United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health Science
Laboratory QA/QC Division
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Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 17.02

Effective Date: 2/10/05

Description and purpose of change(s):

This method revision incorporates testing of raw and cooked meat and poultry products for species determination. Raw species testing was formally included in chapter MLG 21. That chapter is now obsolete following issuance of MLG 17.02. Chapter 20 described a quality assurance plan for a piece of equipment that is no longer used. Both obsolete chapters are now archived.

Cooked species samples continue to be tested via ELISA-TEK™ Cooked Meat Speciation Kits as was described in the previous revision of this chapter.

For raw species, the DTEK™ Immunostick Meat Speciation Testing Kit or Tepnel Biosystems F.A.S.T. Immunostick Meat Species Screening Kit is now utilized as a screen test for available species. Any sample with a positive screen result for an undeclared species are then cooked and subjected to confirmatory testing using the ELISA-TEK™ Cooked Meat Speciation Kits 4-well confirmation procedure.

The agar gel diffusion test found in Part C is no longer performed. The test may be useful in the event that ELISA-TEK ™ Cooked Meat Speciation Kits are unavailable or that speciation of poultry samples is required.

The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use.

United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

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Title: Identification of Animal Species in Meat and Poultry Products		
Revision: 02	Replaces: MLG 17.01 and MLG Chapter 21 Animal Species Determination, Immunological	Effective: 2/10/05

Procedure Outline

PART A
Identification of Animal Species in Cooked and Canned Meat and Poultry

PART B
Commercial ELISA Immunostick Screen Test Kit for Raw Species

PART C
Agar Gel Immunodiffusion Test

PART A
Identification of Animal Species in Cooked and Canned Meat and Poultry

17.1 Introduction

17.1.1 General

This section 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.

The present assay was developed by using unheated skeletal muscle tissue that was fractionated then isolating the antigenic molecules, 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.

Because cross reactions with raw tissue extracts might exceed the cutoff value, raw tissue can only be analyzed using this test by following the cooking directions in Part B, Section 17.14.

17.1.2 Limits of Detection

a. This method, as evaluated with a specific lot of ELISA-TEK™ Cooked Meat Speciation Kits and extracts of each of the listed species sample extract,

was found to detect at least the following percentage of adulteration in a mixed extract of the target and another species:

Beef	1%
Pork	4%
Chicken	4% (Poultry kit)
Turkey	4% (Poultry kit)
Sheep	4%
Horse	4%
Deer	1%

b. Specificity was 100% for the seven species.

c. Sensitivity and specificity may vary from lot to lot. The manufacturer's Certificate of Analysis and laboratory acceptance testing is required for each lot of the test kit.

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^{o}C)/Freezer ($\leq$-10^{o}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

510621 Detection of Cooked Beef
510631 Detection of Cooked Pork
510641 Detection of Cooked Poultry for Chicken and Turkey
510661 Detection of Cooked Deer*
510651 Detection of Cooked Sheep*
510611 Detection of Cooked Horse*

*Note: These species and certain other species are tested by FSIS Laboratories only by special request.

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 before the preparation of each sample.

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, the sample size in step "b" may be increased with a proportional increase in the amount of diluent added in step "c" to obtain a representative sample.

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 homogenized sample 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°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 10,000 X G for 10 minutes (consult centrifuge manual to convert RPM to G).

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 pipette (e.g. Pasteur-type) 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 ≤-10°C.

17.5 Preparation of Cooked Species Tissue Controls

Note: Positive and negative cooked meat species tissue controls are provided with each kit. Cooked species tissue controls, used for lot acceptance of kits, are prepared as follows:

a. Prepare a portion of lean, raw meat by dicing, mincing, or finely chopping tissue.

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 sample 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°C) for 15 ± 1 minute.

f. Remove the flask from the water bath and allow it 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, then dispense into clean vials, label, and store frozen at ≤-10°C.

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 them 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 µl 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) adding deionized water to a total volume of 1L.

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

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.

Plates should be covered during all incubation periods; i.e., with a lab wipe or other protective covering.

Number the strips in sequence on the upper frosted edge. A marker, e.g. Sharpie®, works well. This preserves the identity of the strips should they become detached from the frame. However, if the strips come detached while fluid are present in the wells then the test should be rerun due to possible cross contamination and inadequate reagent volumes.

a. Place 100 µl of normal saline in each of the wells selected as blanks.

b. Place 100 µl of each negative control in each of the 4 wells selected.

c. Place 100 µl of each positive control in each of the 4 wells selected.

d. 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.

e. Mix the plate gently by hand. Allow to stand at room temperature for 60 ± 5 min.

f. 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. 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.

g. Add 25 µl of anti-species biotinylate to the bottom of each microwell of the relevant (same species) antibody sensitized strips. 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.

h. Leave at room temperature for 60 ± 5 minutes.

i. Repeat the washing step (step f).

j. Add 25 µl 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.

k. Leave at room temperature for 30 ± 1 minutes.

l. Repeat the washing step (step f), except wash six times instead of three.

m. Add 50 µl of the Working ABTS Solution to the bottom of each microwell.

n. Cover the plate to protect the ABTS from direct light. Leave at room temperature.

o. 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 the well in row "A," column 1, with continuous movement.

p. Observe the microwells containing the positive controls for visual color change. When color change is 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.

q. When positive control OD values read in the desired range, add 50µl of "Stop Solution" to each microwell. (If the positive control OD values are already above 0.600, save the reading and print. See section 17.8a for further explanation.)

r. Mix the plate gently by hand to distribute the "Stop Solution" and to prevent further color development.

s. 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. Positive controls that are 0.900 or above can cause low level non-reportable results to become positive. Rerun these samples if species result is in violation.

