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METHODS OF THE ALLERGENICS PRODUCTS TESTING LABORATORY

October 1993

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Methods of the
Allergenic Products Testing Lab
LIB, DAPP, CBER, FDA

I. In vitro Test Methods

HYALURONIDASE CONTENT ASSAY USING AGAROSE DIFFUSION

Introduction:

This method assays the hyaluronidase content of allergenic extracts and other solutions. Agarose containing hyaluronic acid is prepared in petri dishes; wells are punched in the agarose and fixed quantities of sample are allowed to diffuse at elevated temperatures. Hyaluronidase present in the sample will digest the hyaluronic acid in the agarose. The hyaluronic acid that is not digested is precipitated with a 10% cetyl peridium chloride and the diameters of the clear circles are related to the enzyme concentration in the sample.

Reagents:

1.Sodium Citrate Buffer (0.05 M,pH 5.3) containing 0.15 M NaCl and 0.02% sodium azide. Store at 4°C in stoppered bottle.

This buffer is prepared from stock solutions of 0.05 M citric acid and 0.05 M trisodiumcitrate, both containing 0.15 M NaCl and 0.02% sodium azide.

a. Citric Acid Solution:
4.803 g citric acid (F.W. 192.13)
4.318 g NaCl
0.1 g sodium azide
Q.S. water to 500 ml;

b. Trisodium Citrate Solution:
7.3525 g trisodium citrate $2H_2O$ (F.W. 294)
4.318 g NaCl
0.1 g sodium azide
Q.S. water to 500 ml

Using a pH meter, bring 300 ml of the trisodium citrate solution to pH 5.3 with the citric acid solution. Approximately 90 ml of citric acid will be required. Do not add any more water to the buffer after the proper ph is obtained.

2. Agarose: 3% weight/volume of Litex type HSC agarose (available from Accurate Chemical and Scientific Corp.) in citrate buffer. Store in 9.5ml aliquots in screw cap tubes at ambient temperature. May be stored up to twelve months.

3. Hyaluronic Acid (from human umbilical cord, Sigma grade 1)
Prepare a 2 mg/ml solution in citrate buffer (200mg hyaluronic acid in 100 ml of citrate buffer). Let this solution stir for 40 hours at 4°C. Store in stoppered bottle at 4°C.

4. Cetylpyridium Chloride: 10% weight/volume in dH_2O. Store at ambient temperature.

5. CBER Reference solutions: Five pre-diluted aliquots of 1 mg/ml stock solution of honey bee venom in 50% glycerol-PBS.

PROCEDURE:

1. Melt aliquot of agarose in boiling water bath.

2. Place hyaluronic acid in water bath and bring to 60°C.

3. Allow agarose to cool to 60°C.

4. Pipet 9.5 ml of hyaluronic acid in a test tube. Add 9.5 ml of the agarose to the test tube and mix gently. Slowly pour the solution into a 9 x 9 cm plastic petri dish on a level surface. Be certain to pour slowly to avoid any air bubbles in the agarose solution. Allow to solidify at room temperature with lid ajar. Cover dish when cool. Store in a sealed humidity chamber until ready for use.

5. Reconstitute the unknowns to 100 mg/ml venom protein with 50% PBS/Glycerol solution.

6. Punch 18 holes in the agarose using a sharpened metal tube with a 3 mm diameter opening.

7. Holes should be filled in the following manner:.

each dilution of reference in duplicates	10 holes
each of the unknowns in duplicates	8 holes

8. Fill the holes with approximately 10 µl of each sample including the references. When filling the holes, make sure the surface is level to avoid uneven filling.

9. Allow the plate to sit at room temperature for about 1/2 hour to allow samples to diffuse in the agarose.

10. Place covered petri dish in a humidity chamber (a closed area with a moist towel underneath the dish) and incubate at 32-37°C for 20 hours.

11. Pour 10 ml of the 10% cetyl pyridium chloride solution over the surface of the agarose. Allow to sit for at least 10 minutes at room temperature.

12. Measure the diameters of the clear circles to the nearest 0.1 mm with calipers.

CALCULATIONS:

1. Calculate the mean diameter of the CBER standards. Using these values, compute a linear regression curve using log units hyaluronidase/ml venom protein vs. mean diameter. The slope should be 4.5 ± 1.5 with a correlation coefficient ≥0.95.

2. Calculate the mean diameters of the unknowns and use these values to calculate the concentration of hyaluronidase (in units/ml) from the regression curve.

12/90 ABS

PHOSPHOLIPASE CONTENT ASSAY USING AGAROSE DIFFUSION

Introduction:

This method assays the phopholipase content of allergenic extracts and other solutions. Agarose containing egg yolk is prepared in petri dishes; wells are punched in the agarose and fixed quantities of sample in the wells are allowed to diffuse at elevated temperatures. The diameters of the clear circles are related to enzyme concentration in the sample.

Reagents:

1. Tris Buffer (0.02 M, pH 7.5) containing 0.05% sodium azide. Prepare fresh from stock soluions.

   This buffer is prepared from stock solutions of TRIS and HCl. The stock solutions are prepared as follows:

   0.2 M TRIS solution:
   24.2 g TRIS
   Q.S. water to 1000 ml

   HCl solution:
   0.2 M HCl from concentrate

   Mix 40 ml of the TRIS solution with 50 ml of HCl. Adjust pH to 7.5 with concentrated HCl if needed. Q.S. water to 200 ml.

2. AgarDifco Noble 0.6% in buffer I, containing 0.1% Azide: 3 ml of 1 M $NaNH_3$ in 200 ml

3. Egg yolk stock:
   Egg yolk supernate: seperate yolk from white using a fresh egg and combine 12 ml of thoroughly disrupted yolk with 36 ml of 0.85% saline. Centrifuge at 2000 rpm/10min/20-25 deg C and discard pellet. Aliquot into 1 ml fractions and freeze at -20°C. (adapted from Haberman and Hardt Anal. Biochem. 50:163, 1972)

4. Calcium Chloride Solution: 2 M

5. 50% Glycerol-Citrate Buffer,.pH 4.8: Sigma Citrate Buffer, pH 4.8 (stock no. 104-4) mixed with an wqual volume of glycerol.

6. CBER Reference Solutions: Five pre-diluted aliquots of 1 mg/ml stock solution of honey bee venom in 50% glycerol-citrate buffer pH 4.8. The stock solution contains 1000 units of phospholipase A by definition.

PROCEDURE:

1. Melt aliquot of agarose in boiling water bath.

2. Allow agarose to cool to 60°C and add to egg yolk and Calcium Chloride solution [1% (v/v) egg yolk stock, 5l/100 ml $CaCl_2$ 2M.] Mix gently until solutions are thoroughly mixed; since the solutions are fairly viscous, this step is essential. Pour the mixture into 9x9 cm plastic petri dish on a level surface and allow to cool with lid ajar. cover dish when cool. Store plates in a sealed humidity chamber until ready for use.

3. Reconstitute the unknowns to 100 µg/ml venom protein with the 50% citrate-glycerol buffer pH 4.8.

4. Punch 21 holes (2 mm) in agarose plate and fill wells with samples. Do not allow the solutions to overflow the wells.

Use the attached grid for a suggested pattern. Wells should be at least 8 mm apart horizontally.

The wells should be

each dilution of reference in duplicate	10 holes
each of 5 unknowns in duplicate	10 holes
buffer	1 hole

6. Place covered dish in a humidity chamber and incubate at 32-37°C for 20 hours.

7. Measure the diameters of the clear circles to the nearest 0.1 mm with a caliper or other viewing device.

CALCULATIONS:

1. Calcuate the mean mm diameter of the standards. Using these values, compute a linear regression curve using log (units/ml) vs. zone diameter (mm). Slope ~2.0-3.0, r~0.95

2. Calculate the means of the unknown duplicates and use these values to calculate the concentration of phospholipase A (in units/ml) from the regression curve.

IEF PROCEDURE
For the Qualitative Evaluation of Cat Extracts Using Polyacrylamide Gels

Introduction:
IsoElectric Focusing (IEF) procedures are performed on cat extracts for the qualitative evaluation of:

(1) extracts prepared from new lots of source material with respect to existing reference extracts in terms of presence and location of stained bands in the two preparations
(2) extraction methodology, in terms of the presence or absence of distinct bands in specific areas of the pH gradient
(3) the final container material, after all processing is complete with respect to a reference preparation

The IEF procedures outlined here serve as the basis for the immunoblotting methods that are included in this manual; IEF was selected to serve as a basis for immunoblotting since this method does not involve any denaturation of the native proteins in the extracts, and does not require animal antisera in the analysis.

The following procedure is employed in the LIB and consists of a modification of the procedure described by the gel manufacturer. A number of alternate methods are potentially acceptable, provided that appropriate marker proteins and reference preparations are included in each assay. Gels with similar pH gradients, cross-linking, acrylamide percentage and thickness may be prepared in the laboratory or purchased from other suppliers. For these gels, the optimum assay conditions must be developed and confirmed by the user; the details of the method should be submitted with product laboratory data to CBER.

Reagents, Supplies and Equipment

1. PAG Plates, pH 3.5 to 9.5, wicks, applicators, templates and covers available from Pharmacia-LKB

2. 1M NaOH as cathode buffer

3. 1M H_3PO_4 as anode buffer

4. 1% Methyl Green

5. Fixing solution: 7.5% Sulfosalicyclic Acid/12.5% Trichloroacetic Acid

6. Destaining solution: 8% Acetic Acid/ 25% Absolute Ethanol(95%)

7. Staining solution: 1% Coumassie Brilliant Blue 250 in destaining solution. Prior to use, this solution may be heated to facilitate the Coumassie to dissolve, filtered through Whatman #4 filter paper to remove any particulates and allowed to cool to room temperature.

8. Preserving solution: 10% Gylcerol in destaining solution

9. Flat Bed IEF apparatus

10. Power supply capable of producing a minimum of 500 Volts, with options for controlling amperage

11. Equipment for maintaining 8-10°C running temperatures during the electrophoresis procedure

Procedure:

A. Running Gel

1. Remove gel from packaging and place on the apparatus as indicated by the manufacturer. Many prepared gels may contain some residual non-polymerized acrylamide; care should be taken to protect operators from exposure to this neurotoxin by wearing gloves when handling the gels. Soak electrode strips or cellulose wicks in appropriate buffers, and place on the gel; these wicks should be fully saturated with electrode buffer, but excess buffer should not flow onto gel surface. Sample applicators, made of paper or plastic, (such as a sample application mask) are the placed on the gel surface. The sample should not be applied in an area where protein bands may potentially focus. To determine an appropriate application site for a particular extract, the extract should be subjected to an initial assay where sample applicators containing the test extracts are placed in different areas of the gel. From these results, an area of the gradient where no bands are detected by staining or immunoblots may be selected.

2. Using a micropipettor, add a sufficient quantity of cat extract to the gel to achieve a total application of 0.6-0.8 units of Fel d I to the sample applicators or application masks. Each assay must include the two CBER reference extracts (Cat Hair Reference and Cat Pelt Reference). Marker proteins of known pI's may be used to assure that the pH gradient is attained during the assay. Paper sample applicators supplied by Pharmacia-LKB will permit the application of 18-20 µl per paper.

3. As a visible marker, place 20 µl of a 1% solution of Methyl Green in deionized water on a sample applicator that is positioned just left of the anode. This dye will separate into three colors as the gradient is established; when all three colored bands have migrated to within 1 mm of the cathode, the gradient has been fully established.

4. For the purposes of evaluating allergenic extracts in this system, the following running conditions are suggested:

   Maximum voltage = 500 volts
   Maximum current = 50 milliamps
   Power (non limiting) = 30 watts
   Time = 75-90 minutes
   Temperature = maintained at 8-10°C

B. Staining gel

1. At the completion of the run, when all three components of the methyl green dye have migrated to the cathode, remove the gel from the apparatus and place in the fixing solution for 15-30 minutes at room temperature. The fixing step should be initiated as quickly as possible, since the discontinuation of the electric current will permit the focused bands to diffuse.

2. After fixing is completed, gels are washed briefly in destaining solution to remove excess fixing solution and restore the gel to an optimum pH for staining.

3. Gels are transferred to the staining solution and stained at room temperature for 4 to 18 hours.

4. Gels are destained with repeated changes of the destaining solution until the background is clear. In some instances, stain that has precipitated on the gel surface can be removed by gently rubbing the gel surface with a wet tissue. Destaining can be accelerated with very gentle heating, such as the application of heat from a light box, X-Ray Viewer or similar apparatus.

