beads.
Nalgene Rapid-Flow Filter Units	Thermo Fisher Scientific, 0.2 µm CN membrane 127-0020	Filter solutions.
pH Meter	General Lab Supplier	Check solution pH.
Plate Sealing Film	Bio-Rad, MSA5001	Cover plates and prevent contamination.
Wide-mouth Polypropylene Bottles, 250 mL	General Lab Supplier	Storage of reference stock standards.
Shaking Water bath	General Lab Supplier	Facilitates extraction of allergens from the sample for reduced denatured extraction.
Freezer, -10 °C and -20 °C	General lab supplier	Storage of standards, reagents, and samples
Refrigerator, 2-8 °C	General lab supplier	Storage of standards, reagents, and samples

Repeating pipettes and tips, 2 µL - 20 µL, 20 µL to 1000 µL, 500 µL to 2500 µL	General lab supplier	Dispense standards and reagents.
Serological pipette, disposable, 1/2 mL	General lab supplier	Transfer solutions
Timer	General lab supplier	Measure time
Glassware, Class A	General lab supplier	Measuring standards and reagents

Instrumentation

Table 2: Instrumentation

Instrument	Supplier and Model Number	Purpose
BioPlex Instrument	Bio-Rad Laboratories Inc., Model 200 with Bioplex Manager™	Read MagPlex beads.

Reagents

Table 3: Reagents

Reagent	Supplier and Part Number
xMap® FADA KIT a. 250X MagPlex Capture Bead Set b. 100X AssayCheX™ c. 250X Detection Antibody Set d. 333X SAPE Reporter e. 200x reference material/calibrant solution	Radix Biosolutions Store 200x reference material/calibrant solution at < -20 °C and the rest of the kit at 2-8 °C in the dark.
10 mM Phosphate Buffered Saline (PBS)	Sigma-Aldrich, P3813-10PAK
Tween - 20	Sigma-Aldrich, P9416-100mL
BD Difco™ Skim Milk Powder	Fisher Scientific, DF0032-17-3
Sodium Phosphate Monobasic Monohydrate	Sigma-Aldrich, S9638, Monobasic Monohydrate – ACS Reagent Grade ≥ 98%
Sodium Phosphate Dibasic Anhydrous	SAFC, RES20908, Pharma Grade
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, L6026
Beta-mercaptoethanol	Sigma-Aldrich, M6250

Reference Materials

Table 4: Standards

Standard	Supplier and Part Number
200x reference material/calibrant solution from xMAP® FADA Kit	Radix Biosolutions, KIT Store in freezer at < -20 °C. *Solution expires at kit expiration date.*
Gluten from Wheat[a]	Sigma-Aldrich, G5004-500G
Peanut Flour[b]	NUTS.COM, Peanut Flour 7275
Soy Flour[a]	Sigma-Aldrich, S9633-500G
Whole Egg Powder[b]	Kalustyans.com, Whole Egg Powder House brand
Nonfat Dry Milk Powder[c]	Fisher Scientific, BD Difco™ Skim Milk 232100, Fisher Cat. No. DF0032-17-3
Almond Flour[b]	NUTS.COM, Almond Flour 3030
Brazil Nut Flour[b]	OHNUTS.com, Ground Brazil Nut Flour SKU 1492
Cashew Flour[b]	NUTS.COM, Cashew Flour 3039
Coconut Flour[b]	NUTS.COM, Organic Coconut Flour (Gluten Free) 9953
Crustacean, raw uncooked[b]	General supplier
Hazel Nut Flour[b]	NUTS.COM, Hazelnut Flour 3040
Macadamia Nut[b]	NUTS.COM, Macadamia nut Flour 1020
Pine Nut Flour[b]	NUTS.COM, Mediterranean Pinenuts (Pignolias) 3032
Pistachio Nut Flour[b]	NUTS.COM, Pistachio Flour 3045
Walnut Flour[b]	NUTS.COM, Walnut Meal 9761

[a] Expires after date listed on Certificate of Analysis. Store at < -10 °C.

[b] Expires after one year. Store double bagged at < -10 °C.

[c] Expires after date listed on the Certificate of Analysis. Store at 2-8 °C.