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.

c. Test samples determined to be presumptive positive for an undeclared species must be repeated using 4 well replicates. A test sample analyzed 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.

d. 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 Quality Control Procedures

Each species analysis is run with a positive and negative control supplied in each kit. Lot acceptance should be performed on each lot of species kits received.

a. Control testing should be performed as in Section 17.5 steps "a-h".

b. Store all kit components at refrigerator temperature (2-8^{o}C) when not in use, to preserve and maintain reactivity of immunoreagents.

c. Observe the manufacturer's expiration date of all test kit components. Kits should not be used beyond the expiration date. Do not mix components from one lot with components from another lot.

d. Dilution of ABTS into the Peroxide Citrate Buffer should be done just prior to use.

e. Kit components should be allowed to equilibrate to room temperature before commencing test procedure.

f. The 5 gram test sample used for extraction must be representative of the entire original sample in order to insure that test results accurately reflect the true composition of the original sample.

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.

Berger, R. G., R. P. Mageau, B. Schwab, and R. W. Johnston. 1988. Detection of poultry and pork in cooked and canned meat foods by enzyme-linked immunosorbent assays. J. Assoc. Off. Anal. Chem. **71**:406-409.

PART B
Commercial ELISA Immunostick Screen Test Kit for Raw Species

17.10 Introduction

17.10.1 General

A commercial ELISA Immunostick Screen Test is employed for presumptive identification of species composition of raw meat and poultry tissues. This procedure is an Enzyme-Linked Immunosorbent Assay (ELISA) method that incorporates the use of NUNC dip-stick paddles (immunosticks) as the solid phase and the use of pre-dispensed, standardized reagents in color coded tubes. It is currently marketed and distributed in the U.S. in a complete (25 test) kit form and is referred to as a commercial ELISA Immunostick Raw Meat Species Screening Test Kit.

This raw meat species screen test is a double antibody "sandwich" ELISA procedure with antibody specificity directed against the various species albumins which are contained in meat tissues. Specific antibody-sensitized immunosticks are allowed to capture a homologous species' albumin from sample tissue extracts, then the immunosticks react with the second peroxidase labeled antibody of the same specificity, followed by a final reaction step in an ABTS/H_20_2 chromogen/substrate solution. A short incubation period and a brief tap water rinse are performed between each of the first two steps. A positive reaction, indicating the presence of the test species tissue in the sample, is evidenced by a distinct green color formation in the last reagent tube. All positive screen test results which represent sample violations are to be confirmed by the enzyme-linked immunosorbent assay (ELISA) for Cooked Species (See Part A). The heating of raw meat and poultry products results in the isolation of heat–resistant antigens that are immunoreactive with the antibodies found in the Cooked ELISA kits.

Each of the Immunostick Screen Kits contains all the necessary reagents, controls and accessories to easily perform the screen test with the production of very accurate results.

17.10.2 Limits of Detection

a. This method, as evaluated with a specific lot of ELISA Technologies, Inc. Commercial ELISA Immunostick Raw Meat Species Screen Test Kits for each of the listed species, was found to detect the following percentage or greater of adulteration in a mixed extract of the target and a nontarget species:

Beef	1%
Pork	1%
Chicken	1% (Poultry kit)
Turkey	4% (Poultry kit)
Sheep	1%
Horse	1%

b. Specificity was 100% for the six species.

c. Sensitivity and specificity may vary from lot to lot. A manufacturer's Certificate of Analysis and laboratory acceptance testing is required for each lot of the test kit.

17.11 Reagents and Equipment

a. Commercial ELISA Immunostick Raw Meat Species Screen Test Kits. Color codes for individual species kits used are as follows (Table 1):

Table 1. Color Codes for Commercial ELISA Immunostick Screen Test Kits.

Color Code	Species
Red	Beef
Yellow	Pork
Blue	Poultry*
Orange	Horse**
Green	Sheep**

* NOTE: The ELISA Immunostick Poultry screen test does not differentiate between chicken and turkey. Upon special request this can be accomplished by performing the traditional agar-gel immunodiffusion procedure (Part C). Since the AGD procedure has less than the required sensitivity, its use should be limited to whole meat or poultry tissues or a mixed meat/poultry emulsion where the poultry component is known to constitute over 5% of the final meat block.