5. Gels may be photographed at this stage, prior to preserving for the maintenance of a permanent record. Photography should be performed with high contrast film; use of a yellow filter will enhance the visibility of the blue stained bands.

6. The completely destained gels are preserved by placing in preserving solution for one hour at room temperature. Gels are allowed to air dry until the gel surface becomes slightly tacky; then, clear plastic film supplied with the gels is applied to the surface with care to prevent the formation and trapping of air bubbles. Excess plastic is trimmed and the gels are stored in plastic sleeves, away from bright light.

C. Confirmation of Cat Extracts:

The use of 0.6 units Fel d I/ml of CBER reference cat extracts (40 µl E2-Cat Pelt Reference and 30 µl E2-Cat Hair Reference) will produce IEF patterns with defined bands that stain with Coomassie Brilliant Blue R-250. Standardized cat extracts should be focused with both reference preparations as a qualitative comparison of non-Fel d I antigens.

Boyle 10/92
Braun 4/93 (revised)

IEF PROCEDURE
For the Qualitative Evaluation of Allergenic Extracts Using Polyacrylamide Gels

Introduction:

IsoElectric Focusing (IEF) procedures are performed on allergenic extracts for the qualitative evaluation of:

(1) extracts prepared from new lots of source material with respect to existing reference extracts in terms of presence and location of stained bands in the two preparations

(2) extraction methodology, in terms of the presence or absence of distinct bands in specific areas of the pH gradient

(3) the final container material, after all processing is complete with respect to a reference preparation

The IEF procedures outlined here serve as the basis for the immunoblotting methods that are included in this manual; IEF was selected to serve as a basis for immunoblotting since this method does not involve any denaturation of the native proteins in the extracts, and does not require animal antisera in the analysis.

The following procedure is employed in the LIB and consists of a modification of the procedure described by the gel manufacturer. A number of alternate methods are potentially acceptable, provided that appropriate marker proteins and reference preparations are included in each assay. Gels with similar pH gradients, cross-linking, acrylamide percentage and thickness may be prepared in the laboratory or purchased from other suppliers. For these gels, the optimum assay conditions must be developed and confirmed by the user; the details of the method should be submitted with product laboratory data to CBER.

Reagents, Supplies and Equipment

1. PAG Plates, pH 3.5 to 9.5, wicks, applicators, templates and covers available from Pharmacia-LKB

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3. 1M H_3PO_4 as anode buffer

4. 1% Methyl Green

5. Fixing solution: 7.5% Sulfosalicyclic Acid/12.5% Trichloroacetic Acid

6. Destaining solution: 8% Acetic Acid/ 25% Absolute Ethanol(95%)

7. Staining solution: 1% Coumassie Brilliant Blue 250 in destaining solution. Prior to use, this solution may be heated to facilitate the Coumassie to dissolve, filtered through Whatman #4 filter paper to remove any particulates and allowed to cool to room temperature.

8. Preserving solution: 10% Gylcerol in destaining solution

9. Flat Bed IEF apparatus

10. Power supply capable of producing a minimum of 500 Volts, with options for controlling amperage

11. Equipment for maintaining 8-10°C running temperatures during the electrophoresis procedure

Procedure:

A. Running gel

1. Remove gels from packaging and place on the apparatus as indicated by the manufacturer. Many prepared gels may contain some residual non-polymerized acrylamide; care should be taken to protect operators from exposure to this neurotoxin by wearing gloves when handling the gels. Soak electrode strips or cellulose wicks in appropriate buffers, and place on the gel; these wicks should be fully saturated with electrode buffer, but excess buffer should not flow onto gel surface. Sample applicators, made of paper or plastic, (such as a sample application mask) are the placed on the gel surface. The sample should not be applied in an area where protein bands may potentially focus. To determine an appropriate application site for a particular extract, the extract should be subjected to an initial assay where sample applicators containing the test extracts were placed in different areas of the gel. From these results, an area of the gradient where no bands are detected by staining or immunoblots may be selected.

2. Using a micropipettor, add the appropriate quantity of extracts to the sample applicators or application masks. The paper sample applicators supplied by Pharamcia-LKB with the gels permit the application of 18-20 µl per paper.

3. Each assay must include a reference extract. Marker proteins of known pI's may be run to assure that the pH gradient is attained during the assay. Application volumes should be applied to the gel to attain equal quantities of ninhydrin protein in the extracts to be compared. NOTE: If evaluating cat extracts, please refer to the IEF Procedure for Qualitative Evaluation of Cat Extracts which is included in this manual. Source material extracts may be applied using equal weight-to-volume ratios as a guide for rough comparisons. As a visible marker, place 20 µl of a 1% solution of Methyl Green in deionized water on a sample applicator that is positioned just left of the anode. This dye will separate into three colors as the gradient is established; when all three colored bands have migrated to within 1 mm of the cathode, the gradient has been fully established.

4. For the purposes of evaluating allergenic extracts in this system, the following running conditions are suggested:

   Maximum voltage = 500 volts
   Maximum current = 50 milliamps
   Power (non limiting) = 30 watts
   Time = 60-75 minutes
   Temperature = maintained at 8-10°C

B. Staining gel

1. At the completion of the run, when all three components of the methyl green dye have migrated to the cathode, remove the gel from the apparatus and place in the fixing solution 15-30 minutes at room temperature. The fixing step should be initiated as quickly as possible, since the discontinuation of the electric current will permit the focused bands to diffuse.

2. After fixing is completed, gels are washed briefly in destaining solution to remove excess fixing solution and restore the gel to an optimium pH for staining.

3. Gels are transferred to the staining solution and are stained at room temperature for 4 to 18 hours.

4 Gels are destained with repeated changes of the destaining solution. In some instances, stain that has precipitated on the gel surface can be removed by gently rubbing the gel surface with a wet tissue. Destaining can be accelerated with very gentle heating, such as the application of heat from a light box, X-Ray Viewer or similar apparatus.

5. Gels may be photographed at this stage, prior to preserving for the maintenance of a permanent record. Photography should be performed with high contrast film; use of a yellow filter will enhance the visibility of the blue stained bands.

6. The completely destained gels are preserved by soaking in the preserving solution for one hour at room temperature. Gels are allowed to air dry until the gel surface becomes slightly tacky; then, clear plastic film supplied with the gels is applied to the surface with care to prevent the formation and trapping of air bubbles. Excess plastic is trimmed and the gels are stored in plastic sleeves, away from bright light.

C. Validation:

As an example, the use of 100 µg of CBER reference pollen extracts when reconstituted to 100,000 BAU/ml and listed below will produce IEF patterns with defined bands that stain with Coomassie Brilliant Blue R-250 in both the acid and basic areas of the pH gradient under these conditions.

Extract Common Name	Scientific Name	Lot#	Protein per Volume vial(mg)	Sample (µl) added to gel
June (Kentucky Blue)	*Poa pratense*	E3-Jkb	2.7	37
Perennial Rye	*Lolium perenne*	E10-Rye	8.01	12
Timothy	*Phleum pratense*	E6-Ti	6.56	15

Anderson 6/89
Braun (Revised 5/93)

RID ASSAY FOR Amb a I

Introduction:
This assay measures the quantity of Amb a I in allergenic extracts by using radial immunodiffusion. A dose response curve is obtained using four dilutions of a reference preparation and a specific antiserum. The diameter of the circles produced by the dilutions of the reference may be used to calculate the best-fit regression line of the form $y = a[\log(x)] + b$ where y is the diameter of the circle in mm and x is the content of the reference preparation in units/ml. From this calculated line, it is then possible to determine the content of Amb a I in an unknown sample.

Reagents and Equipment:
1. Reagents provided by LIB:
   a. Anti-Amb a I serum- re-hydrate with distilled water as directed.

   b. Reference Amb a I- four dilutions in PBS in 50% glycerin (PBSG).

2. Other reagents and equipment:
   a. Agar- an aqueous solution containing 1% Noble agar (or equivalent) and 1% Sodium Azide.

   b. Acetic acid-10% solution

   c. Agar gel cutter- 3 mm in diameter

   d. Level surface- as determined with a spirit level

   e. Micrometer-capable of measuring 0.1 mm

   f. Microscope slides- 1 by 3 inches, pre-coated with 0.01% agar and air dried before use

   g. Petri dishes and filter paper- or equivalent to provide a humid incubation chamber (a petri dish containing a wet filter paper disc that is approximately the size of the bottom of the dish)

   h. Staining dishes for dipping, washing and holding the slides.

   i. Water bath capable of maintaining a constant temperature: 55° C

Procedure:
1. Prepare the solution of 1% agar and 1% sodium azide by placing in a boiling water bath until the solution is clear. Allow the temperature to equilibrate in a 55 °C water bath until needed. The vessel containing the solution should be stoppered to prevent evaporation. Solution may be made up in bulk, stored in aliquots and reheated as necessary.

2. Pipette 2 ml of the hot agar solution into a 100 X 13 mm test tube; add 0.2 ml of anti-Amb a I serum to the agar and stopper or cover the tube with parafilm. Mix the serum and agar by inverting the tube several times; DO NOT VORTEX OR SHAKE VIGOROUSLY. When the solution is thoroughly mixed, pour onto a pre-coated microscope slide on a level surface.

3. After the agar has hardened, place the slide on a sheet of graph paper that contains a template with five evenly spaced dots. Push the gel cutter into the agar at each dot, and remove the agar plug completely by aspiration. Fill each well with approximately 8 µl of the appropriate sample; wells should be filled so that the miniscus is no longer apparent. DO NOT OVERFILL THE WELLS.

4. Place the slide into the humidified chamber. Incubate the slides at 20-25°C (room temperature) for at least 48 but not longer than 72 hours. At the completion of this period, use the microscope staining dishes to dip the slides in a 10% acetic acid solution for approximately 2 minutes. Transfer the slides immediately into dH_2O, again using the staining dishes. Measure the diameters of the precipitin circles by illuminating the slide against a dark or black background and measuring the diameter to the nearest 0.1 mm.

Calculation of the Standard Curve:
The standard curve is determined using the labeled values of Amb a I of the reference dilutions. Each dilution should be analyzed in quadruplicate and the average diameter for each dilution computed. From these values, calculate the best fit regression line of the formula $y = a[\log(x)] + b$, where y is the average diameter in mm and x is the labeled value of Antigen E for that preparation in units/ml. The correlation coefficient of the regression line must be greater than or equal to 0.9. The reference dilutions should be run on every test. CBER re-evaluates the standard curve every six months.

Assay of Extracts:
In addition to the references, an in-house standard containing approximately 20 units of Amb a I should be tested in quadruplicate whenever unknown samples are assayed. The test extract shall be diluted in 50% Glycerol an estimated 10-20 units Amb a I per ml for analysis, and shall be tested in quadruplicate. The average diameter of the test sample shall be calculated and the content of Amb a I is computed from the best fit regression line. The diameter of the test extract must be within the limits of the current standard curve.

Test Limits:
For release of product lots, the assayed value of the extract shall be within ±25% of the labeled value when the test is repeated at CBER.

MEB 4/93 (revised)

RID ASSAY FOR Fel d I

Introduction:
This assay measures the quantity of Fel d I in allergenic extracts using radial immunodiffusion. A dose response curve is obtained using dilutions of a reference preparation and a specific antiserum. The diameter of the circles produced by the dilutions of the reference may be used to calculate the best-fit regression line of the form $y=a[\log(x)]+b$ where y is the diameter of the circle in mm and x is the content of the reference preparation in units/ml. From this calculated line, it is then possible to determine the content of Fel d I in an unknown sample. At present, there is no assignment of a microgram (µg) value to a unit of Fel d I.

Reagents and Equipment:
1. Reagents provided by the LIB:
   a. Anti-Fel d I serum-rehydrate with distilled water as directed.

   b. Reference Fel d I-four dilutions in 50% glycerin in PBS (PBSG).

2. Other reagents and equipment:
   a. Agar: an aqueous solution containing 1% Noble agar (or equivalent) and 1% Sodium Azide.

   b. Acetic acid: 10% solution

   c. Agar gel cutter: 3 mm in diameter

   d. Level surface: as determined with a spirit level

   e. Micrometer: capable of measuring 0.1 mm

   f. Microscope slides: 1 by 3 inches, precoated with 0.01% agar and air dried before use

   g. Petri dishes and filter paper or equivalent to provide a humid incubation chamber

   h. Staining dishes for dipping, washing and holding the slides.

   i. Water bath capable of maintaining a constant temperature of 55° C

Procedure:

1. Prepare the solution of 1% agar and 1% sodium azide by placing in a boiling water bath until the solution is clear. Allow the temperature to equilibrate in a 55° C water bath until needed. The vessel containing the agar should be stoppered to prevent evaporation. Solution may be made up in bulk, stored in aliquots and reheated as necessary.