Extraction and Analysis

Solution Preparation

Table 5: Preparation of Solutions

Solution	Procedures
PBST (10 mM PBS/ 0.1% Tween-20[v/v])	1) Pour 1 packet of PBS into 1 L volumetric flask, dissolve with approximately 900 mL deionized water. 2) Add 1 mL of Tween-20 with a pipettor, and mix. 3) Dilute to volume with deionized water and filter using a 0.2 µm CN membrane filter. 4) Store at 2-8 °C. *Solution expires 1 year after preparation.*
200mM Sodium Phosphate	1) Weigh 13.8 g of sodium phosphate monobasic monohydrate and 14.2 g of sodium phosphate dibasic anhydrous and bring to 1 L with deionized water in a 1 L volumetric flask. 2) The pH must be within the range 6.7 to 6.9. 3) Store at 2-8 °C. *Solution expires 1 year after preparation.*
UD Buffer (105 mM Sodium Phosphate/ 75 mM NaCl/ 2.5% nonfat dry milk/ 0.05% Tween-20)	1) Combine 500 mL of PBST, 500 mL of 200 mM sodium phosphate, and 25 g of Difco skim milk powder. 2) Stir until dissolved. 3) Store at 2-8 °C. *Solution expires 1 day after preparation.*
PBS (10 mM PBS):	1) Pour 1 packet of PBS into a 1 L volumetric flask. 2) Add approximately 900 mL deionized water and mix to dissolve. 3) Dilute to volume with deionized water and filter using a 0.2 µm CN membrane filter. 4) Store at 2-8 °C. *Solution expires 1 year after preparation.*
Reduced Denatured Extraction Buffer (0.5% SDS/2% β-mercaptoethanol in PBS)	1) Weigh 1 g of sodium dodecyl sulfate (SDS) then add to a 200 mL volumetric flask. 2) Add about 100 mL of PBS and mix to dissolve. 3) Pipet 4 mL of β-mercaptoethanol into the solution and dilute to volume with PBS. 4) Equilibrate solution to room temperature before use. Stir to redissolve any SDS. 5) Store at 2-8 °C. *Solution expires 1 year after preparation.*

Denatured Diluent (for serial dilution of denatured calibrants)	1) Add 1 mL of reduced denatured extraction buffer to 199 mL of PBST and mix. 2) Store at 2-8 °C. *Solution expires 1 year after preparation.*

Standard Preparation

Table 6: Single-analyte Stock Standards

Solution	Procedure
Single-analyte stock standards solutions.	1) Each single-analyte stock solution is made to 100 mL volume in 250-mL wide-mouth polypropylene bottles using PBST as the diluent. i) amount to weigh (g) = stock concentration in µg/mL / (100 x % protein in reference material) ii) Measure 100 mL PBST gravimetrically (99 – 101 g) 2) Stock solutions are to be stirred continuously for a minimum of 1 hour and 45 minutes. Maintain continuous stirring and aliquot from the single-analyte stock solutions into the reference working solutions in Table 7 within 2 hours and 15 minutes of the start of preparation. *Stock solutions are to be used on the day of preparation.* **(**See Appendix Table 18 for example calculation of standards.)

Table 7: Reference Working Solutions

Solution	Procedure
Four Analyte (Egg, Milk, Peanut, and Soy) Working solution	1) While maintaining continuous stirring, the single-analyte stock standards solutions are aliquoted to make a 100 mL portion of mixed analyte working solution. 2) The working solutions are made in a 250-mL wide-mouth polypropylene bottle with PBST as diluent. 3) Combine aliquots of the four stock solutions and PBST to achieve a final concentration of 11.8 µg/mL and a total solution mass of 100 g. Assume 1 mL weighs 1 g. (See Appendix Table 19 for example calculation.) 4) While keeping the working solution stirred, aliquot into low-protein binding microcentrifuge tubes and freeze. 5) Working solutions in microcentrifuge tubes are single use only. Discard any excess after thawing and spiking. 6) Store in freezer at < -20 °C. *Solution expires 80 days after preparation.*

Tree Nut (Almond, Brazil Nut, Cashew, Coconut, Hazelnut, Macadamia Nut, Pine Nut, Pistachio, and Walnut) Reference Working Solution	1) While maintaining continuous stirring, the single-analyte stock standards solutions are aliquoted to make 100 mL portions of mixed analyte working solutions. 2) The working solutions are made in 250-mL wide-mouth polypropylene bottles with PBST as diluent. 3) Combine aliquots of the nine stock solutions and PBST to achieve a final concentration of 11.8 µg/mL and a total solution mass of 100 g. Assume 1 mL weighs 1 g. (See Appendix Table 20 for example calculation.) 4) While keeping the working solutions stirred, aliquot into low-protein binding microcentrifuge tubes and freeze. 5) Working solutions in microcentrifuge tubes are single use only. Discard any excess after thawing and spiking. 6) Store in freezer at < -20 °C. *Solution expires 30 days after preparation.*
Crustacean Working Solution	1) While maintaining continuous stirring, the single-analyte stock standard solution is aliquoted to make 100 mL portions of the crustacean working solution. 2) The working solutions are made in 250-mL wide-mouth polypropylene bottles with PBST as diluent. 3) Combine the amount of stock solution of crustacean and PBST to achieve a final concentration of 11.8 µg/mL and a total solution mass of 100 g. (See Appendix Table 21 for example calculation.) 4) While keeping the working solutions stirred, aliquot into low-protein binding microcentrifuge tubes and freeze. 5) Working solutions in microcentrifuge tubes are single use only. Discard any excess after thawing and spiking. 6) Store in freezer at < -20 °C. *Solution expires 30 days after preparation.*