**NOTE: These species and others shall be performed in FSIS Laboratories by special request only.

Each individual species kit contains the following items:

i. Twenty-five color coded, white plastic immunosticks sensitized with specific anti-species capture antibody in tubes of preservative buffer solution.

ii. Twenty-five color coded tubes containing species specific antibody-enzyme conjugate reagent.

iii. Twenty-five tubes (non-color coded) containing color development buffer reagent.

iv. One vial of concentrated ABTS color reagent.

v. One vial of aqueous sodium fluoride stop solution.

vi. One vial of positive control solution (homologous species albumin).

vii. Product insert test kit instruction pamphlet.

b. Adjustable pipette and appropriate disposable pipette tips.

c. Stomacher® 400 or 3500, or equivalent.

d. Whirl-Pak® polyethylene bag, 6 oz size (7.5 x 17 cm), or equivalent.

17.12 Raw Sample Preparation

All types of raw meat and poultry product samples are prepared as follows:

a. Weigh out 1 ± 0.1 g of thawed, diced, raw sample product which is a homogeneous, representative portion of the whole sample.

b. Place in a 6 oz Whirl-Pak® bag, or equivalent.

c. Add 9 ± 0.1 ml of distilled water.

d. Place the bag and its contents in a Stomacher® and stomach for a period of 60 seconds. Allow the extract to settle for 2-3 minutes, until a particle-free liquid layer is formed in the top portion of the bag's contents, or centrifuge at 10,000 X G for 10 minutes (see centrifuge manual for proper speed setting). Use the upper liquid layer as the sample extract in the following test procedure.

17.13 Test Procedure

The following procedure uses minor modifications from the manufacturer's test kit instruction insert. These procedural modifications are designed to improve the accuracy, precision, and reproducibility of test results. The subsequent instructions represent the testing of one sample with one species test procedure. Obviously multiple samples and/or species tests may be performed simultaneously, as long as one is careful to keep track of reaction times, washing steps, various reagent steps, etc., relative to each given test sample.

a. Remove the appropriate color coded species immunostick tube, antibody-enzyme conjugate reagent tube, and color development buffer tube (a set of 3) from refrigerated storage and allow equilibrating to room temperature.

b. Label immunostick caps and all tubes with appropriate sample identification codes.

c. Prepare the color development buffer reagent tube (non-color coded) for later use by adding 40 µl of ABTS concentrate to this tube, replacing the cap, then mixing in a gentle but complete manner.

d. Obtain the first color coded immunostick tube, unscrew the cap and remove the immunostick-paddle. Add 200 µl of prepared sample extract to the liquid in the tube, replace the immunostick-paddle in the tube, and mix contents by rotating the cap rapidly for at least 4-6 times then tighten the cap. DO NOT INVERT tubes to accomplish mixing at any stage in this procedure. Only handle the paddle by its attached cap at all times. DO NOT TOUCH paddle with fingers.

e. Allow this tube to stand for 10 minutes at room temperature.

f. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for a minimum of 10 seconds, then shake to remove excess water.

Note: Water dispensed from a squeeze bottle can also be used to carefully perform this wash step.

g. Place the washed immunostick-paddle into the second color coded tube of antibody-enzyme conjugate reagent. Mix contents by rotating the cap rapidly for at least 4-6 times and tighten the cap.

h. Allow this antibody-enzyme reagent tube to stand for 10 minutes at room temperature.

i. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for a minimum of 30 seconds, then shake to remove excess water.

Note in step “f” above also applies here.

j. Place the washed immunostick-paddle into the final, non-color coded, tube of ABTS prepared (step c) color development buffer reagent, mix contents by rotating the cap rapidly for at least 4-6 times and tighten the cap.

k. Allow the color development reagent tube to stand for 10 minutes at room temperature.

l. Add 200 µl of sodium fluoride stop solution to this color development tube, leave the paddle in, and mix well to stop the reaction.

m. Observe the above tube with the white paddle in it for the presence of any discernable green color in the solution or on the paddle surface. A green color indicates a positive test and the presence of the test species in the original meat sample. A colorless solution around the white paddle indicates a negative test and the absence of the test species in the sample.

All ELISA immunostick positive species results which represent sample violations shall be confirmed by performing the Cooked ELISA test found in Part A of this chapter.

17.14 Confirmation Sample Preparation

a. Weigh out 5 ± 0.5 g of thawed, diced, raw sample product which is a homogeneous, representative portion of the whole sample and add 15 ± 0.5mls of normal saline or distilled water.

b. Stomach for 60 seconds and let stand for 1 hour at room temperature.

c. Transfer contents to a screw cap test tube, loosen cap and autoclave for 15 ± 1 minutes at 100 ± 1^{o}C, or boil for 15 ± 1 minutes. Let cool, then transfer to a centrifuge tube and centrifuge at 10,000 X G for 10 minutes (see centrifuge manual for proper speed conversion). Use the supernatant for the Cooked ELISA procedure (Part A) and analyze using the 4 well confirmation procedure. See Section 17.8.c.