2. Pipette 2 ml of the hot agar solution into a 100 X 13 mm test tube; add 0.1 ml of anti-Fel d I serum to the agar and stopper or cover the tube with parafilm. Mix the serum and agar by inverting the tube several times; DO NOT VORTEX OR SHAKE VIGOROUSLY. When the solution is thoroughly mixed, pour onto a precoated microscope slide on a level surface.

3. After the agar has hardened, place the slide on a sheet of graph paper that contains a template with five evenly spaced dots. Push the gel cutter into the agar at each dot, and remove the agar plug completely by aspiration. Fill each well with approximately 8 µl of the appropriate sample; wells should be filled so that the miniscus is no longer apparent. DO NOT OVERFILL THE WELLS.

4. Place the slide in a humidified chamber (a petri dish containing a wet filter paper disc that is approximately the size of the bottom of the dish). Incubate the slides at 20-25°C (room temperature) for at least 48 but not longer than 72 hours. At the completion of this period, use the microscope staining dishes to dip the slides in a 10% acetic acid solution for approximately 2 minutes. Transfer the slides immediately into dH_2O, again using the staining dishes. Measure the diameters of the precipitin circles by illuminating the slide against a black background and measuring the diameter to the nearest 0.1 mm.

Calculation of the Standard Curve:

The standard curve is determined using the labeled values of Fel d I of the reference dilutions. Each dilution should be analyzed in quadruplicate, and the average diameter for each dilution computed. From these values, calculate the best fit regression line of the formula $y = a[\log(x)]+b$, where y is the average diameter in mm and x is the labeled value of Fel d I for that preparation in units/ml. The correlation coefficient of the regression line must be greater than or equal to 0.9. The reference dilutions shall be run on every test. CBER re-evaluates the standard curve every six months.

Assay of Extracts:

In addition to the reference dilutions, an in-house standard containing a known amount of Fel d 1 per ml or a sample provided by CBER shall be tested in quadruplicate whenever unknown samples are assayed. The test extract shall be diluted in 50% Glycerol if necessary and shall be tested in quadruplicate. The diameter of the test extract must be within the limits of the current standard curve. Using the average diameter, the content of Fel d I is computed from the best fit regression line.

Test Limits:

For release of product lots, the assayed value of the extract shall be within ±25% of the labelled value when the test is repeated at CBER.

Assignment of Biological Allergy Units (BAU):

If a cat extract contains between 10 and 19.9 units of Fel d I/ml, the extract shall be labeled 10,000 BAU/ml. If the extract contains between 5 and 9.9 units of Fel d I/ml the extract shall be labeled 5,000 BAU/ml.

MEB 4/93 (revised)

ELISA COMPETITION ASSAY

Quantitative determination of relative potency of allergenic extracts

PRINCIPLE

Reference extract is coated on a 96 well microplate. Serial dilutions of the test extract, reference extract, and quality control reference extract are added to wells. Serum pool is added to the wells initiating a competition between the extract coated on the plate and the free extract. After washing, horseradish peroxidase labeled anti-Human IgE (conjugate) is added to the wells. The conjugate binds to the serum antibody that is attached to the coating antigen on the wells. Excess conjugate is washed away, and tetramethylbenzidine substrate is added to produce a color. The intensity of the color is directly proportional to the amount of serum antibody bound to the coating antigen and inversely proportional to the amount of soluble competing antigen added. The reaction is stopped with 1M H_3PO_4 and the optical density (OD) is read at 450 nm. Relative potency (RP) of allergenic extracts is determined by a parallel line bioassay method.

Standardization of allergenic extracts provides lot-to-lot consistency in patient treatment. One method of standardizing allergenic extracts is to correlate the potency of the test extract relative to a reference extract. The ELISA Competition Assay is a safe, accurate, reproducible potency test.

REAGENTS, SUPPLIES, AND EQUIPMENT (and suggested sources)

1. COSTAR 3590 microwell plate (Cat.#3590) available from COSTAR.

2. Dulbecco's PBS, 2X (Cat.#12-107A) available from S & S Media, Inc.

   Working DPBS (1X): Mix equal volumes of DPBS (2X) and distilled water (dH_2O).

3. Reference extract and serum pool (both available from CBER) and test extract(s).

4. Brij 35 (Cat.#430AG-6) available from Sigma Chemical Company.

5. B114. Add 1.7 ml of Brij 35 to 1000 ml of working DPBS. Store at 2-8°C.

6. Albumin, Bovine (Cat.# A-2153) available from Sigma Chemical Company.

7. 1% BSA (blocking solution). Dissolve 0.25 grams of Albumin, Bovine in 25 ml of working DPBS. This volume is adequate for blocking one microplate.

8. Goat Anti-Human IgE, HRP-labeled, 1.0 mg (Cat.#074-1004) available from Kirkegaard & Perry Laboratories, Inc. Store at 2-8°C.

   Working reagent: Add 1.0 ml of 50% glycerol/dH_2O. Mix well. Store at 2-8°C.

9. Microwell TMB Substrate System (Cat.#50-76-00) available from Kirkegaard & Perry Laboratories, Inc. This is a two component system consisting of TMB Peroxidase Substrate and Solution B. Store at 2-8°C.

   Working Solution: Remove aliquots of each component and allow to reach room temperature. Just before use, mix equal volumes of the TMB Peroxidase Substrate and Solution B.

10. 1M H_3PO_4. Prepare by adding 55.2 ml of H_3PO_4 (85% by wt) to 944.8 ml distilled water.

11. Albumin (Human), (Cat. # 1653), available from Sigma Chemical Company. Dilute to 5% HSA in B114. Mix well and aliquot. Store at -20°C or colder.

12. Glycerol (Cat.#5092) available from Mallinckrodt. To make 50% glycerol: Add equal volumes of glycerol and dH_2O.

13. Dynatech MR 5000 or other microplate reader capable of accurately measuring absorbance at 450 nm.

14. Positive displacement pipets, for the accurate delivery of solutions containing 50% glycerol. Volumes to include 10 - 200 µl.

15. Multichannel pipettors, for the accurate delivery of 50 µl, 100 µl, and 200 µl.

16. Timer.

17. Bio-Tek ELP-35 or other automatic plate/well washer, or a squeeze bottle for manual washing. Washing consists of 3 washes using an automated plate washer or 5 washes for manual method.

18. Test tubes: 12 X 75 mm (Cat.#60825-550) available from VWR.
    13 X 100 mm (Cat.#60825-571) available from VWR.
    18 X 150 mm (Cat.#79-6322-18) available from PGC Scientific.

19. Staticmaster Brush (Cat.#3620-B30), available from Thomas Scientific.

20. Plate sealing film (Cat.#62406-092), available from VWR.

PROCEDURE FOR OPTIMIZING DILUTIONS OF COATING EXTRACT, SERUM POOL AND CONJUGATE

A. Perform checkerboard titrations to determine optimal dilutions of coating extract and serum pool.

1. Prepare serial three-fold dilutions of the coating extract in glass test tubes. For example:

Dilution	coating extract	DPBS, ml
1.25	150 µl	3.6
1:75	1.0 ml of 1:25 dilution	2.0
1:225	1.0 ml of 1:75 dilution	2.0
1:675	1.0 ml of 1:225 dilution	2.0
1:2025	1.0 ml of 1:675 dilution	2.0
1:6075	1.0 ml of 1:2025 dilution	2.0

2. Add 100 µl of each dilution to duplicate columns of the microwell plate, i.e., 100 µl of the 1:25 dilution to A1 & 2 through H1 & 2; 100 µl of the 1:75 dilution to A3 & 4 through H3 & 4. Continue with all dilutions.

3. Tap the plate gently to dislodge bubbles in the wells and cover the plate with sealing film. Incubate at room temperature (RT) for 3 hours or overnight at 2-8°C.

4. Near the completion of the incubation step, prepare 1% BSA.

5. Wash plate three times on automated plate washer, (or 5 times manually) and blot on absorbent paper.

6. Add 200 µl of 1% BSA to all wells, tap gently to dislodge bubbles and cover with sealing film. Incubate at RT for 1.5 hours or overnight at 2-8°C.

7. During the RT incubation or the next morning if overnight incubation is done:

   a) prepare serial two-fold dilutions of the serum pool in glass test tubes. For example:

Dilution	Serum Pool	B114, ml
1.25	150 µl	3.6
1:50	1.5 ml of 1:25 dilution	1.5
1:100	1.5 ml of 1:50 dilution	1.5
1:200	1.5 ml of 1:100 dilution	1.5
1:400	1.5 ml of 1:200 dilution	1.5
1:800	1.5 ml of 1:400 dilution	1.5
1:1600	1.5 ml of 1:800 dilution	1.5

   b. Make a 1:100 dilution of 5% HSA by adding 15 µl of 5% HSA to 1.485 ml of B114.

8. After incubation, wash the microplate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

9. Add 50 µl of B114 to all wells. Add each serum pool dilution and diluted 5% HSA to separate rows, i.e., 50 µl of the 1:25 serum pool dilution is added to wells A1 through A12; 50 µl of the 1:50 serum pool dilution is added to wells B1 through B12. Continue with all serum pool dilutions and add 50 µl of the diluted 5% HSA to wells H1 through H12.

10. Tap the plate gently to dislodge bubbles and cover the plate with sealing film and incubate for 2 hours at RT or overnight at 2-8°C.

11. After incubation, make a 1:500 dilution of the conjugate in a glass test tube by adding 0.02 ml of conjugate to 9.98 ml of B114.

12. Wash the plate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

13. Add 100 µl of the 1:500 dilution of conjugate to each well. Tap the plate gently, cover the plate with sealing film and incubate 2 hours at RT or overnight at 2-8°C.

14. Wash the plate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

15. Prepare the substrate solution by mixing equal volumes of TMB Peroxidase Substrate and Solution B (5.5 ml of each solution is adequate for one plate). Add 100 µl of the working substrate solution to each well. Tap the plate gently and incubate at RT for 20 minutes.

16. After 20 minutes, add 100 µl of 1M H_3PO_4 to each well. Tap gently to mix.

17. Read the OD on a microplate reader at 450 nm with an air blank.

18. Plot the serum pool dilutions on a graph with OD on the y-axis and coating antigen dilutions on the x-axis. Choose the coating extract dilution where the data levels off. Choose the highest dilution of serum pool that gives an adequate signal at the chosen coating extract dilution.

B. Perform checkerboard titration to determine optimal dilution of conjugate.

1. Coat 2 rows of a microplate with 100 µl of diluted reference extract (use the previously determined optimal dilution).

2. Tap the plate gently to dislodge bubbles in the wells and cover the plate with sealing film. Incubate at RT for 3 hours or overnight at 2-8°C.

3. Near the completion of the incubation, prepare 1% BSA.

4. Wash plate three times on automated plate washer, and blot on absorbent paper.

5. Add 200 µl of 1% BSA to all wells, tap gently to dislodge bubbles and cover with sealing film. Incubate at RT for 1.5 hours or overnight at 2-8°C.

6 Prepare the diluted serum pool (use the previously determined optimal dilution).

7. Dilute the 5% HSA in the same manner as the serum pool. (If the optimal serum pool dilution is 1:100, then dilute the 5% HSA 1:100.)

8. After incubation, wash the microplate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

8. After incubation, wash the microplate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.
9. Pipet 50 µl of diluted serum pool to each coated well in the first row. Pipet 50 µl of diluted 5% HSA to each coated well in the second row. Pipet 50 µl of B114 to all the wells in both rows.

10. Tap the plate gently to dislodge bubbles and cover the plate with sealing film and incubate for 2 hours at RT or overnight at 2-8°C.

11. Prepare serial two-fold dilutions of the conjugate. For example:

Dilution	Conjugate	B114, ml
1:500	10 µl	5.0
1:1000	1.0 ml of 1:500 dilution	1.0
1:2000	1.0 ml of 1:1000 dilution	1.0
1:4000	1.0 ml of 1:2000 dilution	1.0
1:8000	1.0 ml of 1:4000 dilution	1.0
1:16000	1.0 ml of 1:8000 dilution	1.0

12. Wash the plate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

13. Add 100 µl of each dilution to duplicate columns of the microwell plate, i.e., 100 µl of the 1:500 conjugate dilution to A1 & 2 and B1 & 2, 100 µl of the 1:1000 conjugate dilution to A3 & 4 and B3 & 4. Continue with all dilutions.