Gluten Working Solution	1) While maintaining continuous stirring, the single-analyte stock standards solutions are aliquoted to make 100 mL portions of the gluten working solution. 2) The working solutions are made in 250-mL wide-mouth polypropylene bottles with PBST as diluent. 3) Combine the amount of stock solution of gluten and PBST to achieve a final concentration of 11.8 µg/mL and a total solution mass of 100 g. (See Appendix Table 22 for example calculation.) 4) While keeping the working solutions stirred, aliquot into low-protein binding microcentrifuge tubes and freeze. 5) Working solutions in microcentrifuge tubes are single use only. Discard any excess after thawing and spiking. 6) Store in freezer at < -20 °C. *Solution expires 30 days after preparation.*

Table 8: PBST Standard Preparation (All Allergens Except Std 5c and Std 7c for Coconut)

Solution	Procedure
PBST Std 7	1) Add 5 µL of 200x reference material/calibrant solution from xMAP® FADA Kit to 995 µL of PBST in a 1.5 mL microcentrifuge tube. 2) Vortex. 3) Store at 2-8 °C. *Solution expires 1 week after preparation.*
PBST Standard Calibrants	1) Standards 4 and 5 are prepared from PBST Std 7 as shown in the Table 9. 2) Pipet into 1.5 mL microcentrifuge tubes. 3) Vortex. 4) Store at 2-8 °C. *Solution expires 1 week after preparation.*

Table 9: PBST Standards 4 and 5 Preparation

Standard	µL of PBST Std 7	µL of PBST
Std 5	278	622
Std 4	154	746
Std 0	0	400

(See Appendix Table 23 for Standard Calibrants Equivalent Spike Levels in PBST Buffer Extract)

Table 10: PBST Standard Preparation for Coconut

Solution	Procedure
PBST Std 7c	1) Add 5 µL of 200x reference material/calibrant solution from xMAP® FADA Kit to 384 µL of PBST in a 1.5 mL microcentrifuge tube. 2) Vortex. 3) Store at 2-8 °C. *Solution expires 1 week after preparation.*
PBST Std 5c	1) Add 133 µL of Std 7c to 267 µL of PBST in a 1.5 mL microcentrifuge tube. 2) Vortex. 3) Store at 2-8 °C. *Solution expires 1 week after preparation.*

(See Appendix Table 23 for Standard Calibrants Equivalent Spike Levels in PBST Buffer Extract)

Sample Preparation

Samples must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (≤ -10 °C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, temper (partially thaw) the sample while keeping it as cold as possible. Hardware used for grinding, blending, and cutting is to be washed with dish or lab-grade detergent before and after being used for the processing of allergen samples. Grind sample in the blender or vertical cutter-mixer until homogeneous. Store homogenized samples frozen at < -10 °C.

Figure 3: Homogenized RTE sample. Photo courtesy of Marie Kelleher, USDA-FSIS

Allergen Extraction

Samples

Weigh 1.00 ± 0.05 g of homogenized sample into a labeled 50 mL centrifuge tube, as shown in Figure 4.

QUALITY CONTROL

For Screening

1. Weigh two 1.00 ± 0.05 g portions of blank tissue. One for the blank (negative control) and one for the recovery (positive control). Weigh one additional portion for a check sample, if necessary.
2. Prepare the positive control by fortifying sample at 5 ppm by spiking 425 µL of the working solutions.

For Confirmation

1. Weigh two 1.00 ± 0.05 g portions of blank tissue. One for the blank (negative control) and one for the Direct Comparison Control (positive control). Weigh one additional portion for a check sample, if necessary.
2. Prepare the positive control by fortifying sample at 5 ppm by spiking 425 µL of the working solutions.

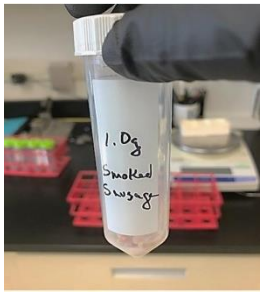

Figure 4: Weighed homogenized sample. Photo courtesy of Marie Kelleher, USDA-FSIS

KEY DEFINITIONS

Blank (negative control): A sample that is negative of all analytes.

Recovery (positive control): A sample that is prepared by the addition of analytes that have a concentration level comparable to the MLA. Samples are compared to the recovery.

Direct Comparison Control: A previously analyzed negative control sample with a matrix similar to the suspect positive sample.

Extraction of Allergens

1) Samples are extracted using different extraction assays based on the materials or ingredients present in the sample.

