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Microbiology Laboratory Guidebook Notice of Change

Chapter new, revised, or archived: MLG 17.01

Effective Date: 7/3/02

Description and purpose of change(s):

The depletion of stocks of antibody developed by FSIS scientists for use in the determination of animal species content of cooked, red meat and poultry products demanded that the agency look for commercially available reagents. ELISA-TEK® species determination kits have been validated and shown to be equivalent to the method described in Chapter 17 of the Microbiological Laboratory Guidebook, 3rd Edition, 1998. The revised chapter describes how the ELISA-TEK® species determination kits will be used in the FSIS Laboratories to analyze cooked and canned meat and poultry products.

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Procedure Outline

- 17.1 Introduction
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17.1 Introduction

This chapter describes a procedure that can identify the species of animal tissue used as ingredients in cooked and canned meat foods. A simple water extraction of samples along with an amplified double antibody sandwich type enzyme-linked immunosorbent assay (ELISA) is employed. With this type of ELISA, capture antibody is bound to the polystyrene plastic of microplate wells. If tissue antigens are present, they are bound by solid phase, species specific, capture antibody in the wells. After washing to remove unbound material, a biotinylated antibody with the same specificity as the capture antibody is added. Biotin is a vitamin that can be covalently bound to antibody with relative ease and with virtually no deleterious effect on the specificity of the antibody molecule. The biotinylated antibody is bound to the solid phase only if antigens of the species in question were previously captured; otherwise the unbound biotinylated antibody is removed by washing. Streptavidin-Horseradish peroxidase conjugate is then added to the wells. Streptavidin is a glycoprotein which has a remarkable affinity for biotin ($K_D = 10^{-15}M^{-1}$). Any biotin in the solid phase complex will bind the streptavidin-enzyme conjugate. Unbound conjugate is removed by washing and a substrate for the enzyme is added. If the species antigens were present in the test sample, a green color will develop as a result of the action of the bound enzyme on the substrate. This assay allows for high sample output and same day results. The procedure must be repeated for each species in question using the appropriate species specific antibodies.

Antigenic molecules, when subjected to high temperatures (such as those encountered in the production of cooked and canned meat products) will denature. The denaturing process includes a randomization of the tertiary structure and reduced solubility. These changes cause a concomitant reduction in the molecules' antigenicity. Hence, standard immunoassays are usually not possible when samples have been subjected to denaturing heat.

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For development of the present assays, unheated skeletal muscle tissue was fractionated and antigenic molecules were isolated which proved to be relatively heat stable. These antigens are highly soluble, acidic glycoproteins. Antisera prepared to them are species specific and are reactive with extracts of cooked and canned meat products containing that species.

Important note:

One should not attempt to quantify the amount of a particular species tissue in a sample based on this assay. Because the antigens are not completely heat stable, a higher temperature and longer cooking time will result in a lower reaction. Therefore the intensity of the reaction is related to the sample processing as well as to the level of antigen present.

A slight cross reaction occurs among red meat species. The colorimetric detection system of the ELISA does not allow distinction between a low-level homologous reaction and a cross-reacting species. This situation has been addressed by establishing an absorbance value that must be exceeded for a sample to be considered positive. The selected absorbance value is significantly higher than any cross-reaction or background color. **DO NOT** test raw products by this assay, as the cross reactions with raw tissue extracts might exceed the cutoff value.

17.2 Equipment and Supplies

- a. Flow (ICN) Laboratories Titertek Multiskan plate reader, or equivalent
- b. Flow (ICN) Laboratories Titertek Microplate Washer, or equivalent manual or automated washer system
- c. Multichannel pipette; 8 channel, adjustable 50-200 ul volume
- d. Repeater pipette with accessory of 1.25 and 2.5 ml capacity combitips (optional)
- e. A variable-volume pipettor with tips
- f. Stomacher[®]
- g. Whirl-pak[®] bags; 6 oz. and 18 oz. sizes
- h. Centrifuge, capable of operation at 15,600 G and appropriate centrifuge tubes
- i. Refrigerator (2-8°C)/Freezer (≤-10°C)
- j. Aluminum foil
- k. Erlenmeyer flasks, 125 ml
- l. Disposable Millipore[®] filters, 0.45 μm, luer lock

17.3 Chemicals and Reagents

- a. ELISA-TEK[™] Cooked Meat Speciation Kits

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- 510621 Detection of Cooked Pork
 - 510631 Detection of Cooked Poultry
 - 510641 Detection of Cooked Sheep
 - 510651 Detection of Cooked Horse
 - 510661 Detection of Cooked Deer
 - 510611 Detection of Cooked Beef
 - (Other species as available and according to need)
- b. Saline, 0.85%

17.4 Sample Preparation and Extraction

Note: Care must be taken at this stage not to cross-contaminate samples; any equipment or utensils used must be either disposable or thoroughly washed between extractions.