14. Tap the plate gently to dislodge bubbles in the wells and cover the plate with sealing film. Incubate at RT for 2 hours or overnight at 2-8°C.

15. Wash the plate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

16. Prepare the substrate solution by mixing equal volumes of TMB Peroxidase Substrate and Solution B (1.5 ml of each solution is adequate for two rows of one plate). Add 100 µl of the working substrate solution to each well. Tap the plate gently and incubate at RT for 20 minutes.

17. After 20 minutes, add 100 µl of 1M H_3PO_4 to each well. Tap gently to mix.

18 Read the OD on a microplate reader at 450 nm with an air blank.

19. Plot the serum pool and 5% HSA on a graph with OD on the y-axis and conjugate dilutions on the x-axis. Choose the conjugate dilution where the data levels off if the 5% HSA gives a low signal. Otherwise, choose the dilution that comes closest to the leveling off area and the 5% HSA signal is relatively low.

PROCEDURE FOR COMPETITION ASSAY

1. Coat the microplate wells (except wells E1, F1, G1 and H1) with 100 µl/well of the optimal coating dilution of reference extract.

2. Tap the plate gently to dislodge bubbles in the wells and cover the plate with sealing film. Incubate at RT for 3 hours or overnight at 2-8°C.

3. Near the completion of the incubation, prepare 1% BSA.

5. Add 200 µl of 1% BSA to all wells, tap gently to dislodge bubbles and cover with sealing film. Incubate at RT for 1.5 hours.

6. During incubation, prepare serial three-fold dilutions of the reference extract and test extract(s). Prepare two separate dilution series of the reference extract - one will be used as the reference and the other will be the quality control reference. Up to three different test extracts of the same allergen can be run on one plate. For example, 3 different lots of Dp mite may be tested on a Dp mite-coated plate; however, 3 tests of the same lot of Dp mite may not be tested on one plate.

   NOTE: Target the AU/ml or BAU/ml of the test extract to that of the reference extract in the first dilution.

   Example: If the reference extract is 100,000 BAU/ml, the 1:50 dilution in the chart below provides a 2,000 BAU/ml solution. If the test extract is targeted to be 10,000 BAU/ml, start with a 1:5 dilution of the test extract to provide a 2,000 BAU/ml solution. If the calculated RP of the test extract is equipotent to the reference extract, the BAU/ml of the test extract is determined by multiplying the AU or BAU/ml of the reference extract by 1/10, since the test dilution (1:5) is 1/10 of the reference dilution (1:50). In this case, the test extract would be 10,000 BAU/ml.

   If the test extract is not equipotent to the reference extract, then retest the sample.

   Dilution series example:

Dilution	Extract	B114, µl
1:50	10 µl	490
1:150	200 µl of 1:50 dilution	400
1:450	200 µl of 1:150 dilution	400
1:1350	200 µl of 1:450 dilution	400
1:4050	200 µl of 1:1350 dilution	400
1:12150	200 µl of 1:4050 dilution	400
1:36450	200 µl of 1:12150 dilution	400
1:109350	200 µl of 1:36450 dilution	400

7. Prepare the selected dilution of the positive serum pool and the 5% HSA in B114 (use the dilution chosen from section I.A for the positive serum pool and the 5% HSA).

   Example: If using a 1:50 dilution of serum: 0.1 ml positive serum pool + 4.9 ml of B114 and 0.01 ml of 5% HSA + 0.49 ml of B114. These amounts are adequate for a plate with reference and control extracts and 3 test extracts.

8. Wash plate three times on automated plate washer (or 5 times manually) , and blot on absorbent paper.

9. Pipet 50 µl of each extract dilution to duplicate wells of the microplate (see the plate example below).

	1	2	3	4	5	6	....	11	12
A	Coating Check	Positive Serum Pool	Reference 1:50	Reference 1:50	Sample #1 1:50	Sample #1 1:50	....	Control 1:50	Control 1:50
B	Coating Check	Positive Serum Pool	Reference 1:150	Reference 1:150	Sample #1 1:150	Sample #1 1:150	....	Control 1:150	Control 1:150
C	Coating Check	Positive Serum Pool	Reference 1:450	Reference 1:450	Sample #1 1:450	Sample #1 1:450	....	Control 1:450	Control 1:450
D	Coating Check	Positive Serum Pool	Reference 1:1350	Reference 1:1350	Sample #1 1:1350	Sample #1 1:1350	....	Control 1:1350	Control 1:1350
E	Reagent Check	5% HSA	Reference 1:4050	Reference 1:4050	Sample #1 1:4050	Sample #1 1:4050	....	Control 1:4050	Control 1:4050
F	Reagent Check	5% HSA	Reference 1:12150	Reference 1:12150	Sample #1 1:12150	Sample #1 1:12150	....	Control 1:12150	Control 1:12150
G	Reagent Check	5% HSA	Reference 1:36450	Reference 1:36450	Sample #1 1:36450	Sample #1 1:36450	....	Control 1:36450	Control 1:36450
H	Reagent Check	5% HSA	Reference 1:109350	Reference 1:109350	Sample #1 1:109350	Sample #1 1:109350	....	Control 1:109350	Control 1:109350

10. Pipet 50 µl of diluted positive serum pool to all wells containing extract dilutions and wells A2, B2, C2, and D2 (Maximum binding). Pipet 50 µl of diluted 5% HSA to wells E2, F2, G2, and H2 (non-specific bind'ng).

11. Pipet 50 µl of B114 into wells A2 through H2 (to bring the total volume of the wells up to 100 µl). Pipet 100 µl of B114 into wells A1 through H1.

12. Tap the plate gently to dislodge bubbles and cover with sealing film. Incubate for 2 hours at RT or overnight at 2-8°C.

13. After incubation, dilute the conjugate with B114 (use the previously determined optimal dilution) in a glass test tube.

14. Wash the plate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

15. Add 100 µl of the diluted conjugate to each well. Tap the plate gently to dislodge bubbles, cover the plate with sealing film and incubate for 2 hours at RT or overnight at 2-8°C.

16. Wash the plate three times on an automated plate washer (or 5 times manually) and blot on absorbent paper.

17. Prepare the substrate solution by mixing equal volumes of TMB Peroxidase Substrate and Solution B (5.5 ml of each solution is adequate for one plate). Add 100 µl of the working substrate solution to each well. Tap the plate gently and incubate at RT for 20 minutes.

18. After 20 minutes, add 100 µl of 1M H_3PO_4 to each well. Tap gently to mix.

19. Read the OD on a microplate reader at 450 nm with an air blank.

CALCULATIONS : Determination of Relative Potency (RP)

Suppose there are M dilutions for the reference and N dilutions for the sample. Convert the dilutions to logarithm to the base 10.

1. Assume for the reference:

   log dil: $X_1, X_2, ..., X_M$

   response Y: $Y_1, Y_2, ..., Y_M$

   Calculate:

   a. $\overline{X} = (\Sigma X_i)/M$; $\overline{Y} = (\Sigma Y_i)/M$

   b. $A_1 = \Sigma(X_i - \overline{X})^2$; $A_2 = \Sigma(Y_i - \overline{Y})^2$

   $A_3 = \Sigma(X_i - \overline{X})(Y_1 - \overline{Y})$

   c Slope = $B = A_3/A_1$

   d. Intercept = $I = \overline{Y} - B \cdot \overline{X}$

   e Corr.. Coefficient = $R = A_3/\sqrt{A_1 \times A_2}$

   f. Variance = $V = (A_2 - B^2 \cdot A_1)/(M-2)$

2. Repeat step 1 using sample data. Note that there are N dilutions in the sample.

3. Subscripts "r" and "s" stand for reference and sample, respectively.

   Calculate:

   a. Pooled variance = $V = [(M-2)V_r + (N-2)V_s]/(M + N-4)$

   b. $C = 1/(A_1)_r + 1/(A_1)_s$

   c. $E = \sqrt{V \cdot C}$

   d. $T = (B_r - B_s)/E$ d.f. = M+N-4

4. For a valid assay or test, there must be:

   a. correlation coefficients greater than or equal to 0.95.

   b. T less than the tabulated t-values at P = 0.01 with (M + N - 4) degrees of freedom.

5. Obtain parallel lines by calculating:

   a. pooled slope = $B = [(A_3)_r + (A_3)_s]/[(A_1)_r + (A_1)_s]$

   b. two new intercepts:

   $I_r = \overline{Y}_r - B \cdot \overline{X}_r$

   $I_s = \overline{Y}_s - B \cdot \overline{X}_s$

6. The log relative potency of sample with respect to the reference when dilutions are used, is given by:

$$\log (RP) = w = (I_r - I_s)/B$$

If, instead of dilutions, concentrations are used, the log (RP) is given by:

$$\log (RP) = w = (I_s - I_r)/B$$

7. Relative Potency is given by: $RP = 10^w$

8. AU/ml or BAU/ml is determined by:

   a. A sample that started with the same initial serial dilution as the reference and has a calculated RP that is equipotent to the reference is designated as having the same AU or BAU/ml as the reference.

   b. For a sample that started at a different initial serial dilution and has a calculated RP equipotent to the reference: multiply the reference AU/ml or BAU/ml by the difference in dilution and that number is assigned to the sample extract.

Example 1. The reference extract is 100,000 BAU/ml and the initial dilution is 1:50. The sample extract is targeted as a 10,000 BAU/ml and the initial dilution is 1:5. The initial dilution of the sample is 1/10 that of the reference, therefore, multiply 100,000 BAU/ml by 1/10 to determine the BAU of the sample (10,000 BAU/ml).

Example 2. The reference extract is 10,000 AU/ml and the initial dilution is 1:50. The sample extract is targeted to 30,000 AU/ml and the initial dilution is 1:150. The initial dilution of the sample is 3 times that of the reference; therefore, multiply the AU/ml of the reference by 3 to determine the AU/ml of the sample (30,000 AU/ml).

QUALITY CONTROL

A. Validity for a Single Plate:

1. Minimum of 4 consecutive values from the linear portion of the curve are used to calculate reference or quality control reference line.

   a. Values used in line must be a graded response.

   b. Values above the mean positive control and below the mean negative control are not to be used for analysis.

2. Reference and quality control reference correlation coefficients must be greater than or equal to 0.95.

3. Slope of the line for quality control reference may not be different from the reference at p=0.01.

4. When the reference is considered to be 1.000, the relative potency of the quality control reference must be between 0.538 and 1.860 (limits for n=1).

If any criteria fails for the reference or the quality control referenced, the plate is considered to be invalid and the assay must be redone.

B. Testing Unknown Samples:

1. The reference-to-reference for each plate on which a sample is tested must meet the Single Plate Validity criteria.

2. Each sample must have a minimum of 4 consecutive values from the linear portion of the curve to calculate the line.

   a. Values used in line must be a graded response.

   b. Values above the mean positive and below the mean negative are not to be used for analysis.

3. The line generated from each sample:

   a. must have a correlation coefficient greater than or equal to 0.95.

   b. must have a slope that is not different from the reference at p=0.01.

If all criteria for unknown samples is met, the relative potency for that sample may be determined. If any criteria fails, the individual determination for that sample is considered to be invalid and must be retested.

5. Samples must have a minimum of three valid relative potency determinations and a maximum of five.

6. Each determination for a sample must be independent, i.e. separate plates and separate dilution series.

7. All valid log relative potency values must be averaged.

8. Calculate the standard deviation of the log relative potencies. The calculated standard deviation should be less than the tabulated value given below. If not, perform more assays (up to 5 total). Each time, recalculate the standard deviation using all log RP's. If the standard deviation is greater than the tabulated value even after performing more assays, the performing laboratory is not in quality control. Reasons for this high variability should be explored and corrective measures taken.

99% fiducial upper limit
for a SD of "N" values of log RP's

n	99% Upper Limit of SD
3	0.2951
4	0.2673
5	0.2505
10	0.2134
20	0.1898

9. If the standard deviation is less than the tabulated value, calculate the geometric mean of the log relative potencies. Calculate the anti-log of the mean log relative potencies and check the table below to determine if the sample is equivalent to the reference.

Two-sided 95% limits for GM
of "n" relative potency values

n	Lower Limit	Upper Limit
1	0.538	1.860
3	0.699	1.431
4	0.733	1.364
5	0.758	1.320
10	0.822	1.217
20	0.870	1.149

NOTES

1. DPBS must be used to dilute the coating antigen. If B114 is used by mistake, the OD readings will be almost the same for all the wells.