If plant material present:		If no plant material present:
UD Buffer Assay:	Reduced Denatured Assay:	PBST Assay:
Screen and confirmation of samples except milk	Screen and confirmation of sample containing milk	Screen and confirmation of samples
i. Add 40 mL of UD buffer to each tube and vortex thoroughly. For the positive controls and check samples subtract the volume fortified from the amount of buffer to be added so the total volume remains 40 mL. ii. Place the tubes on the orbital shaker for approximately 2 hours at room temperature. Then remove, vortex, and proceed to Step 2.	i. Add 10 mL PBST to each tube and vortex thoroughly. For the positive controls and check samples subtract the volume fortified from the amount of buffer to be added so the total volume remains 10 mL. ii. Then add 10mL reduced denatured extraction buffer and vortex again. iii. Place the tubes in a shaking water bath for 30 min at 60 ± 1 °C at approximately 150 RPM and proceed to Step 2.	i. Add 20 mL PBST to each tube and vortex thoroughly. For the positive controls and check samples subtract the volume fortified from the amount of buffer to be added so the total volume remains 20 mL. ii. Place the tubes on the orbital shaker for approximately 2 hours at room temperature. Then remove, vortex, and proceed to Step 2.

2) Transfer 1 mL from each sample to a clean 1.5 mL microcentrifuge tube.

3) Centrifuge for 5 min at 14,000 rcf.

Analysis of Allergens

1) Prepare allergens standards for use. The PBST standards have a 1-week expiration and therefore have the option to be prepared ahead of time. UD buffer and reduced-denatured extraction have only 1-day expiration and are to be prepared the day of use.
   - If using PBST, prepare standard calibrants as in Tables 8 through 10.
   - If using UD buffer, prepare standard calibrants as in Tables 11 through 13.
   - If using reduced-denatured extraction, prepare standards as in Tables 14 and 15.

Table 11: Preparation of UD Standards (All Allergens Except Std 5c and Std 7c for Coconut)

Solution	Procedure
UD Std 7	1) Add 5 µL of 200x reference material/calibrant solution from xMAP® FADA Kit to 995 µL of UD buffer in a 1.5 mL microcentrifuge tube. 2) Vortex. *Solution expires 1 day after preparation.*
UD Standard Calibrants	1) Standards 4 and 5 are prepared from UD Std 7 as shown in the Table 12. 2) Pipet into 1.5 mL microcentrifuge tubes. 3) Vortex. *Solution expires 1 day after preparation.*

Table 12: UD Standard 4 and 5 Preparation

Standard	µL of UD Std 7	µL of UD Buffer
Std 5	278	622
Std 4	154	746
Std 0	0	400

(See Appendix Table 24 for Standard Calibrants Equivalent Spike Levels in UD Buffer Extract)

Table 13: UD Standard Preparation for Coconut

Solution	Procedure
UD Std 7c	1) Add 5 µL of 200x reference material/calibrant solution from xMAP® FADA Kit to 384 µL of UD in a 1.5 mL microcentrifuge tube. 2) Vortex. 3) Store at 2-8 °C. *Solution expires 1 day after preparation.*
UD Std 5c	1) Add 133 µL of Std 7c to 267 µL of UD in a 1.5 mL microcentrifuge tube. 2) Vortex. 3) Store at 2-8 °C. *Solution expires 1 day after preparation.*

(See Appendix Table 24 for Standard Calibrants Equivalent Spike Levels in UD Buffer Extract)

Table 14: Preparation of Reduced Denatured Buffer Standards

Solution	Procedure
1:1 calibrator: reduced-denatured extraction buffer	1) Combine 10 µL of 200x calibrator and 10 µL reduced-denatured extraction buffer in a low-protein binding 1.5 mL microcentrifuge tube. 2) Vortex 3) Incubate 30 min in a water bath at 60 ± 1 °C with shaking. *Solution expires 1 day after preparation.*
Std 7	1) Add 10 µL of 1:1 calibrator: reduced-denatured extraction buffer to 990 µL of Denatured diluent in a 1.5 mL microcentrifuge tube. *Solution expires 1 day after preparation.*
Reduced Denatured Buffer Standard Calibrants	1) Standards 4 and 5 are prepared from Reduced Denatured STD 7 as shown in the Table 15. 2) Pipet into 1.5 mL microcentrifuge tubes. *Solution expires 1 day after preparation.*

Table 15: Reduced Denatured Buffer Standard 4 and 5 Preparation

Standard	µL of Reduced Denatured STD 7	µL of Denatured Diluent
Std 5	278	622
Std 4	154	746
Std 0	0	400

(See Appendix Table 25 for Standard Calibrants Equivalent Spike Levels in Reduced Denatured Buffer Extract)

2) As depicted in Figure 5, pipet 50 µL each of standards, controls, and samples into individual wells in a clean 96-well round bottom plate skipping every other column to reduce cross contamination.