17.15 Quality Control Procedures

Each species analysis should be run with a negative and positive control of the species of interest. This should also be performed when receiving a new lot of test kits. Each species test kit is supplied with a positive control vial (homologous species albumin solution) for these purposes. The negative control for any one particular species test kit may be obtained by using the positive control solution from any of the other heterologous species test kits (e.g., horse albumin solution should always give negative results in all other species kits except horse).

a. Control testing should be performed as in steps “a-m” in Section 17.13.

b. Store all kit components at refrigerator temperature (2-8^{o}C) when not in use, to preserve and maintain reactivity of immunoreagents.

c. Observe the manufacturer's expiration date of all test kit components. Kits should not be used beyond the expiration date. Do not mix components from one lot with components from another lot.

d. The concentrated ABTS color reagent solution tube should be observed over the kit shelf life. If this ABTS concentrate should start to turn a darker shade of green than when it was originally received, this indicates decomposition, and a new tube of ABTS concentrate should be requested from the vendor.

e. Kit components should be allowed to equilibrate to room temperature before commencing test procedure.

f. The one gram test sample used for extraction must be representative of the entire original sample in order to insure that test results accurately reflect the true composition of the original sample.

g. Preparation of the color development buffer reagent tube by the addition of ABTS concentrate (step "c" of Section 17.13, Test Procedure) should only be accomplished just prior to commencing the test procedure. Preparation of this reagent tube should not be done in advance (hours/days) because of the inherent chemical instability of ABTS in buffered substrate for extended time periods.

h. Accurate timings of washing and reaction steps should be performed.

i. Assure that all surfaces of the white immunostick-paddle and cap are adequately washed during the two timed wash steps.

j. Do not use hot or warm water for immunostick-paddle washing, only cold.

k. Since all reactions of this solid phase immunoassay occur on the surfaces of the white immunostick-paddle, it is very important not to touch the paddle surface with fingers or any other physical objects which might interfere with the immunoreactions.

l. When performing different species tests simultaneously on the same sample, be sure to maintain the proper continuity of color coded reagent tubes for each respective test species as you complete the test procedure (e.g., an anti-beef species immunostick [red color code] that has reacted with a beef sample extract, if improperly placed in an anti-pork enzyme conjugate reagent tube [yellow color code], will produce a false negative result).

17.16 Selected References

Anonymous. 1991. Commercial Immunostick Raw Meat Species Screening Kits; product insert instruction pamphlet

Fukal, L. 1991. Review Article. Modern immunoassays in meat-product analysis. *Die Nahrung* 35(5):431-448.

Hsieh, Yun-hwa P., B.B. Woodward, S.H. Ho. 1994. Detection of Species Substitution in Raw and Cooked Meats Using Immunoassays. *J. Assoc. of Food Protection*, Vol.58 (5), pp 555-559.

PART C
Agar Gel Immunodiffusion Test

17.17 Introduction

The test described in this section is included primarily for informational purposes. FSIS laboratories do not routinely perform this analysis.

The agar-gel immunodiffusion procedure described in this section is based upon fundamental principles established previously by Ouchterlony, 1968, and modified for specific application and Agency use by Fugate and Penn, 1971. Agar-gel immunodiffusion is notable for its qualitative ability to demonstrate similarities and resolve differences in related proteins based upon the formation of specific immunoprecipitin lines resulting from the diffusion of specific antigens and antibodies from wells or troughs cut into an agar matrix after they have reached their optimum proportions. As such, this procedure is ideally suited for meat species protein identification. If any

false or "non specific" reactions should occur in a double immunodiffusion assay, it is possible to distinguish them from true positive reactions by carefully observing the immunoprecipitin pattern formed and it's relationship to known antigen extracts. The three basic types of reactions usually observed in double immunodiffusion assays are lines of identity, lines of partial identity, and lines of non-identity. With a little practice and experience these types of reactions can be easily distinguished and their interpretation in relation to resolving the identity and/or relationships of similar proteins can be made in a definitive and reliable manner.

Although several different patterns of wells or troughs may be generally used in an agar-gel to perform double immunodiffusion reactions, the pattern ultimately employed is usually dependent upon the intended, specific application of the assay. Hvass, 1985, used a relatively simple, common, 7 well, circular pattern to differentiate raw meat species, while Fugate and Penn, 1971, used a more complicated pattern consisting of 3 antisera troughs and 24 antigen extract wells. The latter was designed with the intention of demonstrating relationships among more than one species on a single plate and also to provide several identical reaction areas on the same plate showing the identity or non-identity relationship of an unknown meat species sample with known reference species tissue extracts.

17.18 Equipment

a. Dish, Petri, plastic, 15 X 100 mm disposable

b. Pipettes, disposable, capillary, Pasteur type

c. Box, plastic, humidity chamber, or other air tight container used to maintain high humidity

d. Cutter, agar-gel, or template pattern

e. Flask, side arm

f. Tubing, rubber or neoprene, high vacuum type

g. Tubing, brass (Cork borer), 5/32 x 1-3/4 inch (3.95 x 44.5 mm)

h. Applicators, wooden, cotton tipped

i. Pipettes, graduated, serological, assorted sizes

j. Dishes, staining (only if agar is to be dried and stained)

k. Slides, microscope, 1 x 3 inch (2.54 x 7.62 cm); (only if agar is to be dried and stained)

l. Filter paper, Whatman® No. 1 and No. 42

m. Pans, plastic, 6 x 12 x 6 inch (15.2 x 30.5 x 15.2 cm), or other suitable containers (used only if agar is to be air dried and stained)

n. Assorted laboratory flasks, beakers, tubes, etc.