2. Laboratories who submit Product License Applications or Amendments to utilize this method for their relative potency testing will be required to perform a validation study.

   a. Two allergenic extracts will be tested 10 times each (10 different plates). CBER references may be used for the "test" extracts.

   b. The pooled data from each of the products must meet the validity criteria for n=10 and the pooled data from both products must meet the validity criteria for n=20. This data should be included with the PLA.

CAM 9/93

RAST Inhibition Procedure

Introduction

The RAST inhibition test is designed to determine the relative potency of an allergenic product with respect to a reference preparation. The consideration of certain factors is critical to ensure accurate performance of this test; the quality of the test reagents is as important to the assays as the test procedure and the proper calculation of results. This bulletin contains additional details regarding the preparation, characterization and preliminary testing of reagents used in the RAST inhibition test. Elements relating to source materials, formulations of buffers used throughout the assay and the original RAST test procedure are also detailed in this updated procedure. The need to employ reagents of known quality as well as the use of a specific procedure is necessary to permit the test to accurately predict the potential biological activity of the extract *in vivo,* as well as ensuring the reproducibility of the test.

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I. List of Reagents by Procedures

A. Special Buffers

1. **Tris-HCl Buffer, pH 8.0**
   a. 50 ml of 0.2 M Tris base[(hydroxymethyl)amino methane]
   b. 26.8 ml of 0.2 M HCl
   c. Check pH and adjust as necessary
   d q.s. to 200 ml with distilled H_2O

2. **RAST Phosphate Buffer, pH 7.5**
   a. 355 gm Sodium Phosphate, Dibasic, Anhydrous, Na_2HPO_4
   b. 75 gm Potassium Phosphate, Monobasic, KH_2PO_4
   c. 49.5 gm Sodium Azide, NaN_3
   d. pH to 7.5 with IM NaOH or 1 M HCl
   e. q.s. to 6000 ml with distilled H_2O

3 **RAST A Buffer**
   a. 600 ml of RAST Phosphate Buffer, pH 7.5
   b. 12 gm of Bovine Serum Albumin (BSA) (Cohn's fraction V)
   c. 60 ml of Polyoxyethylene-sorbitan monolaurate (TWEEN 20™)
   d. q.s. to 6000 ml with distilled H_2O

4. **RAST B Buffer**
   a. 600 ml of RAST Phosphate Buffer, pH 7.5
   b. 60 ml Polyoxyethylene-sorbitan monolaurate (TWEEN 20™)
   c. q.s. to 6000 ml with distilled H_2O

B. Preparation of extract for solid support coupling

1. Petroleum Ether, boiling point 37-57°C
2. distilled or deionized water

C. Reagents for coupling procedures

1. **Microcrystalline cellulose™**, Brinkmann-Avicel Microcrystalline Cellulose™ Cat #6600550-0, Brinkmann Instruments Inc, Westbury NY 11590
   a. 0.1 N HCl
   b. distilled water
   c. Cyanogen bromide
   d. N, N, dimethyl formamide
   e. I N $NaHCO_3$, pH 8.5
   f. 4 M NaOH
   g. Tris-HCl Buffer
   h. RAST A Buffer

2. **Cellulose Discs**-Type 595, 1/4 inch diameter, Schleicher & Schuell, Inc., Keene, NH 03431
   a. 0.1N HCl
   b. distilled water
   c. Cyanogen bromide
   d. N, N, dimethyl formamide
   e. I N $NaHCO_3$, pH 8.5
   f. 4 M NaOH

g. RAST A Buffer

3. **Matrex™pel 102**, Amicon Corporation, Lexington, MA 02173
   a. Tris-HCl Buffer
   b. RAST A Buffer

4. **Pre-Activated Paper**, ATP™ or BA-85 Nitrocellulose; Schleicher & Schuell, Inc., Keene,NH 03431 or PVDF; Millipore Corporation, Bedford, MA
   a. Initial manufacturer's directions
   b. Tris-HCl Buffer
   c. RAST A Buffer

D. **Iodination Procedure**
1. 0.5 M RAST Phosphate Buffer, pH 7.5
2. 0.1 M RAST Phosphate Buffer, pH 7.5
3. Sodium meta-Bisulfite
4. Chloramine T
5. PD Sephadex G25 M pre-packed column (Pharmacia, Piscataway, NJ 08854) or equivalent
6. Bovine Serum Albumin(BSA)(Cohn's Fraction V)
7. Sodium Iodide I-125
   (Dupont/New England Nuclear, Boston, MA 02118 or equivalent)
8. Anti-Human IgE

E. **RAST Inhibition procedure**
1. RAST A Buffer
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3. I-125 Labeled anti-Human IgE
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II. Preparation and Testing of RAST Reagents

A. Preparation of Extracts for Supports

Prior to the actual coupling. extracts should be tested by an appropriate procedure to assure that these materials are of the highest possible quality. For most extracts, such as those prepared from pollens, the extract to be coupled should have isoelectric focusing (IEF) and immunoblotting patterns that are identical to the CBER Reference preparation. Protein determination of all potential coupling extracts by the ninhydrin assay permits the addition of a known quantity of protein to the support. For extracts where IEF is not appropriate, such as *Dermatophagoides* extracts, equal potency of the coupling extract to the CBER Reference preparation by RAST Inhibition ensures that the all potential allergens will be available.

Supports may be prepared from in-house or commercially purchased extracts if each extract is examined and found to be similar to the CBER Reference with respect to protein content, IEF pattern, immunoblot pattern or Relative Potency by RAST Inhibition in an existing validated system.

Successful solid RAST supports have been prepared with the following extracts; those designated with an * are currently available from CBER:

Short Ragweed	(*Ambrosia artemisifolia*)
Giant Ragweed	(*Ambrosia trifida*)

(continued on next page)

*Timothy Grass	(*Phleum pratense*)
*Perennial Ryegrass	(*Lolium perenne*)
Italian Ryegrass	(*Lolium multiflorum*)
*Redtop Grass	(*Agrostis stoleniferia*)
* Sweet Vernal Grass	(*Anthoxanthum odoratum*)
*Orchard Grass	(*Dactylis glomerata*)
*June Grass	(*Poa pratensis*)
* Meadow Fescue Grass	(*Festuca elatior*)
*Bermuda Grass	(*Cynodon dactylus*)
Velvet grass	(*Holcus lanatus*)
*European Dust Mite	(*Dermatophagoides pteronyssinus*)
*American Dust Mite	(*Dermatophagoides farinae*)

Only extracts of single species of pollen or mite should be coupled to supports.

1. **Defatting of source material:**
When possible, allergenic source materials used to prepare supports for RAST inhibition testing should be defatted. By defatting pollen and other source materials prior to use, filtration of the extracts is less complicated; this operation is significant since an extract without any type of precipitate must be used to prepare the solid supports. The solvent of choice for defatting pollen source materials is petroleum ether (BP 35-57°C) and the safest methodology for performing this process involves the proper use of a Soxhlet apparatus. Source materials should be defatted in cellulose extraction thimbles; care should be taken not to pack the source material tightly in the extraction thimble. Additionally, extraction thimbles of appropriate size for the Soxhlet apparatus selected should be employed.

It is difficult to predict the time necessary for the defatting procedure since different source materials may contain different levels of lipids. As a general guide, CBER source materials are defatted at a rate of four hours in full reflux operation per 10 grams dry weight. If the defatting process removes any color from the source material, the process should be continued until no further color is detectable in the refluxed ether.

An alternative method of defatting involves mixing the source material with petroleum ether or acetone in an Erlenmeyer Flask, allowing the source material to settle and decanting the fluid into an appropriate container. This procedure may be repeated until all color is removed; this may take as many as 5-10 changes of the defatting solution. Ether or acetone should be replaced for each change. Care should be exercised to keep the mixture away from source of sparks or flame, and to prevent inhalation of the fumes. Once the defatting is complete, coarse filter paper may be used to recover the source material.

Following defatting, the source material must be allowed to dry thoroughly to provide an accurate weight. Defatted source material may be covered with a permeable membrane as excess ether or acetone is allowed to evaporate in a well ventilated area.

2. **Extraction of source material:**
The source material should be extracted using methods that produce a full complement of potential allergens in the coupling extract. The optimal extraction method for each source material may be established by comparing extracts made under various conditions from a candidate source material by qualitative Isoelectric Focusing (IEF), Immunoblotting, ninhydrin protein and RAST Inhibition Relative Potency.

Once the optimal extraction conditions for a particular source material have been determined, extracts should be assayed by the ninhydrin method and qualitatively compared to a reference preparation by IEF or other procedures prior to coupling to support.

Extracts containing at least 50% glycerin (e.g., *Dermatophagiodes sp.)* are also suitable for preparation of supports. Freshly prepared extracts may be stored at 0-5°C for 24 to 48 hours after the completion of the extraction process before the coupling procedure is initiated. Alternatively, an extract that does not contain phenol may be frozen at -20°C for up to one year before it is coupled to a solid support. Repeated freezing and thawing of an extract should be avoided and a thawed extract should be filtered and re-examined by IEF prior to use. Extracts may also be stabilized with the addition of sufficient glycerin to produce a reagent that contains at least 50% glycerin; these extracts should be stored continuously at 0-5°C and used within 12 months of extraction. All extracts should be filtered using a 0.45 µm filter prior to coupling.

B. Preparation of Solid Supports

In this phase of the procedure, components of allergenic extracts are immobilized by covalent coupling of the proteins to micro-particulate or other types of solid supports. In most cases, the activation procedure is followed immediately by coupling the allergen to the solid support. Methods necessary to determine the quantity of high quality allergenic extract to adequately sensitize the support are outlined in the next section.

1. Activation of Supports

a. Microcrystalline Cellulose™

The use of Cyanogen Bromide in this procedure requires the use of appropriate safety devices and proper ventilation.

i. Weigh out 0.5 gram of Avicel Microcrystalline Cellulose™ into a 50 ml screw cap centrifuge tube, and wash with 15 ml of 0.1 M HCl by centrifuging the mixture at 2,500 g for 5 minutes.

ii. Wash cellulose three times with distilled water; centrifuge and decant each time.

Perform the following procedures in a fume hood.

iii. Add 30 ml of 0.1 M $NaHCO_3$, pH 8.5 and transfer cellulose into a 100 ml beaker. Chill the mixture in an ice bath.

iv. Place the ice bath on a magnetic stirrer and adjust the pH to 11.0 with 4 M NaOH.

v. Weigh out 1 gram of cyanogen bromide and dissolve in 1 ml of N,N Dimethyl Formamide.

vi. Add all of the cyanogen bromide solution to the cellulose with a capillary pipette.

vii. Adjust the pH of the solution to 11 with 4 M NaOH. Continue to monitor the pH for forty-five minutes, keeping the pH between 10.8 and 11.2.

viii. Pour the mixture into a centrifuge tube; cap and centrifuge for five minutes at 2,500 g at temperatures between 20-25°C.

ix. Decant the supernatant and wash three times with 30 ml of Tris-HCl Buffer by repeating the centrifugation and decanting steps. Discard washings in a fume hood sink and flush with water.

x. Remove final wash and re-suspend the cellulose in 30 ml of Tris-HCl Buffer.

b. Paper Discs

All rinsing solutions and buffers should be at 0-5°C prior to use in order to reduce odors generated during the procedure.

i. Place 1 box of 1000 count 1/4" diameter discs, Type 595 (Schleicher & Schuell, Inc., Keene, NH 03431), in 100 ml distilled H_2O for 30 minutes at room temperature.

ii. Pour mixture of discs and water into Sinterglass funnel and aspirate. Resuspend discs in 100 ml of distilled H_2O at 4° C, washing discs into a 250 ml beaker containing a stirring bar. Initiate gentle stirring with magnetic stirrer.