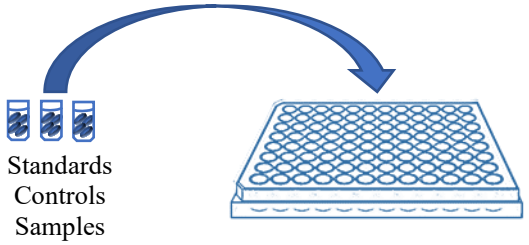

Figure 5: Transfer standards, samples, and controls to wells. Figure courtesy of Marie Kelleher, USDA-FSIS

3) Add 50 µL of the capture bead mix to each filled well, as demonstrated in Figure 6.

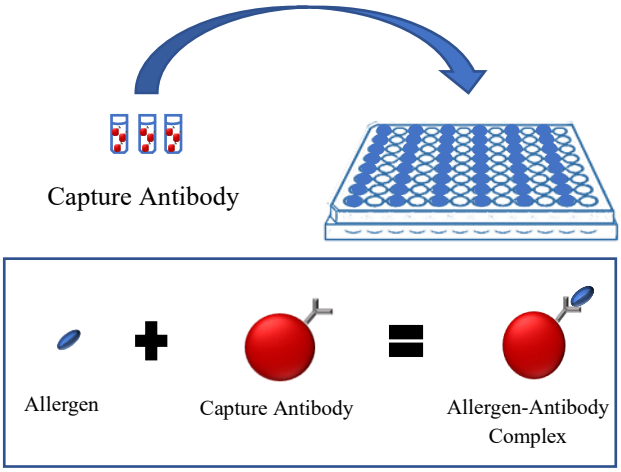

Figure 6: Transfer capture antibody to wells and incubate. Figure courtesy of Marie Kelleher, USDA-FSIS

4) Cover plate with sealing film and incubate in the dark for 30 min on the dry bath at 37 °C at an orbital speed of 600 – 700 rpm.

5) After incubation, attach the round bottom plate to the magnetic plate using rubber bands and wait at least 2 min for the beads to collect at the bottom of the wells. Then carefully discard the liquid by turning the plate assembly upside down using a gentle downward motion. While the plate is still inverted, gently tap it on some clean paper toweling to remove excess liquid.

6) Wash each well with 200 µL PBST three times utilizing the magnetic plate. Discard the liquid each time in the same manner as in step 5.

Capture Bead Mix

1. Calculate the volume of Capture Bead Mix needed using the equations at the bottom of this box.
2. Add the calculated volume of buffer to a 2 mL vial.
3. Add the calculated amounts of capture bead concentrates to vial.
4. Add the calculated amount of 100X AssayCheX™ concentrate to vial.
5. Cap and vortex.
6. Refrigerate until use.
7. Store at 2-8°C.

Solution expires after one day

Calculations

1) A = No. of wells x 55 µL

Where:

A = Total volume in µL needed for Capture Bead Mix solution.

50 µL is required per well. 55 µL is used here to account for possible losses during preparation.

2) Calculate the volume of PBST or UD buffer, capture bead concentrate, and AssayCheX™ concentrate needed using the equations below.

B = A / 250

C = A / 100

E = A - [(B x D) + C]

Where:

B = Volume in µL needed of each capture bead concentrate.

C = Volume in µL needed of AssayCheX™ concentrate.

D = Total number of capture bead concentrates.

E = Volume in µL needed of PBST (or UD Buffer) for capture bead solution.

KEY FACTS

Magnets will attract paramagnetic beads bound to the allergen-antibody complex, leaving contaminants behind in the supernatant. Avoid disrupting beads during PBST washes.

7) As depicted in Figure 7, add 50 µL of the Detection antibody mix to each well and cover plate with sealing film. Incubate in the dark for 30 min at 37 °C at an orbital speed of 600 – 700 rpm.

Figure 7: Transfer detection antibody to wells and incubate. Figure courtesy of Marie Kelleher, USDA-FSIS

Detector antibody solution

1) Calculate the volume of Detector Antibody solution using the equations found in this box.
2) Add the calculated volume of PBST, UD Buffer, or denatured diluent to a 2 mL vial.
3) Add the calculated amounts of Detector Antibody concentrates to vial.
4) Cap and vortex.
5) Refrigerate until use.

Solution expires after one day. Store at 2-8 °C.

Calculations

1. F = No. of wells x 55 µL

   Where:

   F = Total volume in µL for assay solution

2. Calculate the volume of Detector Antibody solution and buffer needed using the equations below.

   G = F / 250

   I = F – (G x H)

   Where:

   G = Volume in µL needed of each Detector Antibody concentrate.

   H = Total No. of Detector Antibody concentrates

   I = Volume in µL needed of PBST, UD Buffer, or denatured diluent for detector antibody solution

8) After incubation, discard liquid and wash each well with 200 µL PBST three times as in steps 5 and 6.