- a. Prepare a cooked or canned meat/meat product for sampling by dicing or finely chopping.
- b. Weigh 5 ± 0.5 g of the diced sample in a 6 oz. Stomacher or whirl-pak bag.
- c. Add 10 ± 0.5 ml of deionized water or normal saline.

Note: If the consistency of a product makes it difficult to identify and separate the meat portion, in order to obtain a representative sample, the sample size in step b may be increased with a proportional increase in the amount of diluent added.

- d. Place bag and contents into a stomacher for approximately 60 seconds. Alternatively for products that tend to emulsify, the mixture may be kneaded or manually homogenized.
- e. Remove from the stomacher and leave undisturbed for at least 1 hour at room temperature.

Note: All samples submitted under the cooked program must be cooked. If it is suspected that the sample is not fully cooked, it is advisable to heat the extract (meat/water mix) in a water bath at $95-100^{\circ}\text{C}$ for 15 ± 1 minutes prior to mixing and centrifugation/filtration.

- f. Pour off some of the sample extract into a centrifuge tube and set the centrifuge at 10,000X G for 10 minutes (consult centrifuge manual to convert RPM to G).

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- g. The clear supernatant of the tissue extract is used in the ELISA.

Note: If the sample has a high fat content, the clear supernatant above the settled meat layer may be beneath a layer of fat. Avoid transferring the fat. It may be appropriate to carefully remove a portion of the aqueous solution using a clean (e.g. Pasteur-type) pipette into a second, clean container prior to analysis.

Note: Sample extracts may be stored at 2-8°C for up to 36 hours. If prolonged storage is required, the extracts must be kept frozen. They will remain stable for several months when stored at $\leq -10^{\circ}\text{C}$.

17.5 Preparation of Cooked Species Tissue Controls (optional)

Note: Positive and Negative Cooked Meat Species Tissue Controls are provided with each kit. Additional species tissue controls may be prepared as follows:

- a. Prepare a portion of lean, raw meat by dicing, mincing, or finely chopping.
- b. Weigh 20 ± 2 g of the diced tissue in a stomacher or whirl-pak bag. Add 60 ± 0.5 ml of normal saline.
- c. Place bag and contents into a stomacher for approximately 10 seconds. Alternately, the mixture may be kneaded or manually homogenized.
- d. Remove from the stomacher and leave undisturbed for at least 1 hour at room temperature.
- e. Place the contents of the bag in a 125 ml. Erlenmeyer flask, seal with aluminum foil, and place in a boiling water bath ($95-100^{\circ}\text{C}$) for 15 ± 1 minute.
- f. Remove the flask from the water bath and allow to cool.
- g. Transfer a portion of the supernatant (being careful to avoid transferring fat) to a centrifuge tube and set the centrifuge at 10,000 X G for 15 minutes.
- h. Filter the supernatant through a 0.45 micrometer filter, aliquot into clean vials, label, and store frozen at $\leq -10^{\circ}\text{C}$.

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17.6 Preparation of Kit Materials

Note: Be sure to use a clean pipette tip for each control and sample to be tested. Pipettors must be verified before use and set to deliver the quantities indicated in the method steps.

- a. Antibody Sensitized Microwell Module: Open the foil pouch (label side up) by cutting between the notches on the end marked "cut here." Open the sealing strip and remove the Microwell Module keeping the wells open side up. Select the desired number of strips and fit into a spare frame. Replace the original frame and remaining strips into the pouch, taking care that the desiccant is present, and reseal the pouch carefully (If necessary, reseal the pouch with adhesive tape or by heat sealing).
- b. Bring the Kit Positive Controls, Anti-species Biotinylates, Streptavidin Peroxide Conjugate, and Stop Solution to room temperature. These reagents are supplied pre-diluted. Mix each by gentle, repeated inversion.
- c. ABTS Concentrate and Peroxide Citrate Buffer: Mix contents of each separate vial by inversion. DO NOT SHAKE. ABTS is supplied as a 25 fold concentrate and must be diluted in Peroxide Citrate Buffer to prepare a working ABTS solution.

Note: Dilutions of ABTS Concentrate should be made just prior to use (e.g. during the avidin peroxidase conjugate incubation).

For 96 test wells add (500 μ l) of ABTS Concentrate to the 12.0 ± 0.1 ml of Peroxide Citrate Buffer. For any other number of test wells, dilute ABTS Concentrate in a 1:25 ratio with Peroxide Citrate Buffer Stopper the vial and mix well by gentle swirling.