The following steps must be done in a fume hood.

iii. Add 2.5 gm of Cyanogen Bromide to solution containing discs and maintain pH at 10.5 with NaOH for 10 minutes.

iv. Pour mixture into funnel and aspirate.

v. Wash 5 times with 1 liter quantities of 0.1 M $NaHCO_3$. Discard wash in a fume hood sink.

vi. Wash 5 times with 1 liter quantities of distilled H_2O.

vii. Wash 4 times with 250 ml aliquots of acetone.

viii. Place discs in a shallow container and cover with porous paper.

ix. Allow discs to dry overnight in a well ventilated area.

x. Store discs in a closed container and desiccated at -20°C.

b. Titration Procedure

If the extract is found to be satisfactory, titrate the solid support to determine the amount of extract necessary to saturate the solid support. The procedure for this operation follows:

i. Prepare the following dilutions of characterized extract in Tris-HCL buffer

Tube 1: 5.0 ml extract. 5.0 ml buffer (Support A)
Tube 2: 2.5 ml extract. 7.5 ml buffer (Support B)
Tube 3: 1.0 ml extract, 9.0 ml buffer (Support C)
Tube 4: 0.5 ml extract, 9.5 ml buffer (Support D)

ii. To each of the four tubes containing extract dilutions, add 0.15 ml Matrex™ or activated Microcrystalline Cellulose™.

iii. Rotate or mix gently overnight at 0-5°C.

iv. Centrifuge at Relative Centrifugal Force (RCF) sufficient to precipitate solid support and decant supernatant.

v. Wash 3 times by resuspending pellet in 5 ml RAST A buffer, followed by centrifugation and decanting the supernatant.

vi. Allow coupled support to equilibrate with RAST A buffer for at least three hours (or overnight) with gentle mixing.

vii. Repeat washing procedure described in step v.

viii. Resuspend solid support in 10 ml RAST A buffer. Store at 0-5°C until used, being careful to resuspend support completely before use.

c. Titration Procedure for Each Test Support

Perform the following procedure for each of the four supports prepared with different dilutions of extract (i.e. testing of four supports would result in 32 tubes, eight tubes per support).

i. Prepare four tubes containing positive sera (30-50 µl of allergic pooled sera) and 450 µl RAST A.

ii. Prepare four tubes containing negative sera or 5% Human Serum Albumin (HSA) (30-50 µl of sera) and 450 µl RAST A.

iii. Add 300 µl of the support to each tube.

iv. Perform a direct RAST test on these tubes:

a. Rotate tubes overnight at room temperature.

b. Wash three times with 2.5 ml of RAST B buffer. by centrifuging at Relative Centrifugal Force (RCF) sufficient to precipitate solid support and decant.

c. Add approximately 2-4 µg of radiolabelled anti-IgE (activity of 20,000-30,000 CPM) in RAST A to each tube in a total volume of 500 µl.

d. Rotate tubes overnight at room temperature.

e. Wash three times with 2.5 µl of RAST B buffer, using the same centrifugation and decanting procedures.

f. Cap tubes and count in a gamma counter.

v. Determine the mean positive and mean negative counts for each support.

An example of the results of the titration is summarized in the following table:

Support	Positive Mean	Negative Mean	Ratio of Positive to negative
A	6500	600	10.8
B	6200	600	10.3
C	4500	400	1 1.3
D	1000	200	5.0

Based on this evaluation, support A, B or C would be considered fully saturated. To conserve materials yet ensure maximal saturation, the relative proportions of extract and activated Microparticulate used to prepare support B would be used on a routine basis for extracts with comparable ninhydrin protein content and IEF pattems.

It is advisable to repeat this procedure for each new support. However, if a large quantity of extract is prepared and tested, it is possible to freeze aliquots of the extract at -20°C for future use and use the proportions indicated in a similar study to prepare several lots of support. Repeated thawing and freezing of the extract should be avoided and extracts prepared in buffers or solutions containing phenol (rather that distilled or deionized water) may precipitate with freezing. Thawed extracts should be re-examined by IEF prior to use.

Similar procedures should be employed to determine the amount of extract necessary to maximally sensitize other types of support media.

3. Allergen Coupling and Support Blocking

This generalized procedure provides a laboratory worksheet for preparation of a solid support. Specific ratios of extract and particulate must be determined from case-by-case titration studies outlined in the preceeding section.

a. Microcrystalline cellulose™

i. Following activation, add a sufficient quantity of allergenic extract to couple the cellulose as determined in titration studies (see section II.B.2.b.).

ii. Rotate for 20 hours at 4°C.

iii. Centrifuge for 15 minutes at 2500 g or at RCF sufficient to precipitate the solid support and decant.

iv. Wash the precipitate with 30 ml of RAST A Buffer, centrifuging to precipitate particulate support. Repeat this procedure three times, fully resuspending the precipitate with each wash.

v. Resuspend in 300 ml of RAST A Buffer and store at 0-5°C.

vi. Test for positive and negative control ratio; then perform a limited Rast inhibition assay to confirm reagent quality.

b. Paper Discs

i. Add the appropriate quantity of allergen solution to each activated disc. If conditions have been tested, batch sensitization of the discs may be performed.

ii. Allow the allergen and disc to couple for 1-3 hours at room temperature or overnight at 0-5°C.

iii. Wash the membrane or discs three times with RAST A Buffer.

iv. Since discs are sensitized individually, testing positive and negative controls must be performed to determine the reproducibility of the discs before use in a RAST inhibition assay.

c. Matrex™-This is the current method used by CBER. All dosage testing on the support will be done prior to supplying the support to the testing facility; the dosage is included with the support.

i. A quantity of allergenic extract containing between 40 and 50 mg protein and an equal quantity of Tris-HCl buffer are mixed thoroughly and vortexed during the addition of 2.0 ml of MATREX™.

ii. Rotate the mixture for at least 20 hours at 0-5°C.

iii. Centrifuge at 2500 x g at 4°C for 30 minutes, or sufficient RCF force and time to precipitate solid support and decant.

iv. Discard the supernant and gently dislodge the pellet.

v. Wash the the allergen-coupled Matrex™ 3 times with 30 ml volumes of RAST A Buffer, pH 7.5 by repeating the centrifugation, decanting the supernatant and adding additional RAST A Buffer.

vi. After decanting the last wash, the coupled support is resuspended in 300 ml of RAST A Buffer and stored at 0-5°C.

vii. Determine quantity of support dose by performing a limited RAST assay.

a.) Label eight tubes for each support dose to be tested. Usually 5 doses are tested starting at 300 µl, and decreasing each time by 50 µl. Therefore, set up 40 tubes.

b.) Pipette into each tube 450 µl RAST A buffer.

c.) Add 30 µl positve serum pool to 20 tubes and 30 µl negative serum pool (5% Human Serum Albumin (HSA)) to 20 tubes.

d.) Add 300 µl coupled support to 4 postive serum pool tubes and 300 µl coupled support to 4 negative serum pool tubes. Continue adding decreasing amounts of matrix to the tubes as above.

e.) Cap and rotate overnight at room temperature.

f.) Remove tubes, centrifuge at 3,000 at 4° C for 15 minutes and decant.

g.) Wash each tube with 2.5 ml of RAST B; repeat centrifugation as in (e.).

h.) Repeat washing procedure (g.).

i.) Add 25 µl containing approximately 12 ng of antibody and 20,000 CPM of I^{125} labeled anti-human IgE and 500 µl of RAST A.

j.) Cap and rotate overnight.

k.) Repeat washing procedure as outlined above (steps 5-7).

l.) Cap and count in a gamma counter.

The tubes containing the positive serum should have at least 5 times the CPM of tubes containing the negative control HSA and the negative control should not exceed 400 CPM. It is preferable to have the negative control less than 200 CPM.

viii.) Perform a RAST Inhibition (see section III) using only the reference and a duplicate with selected dose to see if an inhibition curve is generated. If the curve has leveled off at a value of less than 90, decrease the amount of support added and retest. If the curve appears to be low, increase the amount of extract added to all tubes and retest.

d. Pre-Activated Papers

i. Add the appropriate quantity of allergen solution to each pre-activated disc or to the membrane sheet.

ii. Allow the allergen and disc to couple for 1-3 hours at room temperature or ovemight at O-5°C.

iii. Wash the membrane or discs three times with RAST A buffer.

iv. If discs are sensitized individually, testing positive and negative controls must be performed to determine the reproducibility of the supports before use in a Rast Inhibiion assay.

The shelf life of the coupled supports will vary with individual allergens. A support may be losing allergenic activity if problems such as a loss of reproducibility in the duplicate samples is noticed. Increased numbers of counts bound by the negative control, with a subsequent decrease in the ratio of positive to negative control values also indicates possible loss of activity in the allergen support. All coupled supports that are more than 6 months old should be tested by a direct RAST prior to use in an inhibition procedure, to check background levels and positive controls.

C. Allergic Serum Pools

1. Selection of Allergic Serum Pools

Pools of allergenic human serum used to determine the relative potency of allergenic extracts by RAST inhibition procedures should be assembled from individual sera that have been tested and found to be free of antibody to hepatitis and HIV. Sera or plasma may be taken from individuals with a history of clinical symptoms to the allergen of interest, as well as positive skin tests from prick-puncture procedures with stock concentrate. Direct RAST values of the patient sera should be greater that 5 times background; immunoblotting studies should indicate that the sera has IgE antibody binding to proteins in the electro-focused extracts. Evaluation of a number of patient sera is necessary to identify characteristic IgE binding immunoblotting patterns in a target extract; combining sera with different profiles of IgE binding should permit the development of a serum pool that will recognize numerous IgE binding proteins in RAST inhibition assays. The selection of a serum pool must be coordinated with the optimization of extraction conditions for a particular extract and the preparation of a saturated support with a particular extract. Reference serum pools and supports for testing in-house reagents will be available for testing from CBER as new extracts are proposed for standardization. quantities of serum and support used may differ depending on the properties of each individual system. The numbers used in the following assays are typical of the quantities used in CBER assays.

2. Testing of Allergic Serum Pools

a. Serum testing

i. Dispense 200 µl of support into each of eight test tubes. Microparticulate support must be thoroughly resuspended before dispensing.

ii. Add 50 µl of the positive serum pool into each of tubes 1 to 4, followed by 200 µl of RAST A.

iii. Add 50 µl HSA to tubes 5 through 8, followed by 200 µl of RAST A.

iv. Cap and rotate overnight at room temperature.

v. Remove tubes, centrifuge at 3,000 at 4° C for 15 minutes and decant.

vi. Wash each tube with 2.5 ml of RAST B; repeat centrifugation as in Step 5.

vii. Repeat washing procedure (step 6).

viii. Add 25 µl containing approximately 12 ng of antibody and 20,000 CPM I^{125} labeled anti-human IgE and 500 µl of RAST A.

ix. Cap and rotate overnight.

x. Repeat washing procedure as outlined above (steps 5-7).

xi. Cap and count in a gamma counter.

The positive serum should have at least 5 times the CPM of the negative control HSA and the negative control should not exceed 400 CPM. It is preferable to have the negative control less than 200 CPM.

D. Anti-Human IgE

1. Specifications

A satisfactory anti-human IgE should be directed to the FC fragment or the epsilon chain of human IgE and should be purified by affinity chromatography. In most cases, use of a myeloma protein as the immunogen simplifies this task. Testing has shown that the PS and ND myeloma preparations have similar reactivities. The preparation must be free of antibody directed to IgG class antibodies and other immunoglobulins. Antibodies have been prepared in rabbits and goats (Atlantic Antibodies, Westbrook, Maine 04092). The activity of other anti-human IgE antibodies, both polyclonal and monoclonal, should be determined in direct testing experiments comparing several pools of allergic sera with well characterized supports.

a. Dispense 200 µl of support into each of eight test tubes. Microparticulate support must be thoroughly resuspended before dispensing.

b. Add 50 µl of the positive serum pool into each of tubes 1 to 4, followed by 200 µl of RAST A.

c. Add 50 µl 5% Human Serum Albumin (HSA) to tubes 5 through 8, followed by 200 µl of RAST A.

d. Cap and rotate overnight at room temperature.

e. Remove tubes, centrifuge at 3,000 g at 4 °C for 15 minutes and decant.

f. Wash each tube with 2.5 ml of RAST B; repeat centrifugation as in Step 5.

g. Repeat washing procedure (step 6).

h. Add 25 µl containing approximately 12 ng of antibody and 20,000 CPM of 1^{125} labeled anti-human IgE and 500 µl of RAST A.

i. Cap and rotate overnight.

j. Repeat washing procedure as outlined above (steps 5-7).

k. Cap and count in a gamma counter.

The anti-human IgE should show a similar response, in terms of the ratio of positive to negative controls, as previously used antibody preparations. It is preferable to have the negative control less than 400 CPM.