9) As depicted in Figure 8, add 50 µL of the SAPE mix (Detection Dye) and cover plate with sealing film. Incubate in the dark on the dry bath for 15 min at 37 °C at an orbital speed of 600 – 700 rpm.

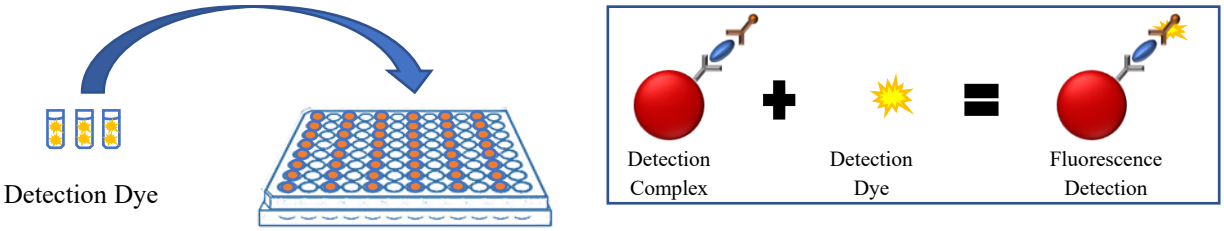

Figure 8: Transfer detection dye to wells and incubate. Figure courtesy of Marie Kelleher, USDA-FSIS

SAPE solution	**Calculations**
1) Calculate the volume of SAPE solution needed using the equations to the right. 2) Add the calculated volume of PBST to a 2 mL vial. 3) Add the calculated amount of 333X SAPE Reporter concentrate to vial. 4) Cap and vortex.	1. J = No. of wells x 55 µL Where: J = Total volume in µL needed for assay solution 2. Calculate the volume of 333X SAPE Reporter concentrate and PBST needed using the equations below. K = J / 333 L = J – K Where: K = Volume in µL needed of 333X SAPE Reporter concentrate L = Volume in µL needed of PBST

10) After incubation, wash each well with 200 µL PBST three times as in steps 5 and 6.

11) Reconstitute standards, samples, and controls with 100 µL PBST and cover plate with sealing film. Mix the solution on the plate shaker until homogenous, as demonstrated in Figure 9.

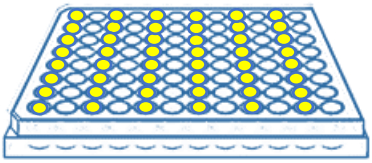

Figure 9: Reconstitute and analyze. Figure courtesy of Marie Kelleher, USDA-FSIS

12) Analyze the plate on an instrument capable of reading MagPlex® beads for fluorescence detection.

Instrumental Analysis

Plate Reader Parameters

1) Bead Map: 100 per region
2) Bead Type: Bio-Plex MagPlex® Beads (Magnetic)
3) Sample size: 50 µL
4) Sample timeout: 45 sec
5) PMT: Low
6) Doublet Discriminator Gates: Low 5000 High 25000
7) Wash: before and after plate run

Figure 10: Fluorescence plate reader. Photo courtesy of Marie Kelleher, USDA-FSIS

Sample Set

The injection sequence below can be modified, as needed, but must include the controls.

Screening Set:

1) Standard curve (Std 0, Std 4, Std 5, Std7, Std 5c (if applicable), Std 7c (if applicable))
2) Negative control
3) Intra-Laboratory Check Sample (If applicable)
4) Positive control
5) Samples, up to a maximum of 40
6) Replating of standard or positive control

INTRA-LABORATORY CHECK SAMPLE
Defined on the CLG website.

Confirmation Set:

1) Standards (Std 0, Std 4, Std 5, Std 7, Std 5c (if applicable), Std 7c (if applicable))
2) Negative control
3) Intra-Laboratory Check Sample (If applicable)
4) Positive control (Direct Comparison Control-DCC)
5) Samples, up to a maximum of 40
6) Replating of a standard or the positive control (DCC)

Reporting of Results

Decision Criteria

Screening

A sample is screened as positive for an allergen if the following criteria are met:

1) The bead count for each bead type must be at least 10.
2) For all allergens, the sample must have a bead response that is greater than or equal to the lowest standard (Std 4) on all beads of interest.
3) The allergen of interest must meet positive screening criteria in the positive control and bracketing quality control sample.
4) The allergen of interest must not meet positive screening criteria in the negative control.

KEY DEFINITION

Bead Count: The number of color-coded magnetic beads with covalently linked antibodies that are specific to an allergen.
Bead Response: Fluorescence intensity (of sample or standard) minus reagent blank (S0).