Clean all glassware, rinse in distilled water, and heat a minimum of two hours at 200 ± 2°C in a dry heat oven to eliminate contamination from prior use.

17.19 Reagents

a. Normal saline, (0.85 percent sodium chloride solution)

b. Buffered saline (0.85 percent sodium chloride solution, pH 7.2 phosphate buffered)

c. Phosphate buffer stock solution - pH 7.2

d. Agar, 1.0 percent (Oxoid® Purified Agar, L28)

e. Tissue extracts from known animal species:

Cut muscle tissue collected from animals (known species) into 10 ± 0.5 g of ground or finely diced portions and freeze until needed. Add 30 ± 1ml normal saline and stomach for 5 – 10 minutes maximum. Let stand a minimum of 90 minutes. Decant liquid and filter through Whatman® No. 42 filter paper. Use immediately. (Note Section Quality Control of key reagents or procedures.)

f. Antisera:

Undiluted anti-horse, beef, pork, sheep, chicken and turkey species serum, or others as may be required.

g. Tissue extracts (unknown samples to be confirmed):
Extract unknown tissue(s) as in (e) above, using 25 ± 0.5 g tissue and 75 ± 1ml normal saline.

h. Staining solution:
Dissolve 2 g acid fuchsin in 500 ± 0.5 ml absolute methyl alcohol; add 400 ± 0.5ml distilled water and 100 ± 0.5 ml glacial acetic acid.

i. Destaining solution:
To 500 ± 0.5 ml absolute methyl alcohol, add 400 ± 0.5ml distilled water and 100 ± 0.5 ml glacial acetic acid.

j. Acidified Distilled Water:
To 1000 ± 1ml distilled water, add 0.2 ml glacial acetic acid.

k. Mounting fluid:
A commercially available material for mounting cover slips permanently.

17.20 Preparation of Agar-Gel Immunodiffusion Plates

17.20.1 Agar Plate Preparation

Re-melt purified agar prepared above and dispense 18-20 ml into the 15 x 100 mm plastic Petri dishes. Allow to solidify and refrigerate for a minimum of 30 minutes. Store no more than 2 weeks under refrigeration in a high humidity atmosphere. Do not use plates showing desiccation or microbial growth. (Note: Quality Control Section 17.23)

17.20.2 Cutting Pattern of Wells and Troughs

Remove the plates from refrigeration and cut the desired pattern by one of the two methods described below:

a. Use a gel cutting tool which has the proper well and trough cutting tubes and has knife edges permanently embedded in a fixture, such as Plexiglas® or other solid substance.

Figure 1 illustrates one such tool. Align the tool carefully on the agar surface to obtain a perpendicular cut, and then press down firmly to cut the agar.

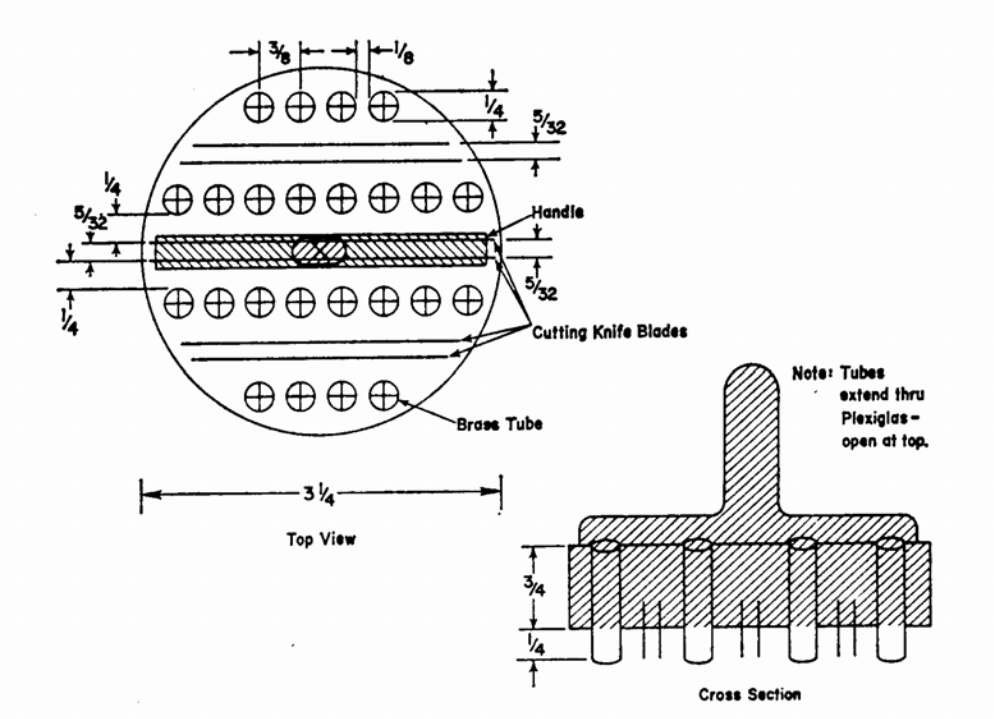

FIG. 1 - Cutting tool used to cut pattern of wells and troughs in agar-gel. (Fugate and Penn, 1971)

b. Using a pattern of the desired arrangement drawn on graph paper, center the plate over the pattern, agar side up. Press a metal tube of acceptable diameter, connected to a vacuum source by a vacuum tube and side arm

flask, through the agar at the indicated places on the pattern. Then cut the troughs with a razor blade or scalpel along the lines of the pattern; or use a tool fashioned with two blades or knife edges the correct distance apart, and with a downward motion cut the agar.