2. Iodination Procedure

a. Make the following two solutions just prior to labeling:

   I. Sodium meta Bisulfite
   40 mg/10 ml of O.5 M RAST Phosphate buffer.

   II. Chloramine-T
   30 mg/ 10 ml of 0.5 M RAST Phosphate buffer.

b. Rehydrate a PD-10 Sephadex G-25 M column (from Pharmacia or equivalent) with 15 ml RAST A Buffer.

c. Take materials and equipment to the labeling area.

d. Put 20 µl of NaI^{125} solution containing 1 mCi into a reaction vial.

e. Add 200 µl 0.5 M PO_4 buffer.

f. Add 20 µl of anti-IgE containing about 20 mg of antibody

g. Add 20 µl Chloramine-T solution and stir for 60 seconds on magnetic stirrer.

h. Add 100 µl of sodium metabisulfite solution and stir for 30 seconds.

i. Add contents of vial to column. Wash vial out twice with 100 µl of 0.5 M PO_4 buffer. Allow solution to enter column before adding buffer.

j. Wash column with 0.1 M PO_4 buffer and collect 10 1 ml aliquots .

k. Count each tube. There will be two peaks; the ^{125}I-labeled-anti-IgE is the first peak and free ^{125}I is the second peak. The first peak may be contained in a single tube; this can be used if the peak is sharp, or pool tubes in the first peak Discard the second peak containing free isotope.

l. Dialyze overnight against one liter of 0. I M PO_4 buffer at 0-5°C. Change the dialysate and continue to dialyze for at least 6 hours more or until 1 ml of dialysate contains only background CPM levels.

m. 20-25 µl of iodinated antibody should contain approximately 12 ng of protein and 20,000-25,000 CPM when used in inhibition assays.

Note: Radiolabeled anti-human IgE is available from several sources including Pharmacia Diagnostics, Piscataway, NJ 08854.

III. The RAST Inhibition Procedure

The following procedure is designed for an automated pipetting system, i.e., Micromedic or similar instrument.

A single assay of a product or a reference consists of the mean of duplicate samples of each allergen dilution. More than one assay of a product may be performed in a single RAST inhibition test, although CBER recommends that testing of a given sample be performed on at least two separate occasions.

Statistics and procedures detail the use of a microparticulate support; use of another type of support will result in some modifications in technique, as well as the determination of appropriate statistics.

Two separate assays of the reference preparation should be included in each RAST inhibition test to insure test validity. If any criteria for the second reference (quality control reference) is invalid (less than 4 points, failure to bracket the 50% inhibition value, r <0.9, Student's t-test failure, or relative potency not within the 5 test limit) the test is considered invalid and no further calcualtions are performed. All test extracts are run a minimun of three times and a maximun of five times ie, separate dilution series, for a valid test. If any criteria for an independant run is not valid (less than 4 points, failure to bracket the 50% inhibition value, r <0.9 or Student's t-test failure) that run is considered invalid and may be repeated.

A. DAY ONE

1. Prepare serial three fold dilutions of reference, test extracts and quality control reference in RAST A buffer. A series of at least six dilutions should be prepared to permit the best-fit line to be computed from a minimum of four points. All valid points must be included in the calculation of the best-fit line.

2. Pipet 50 µl of each dilution into two separate tubes, using an automatic pipetting device and washing the allergen sample into the tube with 450 µl of RAST A Buffer. After this step, all tubes should contain 500.

3. Prepare eight tubes with 500 µl of RAST A Buffer without allergen to be used as the postitive and negative controls.

4. Add 30 µl of positive serum pool to each tube containing allergen dilution and the four positive control tubes.

5. Add 30 µl of 5% HSA or normal serum to the remaining four negative control tubes.

Note: If other types of pipetting devices are employed, the amounts of buffer used in steps 2 through 4 may differ. Adequate washing volumes should be reviewed by the operators.

6. The predetermined amount of support is then added to each tube. The support must be uniformly suspended before dispensing.

7. Cap the tubes and rotate at room temperature overnight.

B. DAY TWO

1. Remove tubes from the rotator and centrifuge with sufficient force to precipitate microcrystalline support. Decant and discard supernatant.

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the

3. Repeat washing procedure described in step 2.

THE FOLLOWING STEPS ARE TO BE DONE UNDER PROPER RADIOISOTOPE CONDITIONS.

4. Add 20-25 µl of ^{125}I- labeled-anti-IgE containing approximately 20,000-30,000 CPM. Add sufficient RAST A so that all tubes contain a final volume of 500 ml.

5. Prepare an additional tube containing only isotope and buffer to verify the number of counts added in the assay and set aside until counts are performed on the following day. Cap and rotate overnight at room temperature, taking proper radioisotope precautions.

DAY THREE: WORK TO BE DONE UNDER PROPER RADIOISOTOPE CONDITIONS.

1. Remove tubes from rotator and centrifuge to precipitate microcrystalline support. Leave the tubes capped during the centrifugation to avoid contamination of the centrifuge. Uncap and decant the tubes carefully, disposing of waste radioactivity in a proper manner.

2. Wash each tube with 2.5 ml of RAST B, following the procedure as in step 2 of day two.

3. Repeat washing procedure.

4. Recap tubes and count in a gamma counter.

VIII. CALCULATION OF RELATIVE POTENCY

1. Calculate the average positive control values (APC).

2. Calculate the average negative control values (ANC).

3. Calculate the average duplicate CPM values for each dilution.

4. For each dilution of allergen, calculate the percent inhibition as follows:

$$\frac{\text{APC} - \text{Average Duplicate CPM dilution}}{\text{APC-ANC}} \times 100 = \%\ \text{inhibition}$$

In each assay of test sample or reference, there should be at least four dilutions for which the inhibition values are not less than 10% or greater than 90%. All points in the range between 10 and 90% inhibition must be included in the calculations; however those values outside of the range are not included. These inhibition values should bracket the 50% value.

5. Calculate individual best-fit lines by linear regression computations for the reference and the test samples, using the formula:

$$Y = A + B[\log_{10}(x)]$$

where:

Y=% inhibition
A=Y axis intercept
B=slope
X=volume of sample in microliters

6. For each test product or reference, the correlation coefficient (r) for the best fit line should be greater than 0.9. If r is less than 0.9, that run is considered invalid, and no further computations are performed on that sample. If the r value for either run of the reference is less than 0.9, the entire assay is invalid.

7. Using Student's t test, check for the parallelism of the regression lines of the reference and each individual test sample. If the lines are not parallel at p =0.01, then the run of this test product is considered to be invalid, and no further computations are performed. Other test samples in the same assay may meet this requirement and computation for these test samples would continue.

8. For each sample using the combined data of reference and sample, calculate the best-fit parallel lines, i.e., compute the common slope and the two new intercepts.

9. Using the slope and the intercepts of the parallel lines, calculate the horizontal distance between these two lines. This is accomplished by subtracting the intercept of the reference from the intercept of the test sample and dividing by the common slope . This distance is the log of the relative potency [log (r.p.)] of the test sample as compared to the reference.

10. Perform at least three independent valid assays on each test product, and calculate the mean and standard deviation of the [log(r.p.)] values. ALL VALID ASSAYS MUST BE INCLUDED. If the standard deviation of the mean of the [log(r.p.)] values is equal to or less than the upper limit calculated from a validation study for the corresponding number of valid assays, the variability of the [log(r.p.)] is acceptable.

11. Determine the geometric mean (G.M.) of the relative potency by taking the antilog of the mean of the [log(r.p.)] values. If the G.M. is between the limits determined from a validation study for the corresponding number of tests, the test material is considered to be equal in potency to the reference extract.

If a lot is not considered to be equal to the reference extract when the data is based on three tests, additional tests may be performed, up to a maximum of five tests. However, all valid tests must be included in the computations and the product must then meet the limits imposed for the total number of valid tests.

In addition, a test is not considered to be valid if the duplicate analysis of the reference preparation fails any statistical parameter of the test. The r.p. of the test reference preparation must fall within the 5 test range, or the test is considered to be invalid.

Statistical Analysis by Dr. Suresh Rastogi
MEB 4/94 (Revised)

Procedure:

1. Prepare triplicate samples and standards in a total volume of 100 µl using the cryotubes. Samples should contain 3-12 total protein. Samples should be prediluted with water to bring the estimated protein concentrations into the range (30-120 µg/ml) of the standard curve. In prediluting samples, a sufficient volume of solution should be pipetted (50 µl or more) to assure accuracy.

NOTE: Special precautions should be observed in prediluting samples containing 50% glycerol in preparation for assay. If a micropipetting device is used, calibrated tips should be employed to assure that the volume taken is correct. It is necessary to wash the pipette with the diluted solution several times after dispensing the sample, whether using a micropipette or a larger volume (0.5-1.0 ml) pipette. Dilutions performed in this manner gave protein values within 4% of the value determined spectrally for a BSA solution. If the pipetting device was not washed, the values obtained were up to 30% lower.

The standard curve should be constructed as follows (triplicate samples):

Vol Stock BSA Sol'n (µl)	Vol Water (µl)	Total Protein (µg)
0	100	0
50	50	6.0
100	0	12.0

The total protein values given are based on a 120 µg/ml stock solution. The protein values used in the calculations should be based on a spectral determination of the actual concentration of the BSA solution (see item 7 under Materials and Reagents).

2. Add 100 µl of 10 M NaOH, cap tubes, gently mix, and incubate at 95°C for 17 hours. Allow tubes to cool to 75°C and centrifuge for 5 min. at 3000 rpm. Uncap tubes and maintain at 75°C for 2 hours to drive off free ammonia.

NOTE - These heating steps are performed in a heating block with 10 mm diamter wells.
Satisfactory results have also been obtained using an oven.

3. Allow tubes to cool to ambient temp. Add the predetermined volume of conc. HCL to each tube to neutralize the reaction mixtures. Normally this volume is 80-95 µl. Gently mix.

NOTE - The volume of 12 M HCl required to neutralize the base can be determined as follows: Prepare several mock reaction mixtures containing 100 µl 10 M NaOH, 300 µl citrate buffer pH 5.0 , and sufficient water to obtain a reading on a pH meter (the volume of water needed is about 1 ml for a semimicro combination electrode). Various amounts of HCl are added to different tubes until a pH of 5.0 is obtained, and this volume is used to neutralize all of the tubes carried through the colorimetric reaction (steps 4-6). The pH of the solutions of the reactions carried through the colorimetric reaction can be checked on a pH meter for consistency, but the apparent pH values are about 0.5 pH units higher because of the presence of organic solvents.

4. Add 600 µl of ninhyrin reagent (see item 3 under Materials and Reagents) to each tube. Mix thoroughly. Cap tubes and place directly in boiling water. The rapid boiling assures uniform heating.

5. Allow tubes to cool to ambient temp. Dilute sample 1:1 in 50% 1-propanpol. Mix well on vortex mixer. Read absorbance at 570nm. Calibrate the spectrophotometer by reading the absorbance of 25% 1-propanol.

6. If absorbance at 570 nm is above range of standard, it can be further diluted with diluent unless a precipitate has formed.

Calculations:

1. Calculate linear regression for absorbance at 570nm vs Protein (µg) for the standard using the mean absorbance at 570nm values for the 3 data points. Criteria for valid assay: slope, 0.045-0.080 (2 SD of the mean); y-intercept <0.4; correlation coefficient >0.98. These criteria are based upon data from 72 assays performed over a 2 year period.

NOTE-A representative standard curve is shown below.

2. Calculate protein content of unknowns from linear regression curve. The absorbance of the unknowns should be within range of standard curve (3-12 µg).

NOTE - If the sample was diluted to bring the absorbance at 570nm into the range of the standard curve, then the absorbance at 570nm should be multiplied by the dilution factor prior to calculating the protein from the extrapolated standard curve.

3. The between test variability is +/-25% (2 SD of the mean) based on data from the repeat tests (N=9) performed on frozen aliquots of the same protein solution over the course of 2 weeks.

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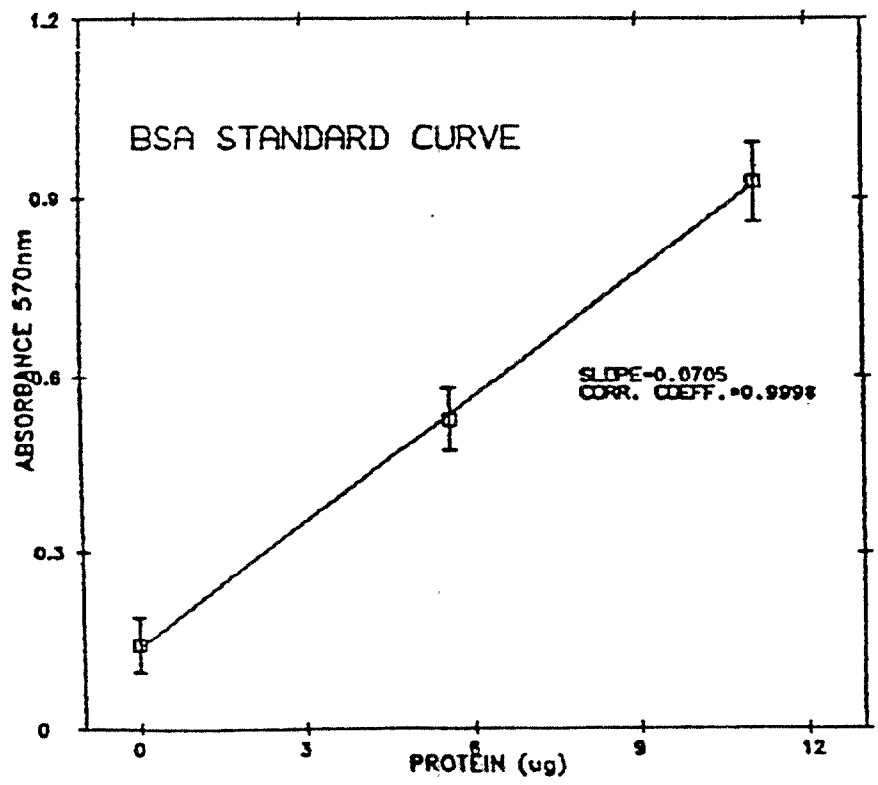


II. Supplemental in vitro Test Methods

14. 15 ml conical tubes, available from Falcon, Cat.No. 2095.