QUALITY CONTROL

Quality Control Procedures for Screening

For set acceptance:

1) Allergen response in the standards must increase with increasing allergen amount.
2) 90% of the monitored analytes in the recovery (positive control) must meet screening criteria.
3) 90% of the monitored analytes in the blank (negative control) must be negative using the screening criteria.
4) The response in the negative control must be less than 50% of the response in the positive control.

Intra-Laboratory Check Samples (If applicable)

1) Acceptability criteria.
    a. 90% of the monitored analytes in a fortified Intra-Laboratory Check must meet screening criteria.
    b. 90% of the monitored analytes in an unfortified Intra-Laboratory Check must be negative using the screening criteria.
    c. FSIS Field Service Laboratories are to refer to internal FSIS Quality Control Procedures when unacceptable values are obtained:
        i. Refer to LW-Q1002, Chemistry Non-Conformance Tables, for how to proceed and whether to take corrections or corrective actions.

Confirmation

A sample is confirmed as positive for an allergen if the following criteria are met:

1) The bead count for each bead type must be at least 10.
2) For all allergens, the positive control (spiked DCC) must have a bead response that is greater than or equal to the lowest standard (Std 4) on all beads of interest.
3) The allergen of interest must meet positive confirmation criteria in the positive control and bracketing quality control sample.
4) The sample must have a bead response that is greater than or equal to the spiked DCC for all beads for the allergen of interest.
5) The allergen of interest must not meet positive control (spiked DCC) criteria in the negative control.

QUALITY CONTROL

Quality Control Procedures for Confirmation

For set acceptance,

1) The allergen response in the standards must increase with increasing allergen amount.
2) 90% of the monitored analytes in the spiked DCC (positive control) must meet confirmation criteria.
3) 90% of the monitored analytes in the blank (negative control) must be negative using the confirmation criteria.
4) The response in the blank must be less than 50% of the response in the spiked DCC for the beads of interest.

Intra-Laboratory Check Samples (If applicable)

1) Acceptability criteria.
    a. 90% of the monitored analytes in a fortified Intra-Laboratory Check must meet screening criteria.
    b. 90% of the monitored analytes in an unfortified Intra-Laboratory Check must be negative using the screening criteria.
    c. FSIS Field Service Laboratories are to refer to internal FSIS Quality Control Procedures when unacceptable values are obtained:
        i. Refer to LW-Q1002, Chemistry Non-Conformance Tables, for how to proceed and whether to take corrections or corrective actions.

Minimum Level of Applicability (MLA)

1) The screening levels for the allergens are listed in Table 16.
2) The confirmation MLA for all allergens in this method is 5 ppm.

Table 16: Allergen Screening Level

Allergen	Screening level (µg protein / g food)		
	PBST extraction	UD Extraction	Reduced Denatured Extraction
Egg	0.677	1.355	N/App
Gluten	0.429	0.857	N/App
Milk	0.169	N/App	0.169
Peanut	0.180	0.360	N/App
Soy	1.072	2.143	N/App
Almond	0.339	0.677	N/App
Brazil Nut	0.339	0.677	N/App
Cashew	0.054	0.107	N/App
Coconut	0.074	0.149	N/App
Crustacean	1.800	3.601	N/App
Hazelnut	0.135	0.271	N/App
Macadamia	0.564	1.129	N/App
Pine Nut	0.941	1.881	N/App
Pistachio	0.339	0.677	N/App
Walnut	1.072	2.143	N/App

References

1) Nowatzke, WL, Oliver, KG, Cho, CY, Ralladbhandi, R, Garber, EAE. (2019) Single Laboratory Validation of the Multiplex xMAP Food Allergen Detection Assay (xMAP FADA) with Incurred Food Samples. J. Agric. Food Chem., 67:484
2) Garber, EAE, Cho, CY, Rallabhandi R, Nowatzke, WL, Oliver, KG, Venkateswaran, KV, Venkateswaran N. (2020) Multi-laboratory validation of the xMAP–Food Allergen Detection Assay: A multiplex, antibody-based assay for the simultaneous detection of food allergens. PLOS One https://doi.org/10.1371/journal.pone.0234899

Safety Hazards

Table 17: Safety Hazards and Recommended Safe Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Allergen Standards	Some individuals may have allergic reactions, which may cause skin and respiratory irritation.	Wear personal protective equipment, avoid skin contact. Handle with extreme caution. Work in a well-ventilated area.
Beta-mercaptoethanol	Potent reducing agent. Poisonous and can cause irritation to mucous membranes.	Wear personal protective equipment, avoid skin contact. Handle with extreme caution. Work in a well-ventilated area.

Contact Information and Inquiries

Inquiries about methods can be submitted through the FSIS website via the AskFSIS or please contact:

Chemistry Section
Laboratory Quality Assurance, Response, and Coordination Staff
USDA/FSIS/OPHS
950 College Station Road
Athens, GA 30605
OPHS.LQAD@usda.gov

This method has been validated, reviewed, approved, and deemed suitable and fit for purpose for use in the USDA FSIS Field Service Laboratories.