Remove the agar plugs in the wells with a metal tube connected to a vacuum source. Experience will dictate how to avoid tearing the agar surrounding the wells. Remove the trough plugs with an applicator stick which has one end shaved to present a shovel edge. Gently push the applicator stick to the dish bottom and guide it along the cut, raising the strip of agar as a plow would.

Remove the remaining agar in the wells and troughs with a cotton tipped applicator very carefully so as to not tear the surrounding agar surface.

17.20.3 Sealing Wells and Troughs

With a Pasteur pipette, place a thin layer of agar on the floor of each well and trough, sealing the bottom edges of the cut agar to the plate. Do not add an excess of agar. Repair torn wells or troughs in a similar way; if necessary, refill the well or trough and recut it. Caution: An overfilled well will distort the agar and the reaction bands.

17.20.4 Charging the Wells

Mark the outside of the plate to identify the location and contents of each well and trough. Using a Pasteur capillary pipette, partially fill the wells with the known and unknown extracts prepared in “e” and “g” of Section 17.19, maintaining a concave meniscus. Overfilling to form a convex meniscus will interfere with diffusion and may cause wells to overflow. Always place the extract of the unknown between known antigens of two different species. Like antigens will form continuous reactant bands in the agar media, and unlike antigens will form discontinuous bands (See Figure 2).

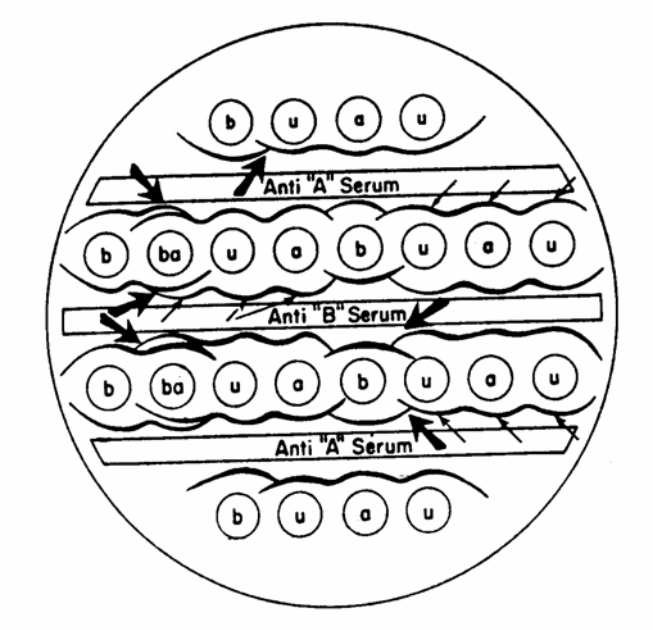

FIG. 2 - Precipitin pattern resulting from heterologous antigen-antisera reactions: "a" antigens derived from species A; "b" antigens derived from species B; "u" antigens derived from unknown; "⇒" are lines of partial identity; "→" are lines of identity. Although atypical, the above pattern results when all antigens react with antisera used. The identification of unknown antigen "u" is accomplished by lines of identity formed with antigen "a." Both "a" and "u" form lines of partial identity with lines formed by antigen "b," which is indicated by a spur reaction. It can be concluded that antigen "u" is derived from species A and is similar but not identical to species B. (Fugate and Penn, 1971)

17.20.5 Charging of Troughs

Fill troughs with the antisera. Use one plate to determine two species only (e.g., beef and sheep, or beef and horse, etc.). Use the top and bottom troughs for one antiserum, and the center trough for the other. (See Figure 2)

17.20.6 Incubation and Observation

Replace the plate covers and allow the plates to remain at room temperature for 1 1/2 to 2 h. Refill the wells and troughs with the appropriate antigens and antisera. Line the bottom of an airtight chamber with wet filter paper or cotton. Incubate the plates in this high humidity chamber at room temperature for 18 to 24 h. To read the plates, direct a light source parallel to the agar surface (i.e., from the side of the plate), and hold the plate over a dark black background. The reactant bands will appear white on a grey surface. If the bands are not fully developed, refill the wells and troughs, and continue incubation in the chamber for an additional 24 to 48 h under refrigeration.

Following incubation, remove the plates from the humidity chamber, discard the remaining reactants and gently wash the plates under a stream of distilled water. Use a soft cotton applicator to remove any film from the agar surface and precipitated matter from the wells and troughs. Dry the bottom of the Petri dish with a soft laboratory tissue and observe the plate for reaction bands. Position the plate in alignment with the worksheet (Figure 3) and draw the reaction bands observed on the plate onto the worksheet.