- d. Wash Solution Concentrate: Wash Solution Concentrate is supplied as a 10 fold concentrate and requires dilution in purified water to prepare a Working Wash Solution.

For 96 test wells use the total contents of the Wash Solution Concentrate (100 ml) by making up to 1 liter in a volumetric flask/cylinder with deionized water.

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For any other number of test wells, dilute the Wash Solution Concentrate in a 1:10 ratio with deionized water.

17.7 Detailed Enzyme Immunoassay Procedure

Note: Sample Extracts are tested using 2 well replicates. Samples containing violative species (see 17.8 for sample status) are re-tested for the violative species using a 4 well format.

Note: Plates should be covered during all incubation periods; ie, with a lab wipe or other protective covering.

- a. Using a marker, number the strips in sequence on the upper frosted edge; this preserves the identity of the strips should they become detached from the frame.
- b. Place 100 µl of normal saline in each of the wells selected as blanks.
- c. Place 100 µl of each Negative Control in each of the 4 wells selected.
- d. Place 100 µl of each Positive Control in each of the 4 wells selected.
- e. Place 100 µl of each Sample Extract in each of the 2 or 4 wells selected. Avoid transferring fat from sample preparations to the wells.
- f. Mix the plate gently by hand. Allow to stand at room temperature for 60 ± 5 min.
- g. At the end of the incubation period, empty the wells by flicking into the sink. Fill and aspirate the wells three times with Working Wash Solution. Invert the aspirated plate and rap lightly several times onto a soft paper towel placed on the lab bench.

Note: When inverting the plate, be sure to squeeze the plastic frame at the center of the long edges to prevent the strips from falling out of the frame.

- h. Add 25 µl of Anti-Species Biotinylate to the bottom of each microwell of the relevant (same species) Antibody Sensitized Strips.

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Note: Observe that the bottom of each well is covered with liquid. If not, gently tap the edge of the plate until this is accomplished. Avoid getting any antibody on the sides of the wells.

- i. Leave at room temperature for 60 ± 5 minutes.
- j. Repeat the washing step (step g.).
- k. Add 25 μ l of Peroxidase Conjugate to the bottom of each well. Again, observe that the bottom of each well is covered with liquid and that no conjugate sticks to the sides of the wells.
- l. Leave at room temperature for 30 ± 1 minutes.
- m. Repeat the washing step (step g), except wash six times instead of three.
- n. Add 50 μ l of the Working ABTS Solution to the bottom of each microwell.
- o. Cover the plate to protect the ABTS from direct light. Leave at room temperature.
- p. Warm up the plate reader and set the program to use a dual wavelength measurement mode. The program should read absorbance (OD) using a 414 nm absorbance filter and a 492 nm reference filter. The results of the second measurement are subtracted from the results of the first measurement. Set the instrument to blank on well A of column 1 with continuous movement.
- q. Observe the microwells containing the Positive Controls for visual color change. When observed, place the plate on the reader carriage. Read and obtain absorbance values for the wells. Continue to read until Positive Control OD values read in a range of 0.450 to 0.500.
- r. When Positive Control OD values read in the desired range, add 50 μ l of Stop Solution to each microwell. (If the Positive Control OD values are already above 0.600, save the reading and print.)
- s. Mix the plate gently by hand to distribute the Stop Solution and to prevent further color development.

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- t. Return the plate to the reader carriage, read the plate, and obtain a printed copy of the OD values for the plate. Save the electronic file.

17.8 Determination of test validity and sample status

- a. The assay is valid if the mean absorbance value of the Positive Control is greater than 0.600 with standard deviation of its replicates no more than 0.060 and the mean of the negative controls is less than 0.060. Otherwise, the test is invalid and should be repeated.
- b. Samples are classified as presumptive positive if the raw OD value of one or both of the two replicate wells is greater than 0.250.

Note: Test samples determined to be presumptive positive for an undeclared species must be repeated using 4 well replicates. A test sample in 4 well replicates is classified positive if the adjusted mean OD is greater than 0.250. The adjusted mean OD value is obtained by subtracting 3 times the standard deviation from the raw mean OD value of the 4 wells. Computer software, such as Excel[®], may be used to calculate the mean and standard deviations from the raw data.

- c. Samples are reported as negative for each species for which the raw OD value of both of the two replicate wells is less than 0.250.

17.9 Selected References

Andrews, C. D., R. G. Berger, R. P. Mageau, B. Schwab, and R. W. Johnston. 1992. Detection of beef, sheep, deer, and horse meat in cooked meat products by enzyme-linked immunosorbent assay. *J. Assoc. Off. Anal. Chem. Int.* **75**:572-576.

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