William K. Shaw, Jr., PhD

Executive Associate for Laboratory Services

Example Calculations for Standard Prepared from Reference Materials

Table 18: Single-analyte Stock Standards Preparation

Analyte	Stock solution concentration (µg/mL)	% Protein in Reference material (example)[1]	Amount to weigh for 100 mL of stock solution (g) (example)[2,3]
Egg	3960	47.0	0.843
Gluten	2500	76.0	0.329
Milk	980	36.0	0.272
Peanut	1050	50.0	0.210
Soy	6260	56.0	1.118
Almond	990	7.14	1.386
Brazil Nut	1980	14.29	1.386
Cashew	320	17.86	0.179
Coconut	440	14.29	0.308
Crustacean	2100	15.93	1.318
Hazelnut	800	14.29	0.560
Macadamia nut	825	6.67	1.238
Pine Nut	2740	19.0	1.442
Pistachio	1980	20.83	0.951
Walnut	1565	13.33	1.174

[1] Example values for % protein in reference material. Data should be taken from Certificate of Analysis or ingredient label.

[2] Example values only; amount to weigh (g) = stock concentration in µg/mL / (100 x % protein)

[3] Measure 100 mL PBST gravimetrically (99 – 101 g).

Table 19: Four Analyte (Egg, Milk, Peanut, and Soy) Reference Working Solution Preparation

Analyte	Amount of Stock Solution (µL)	Working Solution Concentration (µg/mL)
Egg	297	11.8
Milk	1200	11.8
Peanut	1121	11.8
Soy	188	11.8

Add 97.2 mL PBST gravimetrically (96.2 – 98.2 g). See Table 18 for stock solution concentrations.

Table 20: Tree Nut Reference Working Solution Preparation

Analyte	Amount of Stock Solution (µL)	Working Solution Concentration (µg/mL)
Almond	1190	11.8
Brazil Nut	595	11.8
Cashew	3681	11.8
Coconut	2677	11.8
Hazelnut	1473	11.8
Macadamia nut	1428	11.8
Pine Nut	430	11.8
Pistachio	595	11.8
Walnut	753	11.8

Add 87.2 mL PBST gravimetrically (86.2 – 88.2 g). See Table 18 for stock solution concentrations.

Table 21: Crustacean Reference Working Solution Preparation

Analyte	Amount of Stock Solution (µL)	Working Solution Concentration (µg/mL)
Crustacean	561	11.8

Add 99.4 mL PBST gravimetrically (98.4 – 100.4 g). See Table 18 for stock solution concentrations.

Table 22: Gluten Reference Working Solution Preparation

Analyte	Amount of Stock Solution (µL)	Working Solution Concentration (µg/mL)
Gluten	471	11.8

Add 99.5 mL PBST gravimetrically (98.5 – 100.5 g). See Table 18 for stock solution concentrations.

Table 23: PBST Standard Calibrants Equivalent Spike Levels in PBST Buffer Extract

Reference Standard	µg protein / g Food in PBST Buffer		
	STD 7	STD 5	STD 4
Egg	3.950	1.219	0.677
Gluten	2.500	0.772	0.429
Milk	0.988	0.304	0.169
Peanut	1.050	0.324	0.180
Soy	6.250	1.930	1.072
Almond	1.975	0.610	0.339
Brazil Nut	1.975	0.610	0.339
Cashew	0.313	0.097	0.054
Coconut	1.110	0.370	0.074
Crustacean	10.500	3.240	1.800
Hazelnut	0.790	0.243	0.135
Macadamia	3.292	1.015	0.564
Pine Nut	5.486	1.694	0.941
Pistachio	1.975	0.610	0.339
Walnut	6.250	1.930	1.072

Table 24: Standard Equivalent Spike Levels in UD Buffer Extract

Reference Standard	µg protein / g Food in UD Buffer		
	STD 7	STD 5	STD 4
Egg	7.900	2.439	1.355
Gluten	5.000	1.543	0.857
Peanut	2.100	0.648	0.360
Soy	12.500	3.857	2.143
Almond	3.950	1.219	0.677
Brazil Nut	3.950	1.219	0.677
Cashew	0.626	0.193	0.107
Coconut	2.235	0.745	0.149
Crustacean	21.000	6.482	3.601
Hazelnut	1.580	0.488	0.271
Macadamia	6.583	2.032	1.129
Pine Nut	10.972	3.386	1.881
Pistachio	3.950	1.219	0.677
Walnut	12.500	3.857	2.143

Table 25: Standard Equivalent Spike Levels in Reduced Denatured Extraction

	µg protein/ g Food		
Reference Standard	STD 7	STD 5	STD 4
Milk	0.988	0.304	0.169