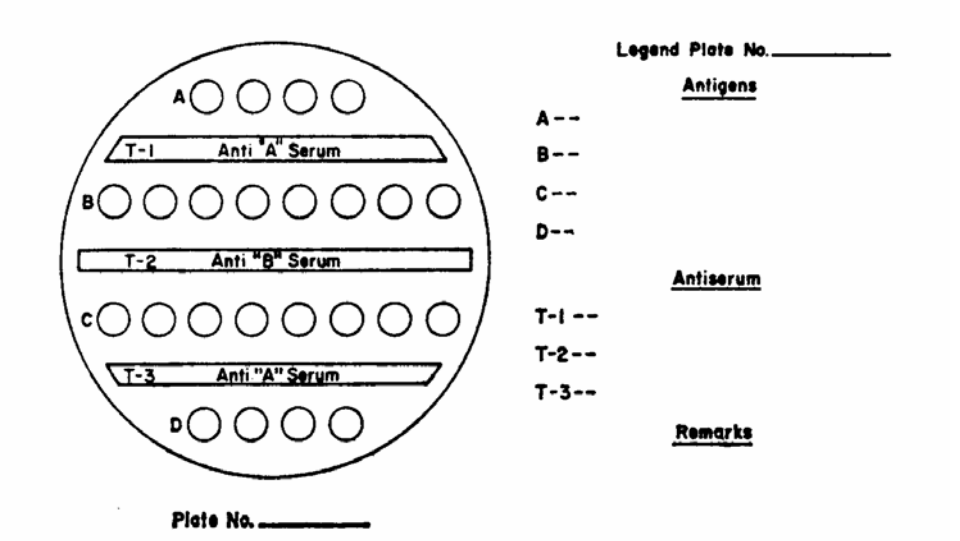

FIG. 3- Worksheet showing well and trough arrangement and antigen-antisera placement (Fugate and Penn, 1971)

17.21 Interpretation of Precipitin Reactions

Interpretation of results depends upon lines formed with known and unknown antigens. Figure 4 (A) illustrates an identity line, i.e., the precipitin line that forms when the antigens are identical. Figure 4 (B) shows partial identity lines, i.e., the lines that form when extracts contain similar but not identical proteins which react with the same antiserum. Figure 2 (page 25) illustrates a typical reaction with an unknown and two known antigens, showing lines of identity and partial identity. Since unknown antigen "u" forms a continuous wave pattern with known antigen "a," lines of identity form. The lines formed by known antigen "b" appear as spurs of those formed by antigen "a" and "u," and are typical lines of partial identity.

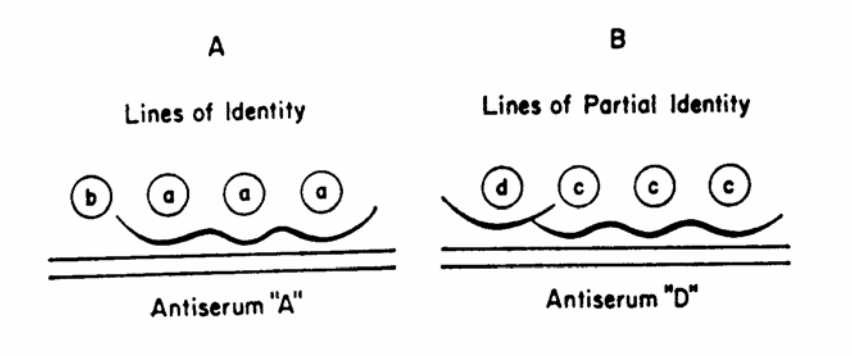

FIG. 4 - Precipitin lines of identity and partial identity. Pic A: lines of identity formed with homologous antigen-antiserum only (antigen "a" vs. antiserum "A"); pic B: lines of partial identity formed when similar antigens react with the same antiserum. Note the typical spur formed, indicating lines of partial identity (antigen "c" and "d" are similar but not identical). (Fugate and Penn, 1971)

Figure 2 also illustrates the pattern of precipitin lines formed when the sample contains tissue antigens from two species (wells "ba"). In the majority of cases, the antisera will not react with heterologous antigens, and lines of partial identity do not form. This occurs when the animal species are closely related (such as bovine and ovine).

Figure 5 illustrates areas containing identical antigen alignment. Four of the 6 areas have antigens reacting with antiserum "A" and 2 of the 4 areas are in position to react with antiserum "B." The 2 remaining areas (2 and 4) are control as well as indicative sites. The mixtures of antigens "a" and

"b" in wells marked "ba" are in position to react with both antisera and illustrate precipitin lines that occur when the sample contains tissues from both species.

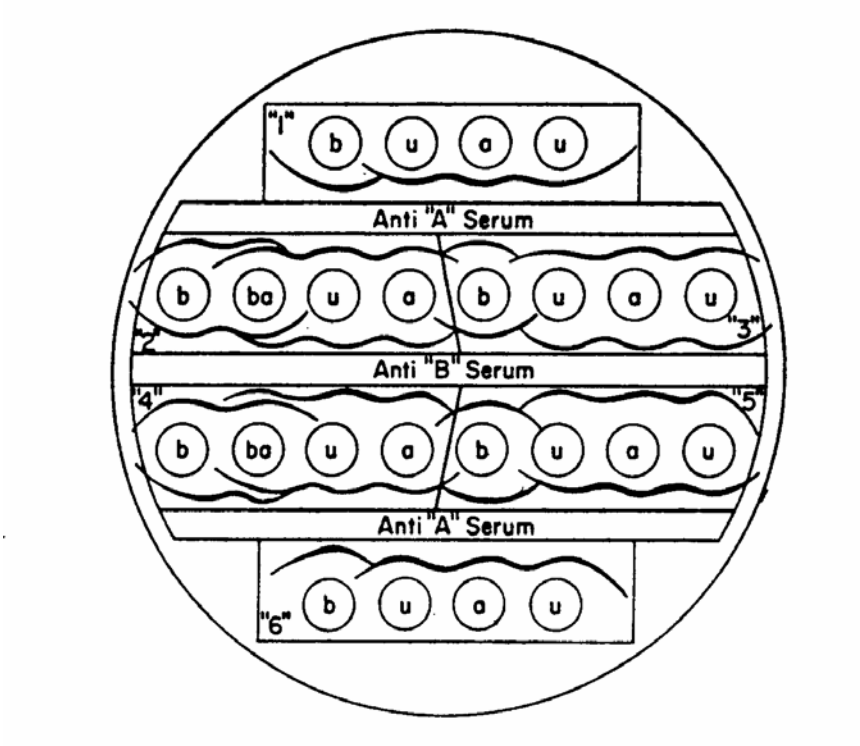

FIG. 5 - Position and reaction sites (6 areas) each consisting of 4 antigen wells. With the exception of areas 2 and 4, antigen placement is identical in each area. Areas 2 and 4 utilize one well each for a mixture of the 2 known antigens (ba), and illustrate precipitin reactions when sample consists of tissues from both species. All areas, except 1 and 6, are positioned to react with both antisera. Interpretation of results from areas 1, 3, 5, and 6 should correlate. Lines enclosing areas indicate portion of plate mounted on slides for preservation. (Fugate and Penn, 1971)

17.21.1 Staining Reaction Bands (Optional)

To keep a permanent record, dialyze to remove free proteins and salts, then dry, stain, and prepare a mount under a cover slip, as follows:

Flood the plate with 500 to 1000 ml pH 7.2, buffered saline in a plastic pan. Replace with fresh buffer twice daily for three days, then once daily for two more days. Finally replace with acidified distilled water and let stand overnight.

Drain off the acidified distilled water, and cut a block of the reaction areas from the agar, and place it onto a 1 x 3 inch (2.54 x 7.62 cm) marked glass slide. Cover the block with a strip of filter paper, and dry in the incubator to a very thin film. Wash gently with a cotton applicator wetted with distilled water to remove adhering bits of the filter paper. Stain the films in acid fuchsin staining solution for 10 minutes. Remove the excess stain and rinse in destaining solution for a period of 15-20 minutes using 2-3 changes, until the agar is clear. Allow the slides to dry, and then mount under cover slips with mounting fluid.

17.22 Photographic Recording of Reaction Bands

One of the easiest methods to obtain a permanent record of the immunodiffusion reaction is to photograph the entire unstained plate. Although there are many ways to achieve this, one of the easiest and quickest is to use a Cordis® Immunodiffusion Camera. This is an instrument with preset optics, light source and Polaroid® Camera which uses Polaroid® Type 084 or 107 black and white film packs. The plate is placed in the instrument, the shutter is tripped, the film tab is pulled from the camera, and within 25 seconds an excellent quality black and white print of the immunodiffusion reaction is produced.

17.23 Quality Control Procedures

17.23.1 Tissue Extracts from Known Species

It is extremely important to establish the authenticity of these reference tissues before they are used, since the basis for the types of immunodiffusion reactions obtained with unknown tissue extracts in the agar gel immunodiffusion test depends upon the use of known species tissue extracts.

17.23.2 Prepared Agar Gel Immunodiffusion Plates

It is usually convenient to prepare a large number of plates at one time for future needs. Care must be taken to prevent deterioration of these plates during storage in the refrigerator. It has been found most useful to stack about 10 plates together in double or triple, air tight,

tightly sealed plastic bags. Any plates showing microbial contamination, desiccation, or salt crystal formation should not be used as they will adversely effect the formation of immunoprecipitin lines.

17.23.3 The Specific Anti-species Sera

Sera used in the immunodiffusion procedure should always be initially checked for their proper reactivity against known, authentic reference tissues prior to their routine use as a diagnostic reagent.

17.24 Selected References

Fugate, H. G., and S. R. Penn. 1971. Immunodiffusion technique for the identification of animal species. J. Assoc. Off. Anal. Chem. 54:1152-1156.

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Ouchterlony, O. 1968. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publishers, Inc., Ann Arbor, MI.