15. 50 ml conical tubes, available from Falcon, Cat.No. 2070.

16. HRP-labeled anti-Human IgE, 1.0 mg/ml, Kirkegaard & Perry Laboratories, Inc. Cat.No. 074-1004.

17. Amersham ECL Western blotting detection reagents, available from Amersham Cat.No. RPN 2106.

18. Gel Support Film for Agarose, available from Bio-Rad, Cat.No. 170-4251.

19. X-ray film, available from Kodak, Cat.No. 165-1579.

20. X-ray film cassettes.

21. Film developing facility and reagents.

22. Timer.

PROCEDURE

A. Optimization of the antiserum and conjugate.

1. Prepare a worksheet to indicate 12 lanes of an allergenic extract and the amounts of antiserum and conjugate that will be added to each lane. For example:

Lane	Antiserum, ml	Conjugate Dilution
1	0.5	1:10,000
2	1.0	1:10,000
3	1.5	1:10,000
4	2.0	1:10,000
5	0.5	1:50,000
6	1.0	1:50,000
7	1.5	1:50,000
8	2.0	1:50,000
9	0.5	1:100,000
10	1.0	1:100,000
11	1.5	1:100,000
12	2.0	1:100,000

2. Isoelectric focus 12 lanes of the allergenic extract. After isoelectric focusing, place the gel and the template on a glass plate or other solid, flat surface. Make sure the gel is lined up correctly on the template and then remove the applicators.

3. Cut a piece of nitrocellulose membrane and overlay the gel with it. Use a Nitrocellulose Marking Pen to mark the application sites appropriately so that each lane can be identified.

4. Place several absorbent tissues on top of the membrane and cover with a heavy weight. Leave undisturbed for a minimum of 1/2 hour.

5. Remove the weight and absorbent tissues, and trim any excess nitrocellulose from the edges with an unused one-sided razor blade. Cut the membrane between each of the lanes.

6. Place the gel/membrane into a container and pour enough blocking solution in to just cover the gel (approximately 200 ml). Allow the gel/membrane to soak for 1-2 minutes.

7. Gently peel the membranes from the gel and gel backing. For each lane, place the membrane (gel side down) on the filter paper backing. Gently peel the membrane up leaving as much gel as possible on the filter paper backing. Wet the membrane periodically in blocking solution to prevent drying. Remove any remaining gel from the membrane by gently wiping with a tissue. All gel must be removed.

8 Place the membranes in fresh blocking solution and set on an orbit shaker for 1 hour minimum.

9. Prepare the antiserum by filtering through a 0.45 micron filter.

10. Place each membrane in a 15 ml conical tube and add enough washing solution to fill the tube halfway.

11. Add the appropriate amount of filtered antiserum to each conical tube according to the worksheet.

12. Cap the tubes and rotate for three hours at room temperature or overnight in the refrigerator.

13. Wash the membranes in one of two ways:

    a. Place the membranes in a container with a large volume (approximately 300-500 ml) of wash/block solution. Rotate on orbit shaker for a minimum of 1 hour.

    b. Discard liquid from conical tubes, fill with wash/block solution and put on rotator for 5 minutes. Repeat for a total of three washes.

14. While the membranes are washing, prepare the dilutions of HRP-labeled anti-Human IgE (conjugate) according to the worksheet.

15. If using a wash container on the orbit shaker, place the washed membranes in conical tubes and fill the tubes with filtered, diluted conjugate according to the worksheet.

    If using conical tubes, discard the wash/block solution after the final wash. Fill the tubes with filtered, diluted conjugate according to the worksheet.

16. Allow the membranes to rotate a minimum of 3 hours at room temperature or overnight in the refrigerator.

17. Wash the membranes as in step 13 above.

18. After the membranes are washed, prepare the detecting reagent by mixing equal volumes of Reagent 1 and Reagent 2. The membranes are incubated with the detecting reagent at room temperature for exactly one minute.

   a. If the membrane is in a conical tube, discard the liquid and add the detecting reagent directly to the conical tube. Cap the tube and roll it on a flat surface to allow full contact between the reagent and all parts of the membrane for 1 minute. At the end of 1 minute, open the tube, remove the membrane and blot gently on absorbent tissue. Place the membrane protein side up (the identification mark will be face down) on Gel Support Film. Cover with another Gel Support Film. (More than one membrane can be put on the Gel Support Film.)

   b. If the membranes are not in conical tube, remove the membranes (up to 4 membranes may be exposed to the detecting reagent at one time) from the wash/block solution and place in a small, clean container. Pour the detecting reagent onto the membranes and start the timer for 1 minute. Gently tilt the container to allow full contact between the detecting reagent and all sections of the membranes. At the end of 1 minute, remove the membranes and blot gently on absorbent tissue. Place the membranes protein side up (the identification mark will be face down) on Gel Support Film. Cover with another Gel Support Film. (More than one membrane can be put on the Gel Support Film.)

19. Place the membranes (sandwiched in Gel Support Film) into an X-ray cassette with the protein side up (the identifying marks will be facing down). Ensure that there is no free detection reagent in the cassette; do not allow the film to get wet.

20. In the darkroom under red safelight, place a sheet of X-ray film on top of the blots. Close the cassette and expose for 1 minute. Remove the film and develop it. Meanwhile, place another unexposed sheet of film in the cassette, close the cassette, and expose for 2 minutes.

21. On the basis of the appearance of the first film, estimate how long to expose further films. Exposure times can vary from 5 seconds to 5 minutes. Exposure times up to 1 hour may be used, although this is generally not necessary.

22. Determine the optimal amount of antiserum and the conjugate dilution based on the appearance of the X-ray film. Choose the combination that yields distinct bands with little background.

3. BIEF-LIGHT PROCEDURE

1. After isoelectric focusing, place the gel and the template on a glass plate or other solid, flat surface. Make sure the gel is lined up correctly on the template and then remove the applicators.

2. Cut a piece of nitrocellulose membrane and overlay the gel with it. Use a Nitrocellulose Marking Pen to mark the application sites with the appropriate labels so that each lane can be identified.

3. Place several absorbent tissues on top of the membrane and cover with a heavy weight. Leave undisturbed for a minimum of 1/2 hour.

4. Remove the weight and absorbent tissues, and trim any excess nitrocellulose from the edges with an unused one-sided razor blade. The membrane may also be cut to separate lanes, if desired. If the same antiserum is going to be added to all the lanes, the membrane may be cut to hold up to 4 lanes per section.

5. Place the gel/membrane into a container and pour enough blocking/washing solution in to just cover the gel (approximately 200 ml). Allow the gel/membrane to soak for 1-2 minutes.

6. Carefully separate the plastic support film from the membrane/gel strip. Place the membrane (gel side down) on the filter paper backing. Gently separate the membrane strips from the gel as the gel adheres to the filter paper backing. Wet the membrane periodically in blocking/washing solution to prevent drying. Remove any remaining gel from the membrane by gently wiping with a tissue. All gel must be removed.

7. Place the membrane (free from gel residue) in fresh blocking/washing solution and set on an orbit shaker for 1 hour minimum.

8. Prepare the antiserum by filtering through a 0.45 micron filter.

9. Place the membrane in a conical tube and add enough washing solution to fill the tube halfway. (If the membrane contains 3 or 4 lanes, use a 50 ml conical tube; if there are 1-2 lanes on the membrane, a 15 ml conical tube may be used.)

10. Add the appropriate amount of filtered antiserum as determined by the optimization process. If more than one lane is contained in the conical tube, be sure to add the right amount of antiserum for each lane. Fill the tube to the top with blocking/washing solution and cap tightly.

11. Allow to rotate for three hours at room temperature or overnight in the refrigerator.

12. Wash the membranes in one of two ways:

    a. Place the membranes in a container with a large volume (approximately 300-500 ml) of blocking/washing solution. Rotate on an orbit shaker for minimum of 1 hour.

    b. Discard liquid from conical tube, fill with blocking/washing solution and put on a rotator for 5 minutes. Repeat for a total of three washes.

13. While the membrane is washing, prepare the appropriate dilution of conjugate as determined by the optimization process.

14. If using a wash container on the orbit shaker, place the washed membranes in conical tubes and fill the tubes with filtered, diluted conjugate according to the worksheet.

    If using conical tubes, discard the blocking/washing solution after the final wash. Fill the tubes with filtered, diluted conjugate according to the worksheet.

15. Allow the membranes to rotate a minimum of 3 hours at room temperature or overnight in the refrigerator.

16. Wash the membrane as in step 12 above.

17. After the membranes are washed, prepare the detecting reagent by mixing equal volumes of Reagent 1 and Reagent 2. The membranes are incubated with the detecting reagent at room temperature for exactly one minute.

   a. If the membrane is in a wash container, remove the membranes (up to 4 membranes may be exposed to the detecting reagent at one time) from the wash/block solution and place in a small, clean container. Pour the detecting reagent onto the membranes and start the timer for 1 minute. Gently tilt the container to allow full contact between the detecting reagent and all sections of the membranes. At the end of 1 minute, remove the membranes and blot gently on absorbent tissue. Place the membranes protein side up (the identification mark will be face down) on Gel Support Film. Cover with another Gel Support Film. (More than one membrane can be put on the Gel Support Film.)

   b. If the membrane is in a conical tube, discard the liquid and add the detecting reagent directly to the conical tube. Cap the tube and roll it on a flat surface to allow full contact between the reagent and all parts of the membrane for 1 minute. At the end of 1 minute, open the tube, remove the membrane and blot gently on absorbent tissue. Place the membrane protein side up (the identification mark will be face down) on Gel Support Film. Cover with another Gel Support Film. (More than one membrane can be put on the Gel Support Film.)

18. Place the membranes (sandwiched in Gel Support Film) into an X-ray cassette with the protein side up (the identifying marks will be facing down). Ensure that there is no free detection reagent in the cassette; do not allow the film to get wet.

19. In the darkroom under red safelight, place a sheet of X-ray film on top of the blots. Close the cassette and expose for 1 minute. Remove the film and develop it. Meanwhile, place another unexposed sheet of film in the cassette, close the cassette, and expose for 2 minutes.

20. On the basis of the appearance of the first film, estimate how long to expose further films. Exposure times can vary from 5 seconds to 5 minutes. Exposure times up to 1 hour may be used, although this is generally not necessary.

NOTES

1. If the background is high, filter all reagents through a 0.45 micron filter. The combined detecting reagent may be diluted 1:4 with blocking/washing solution if high background persists after filtering.

2. Membranes may be stored wet and wrapped in plastic wrap in a refrigerator after immunodetection for reprobing to confirm or clarify results.

CAM 9/93

Blotted Radio-immuno IsoElectric Focusing Procedure (BRIEF)

Introduction:

Blotted Radio-immuno IsoElectric Focusing or BRIEF is used to visualize the IgE binding components in an allergenic extract. This method amplifies the information available from a simple IsoElectric Focusing (IEF) performed in commercially prepared acrylamide gels. Focused extract components are passively transferred to a Nitrocellulose (NC) membrane which is treated with sera from allergic patients. Treatment of the membrane with I-125 labeled anti-human IgE then reveals the separated extract components that bind human IgE. Through the exposure of the treated membranes to X-Ray films and comparison of the films to stained IEF gels, individual extract components may be identified and studied.

Several different types of evaluations can be performed using the BRIEF procedure. Part I describes the use of the BRIEF method for comparing allergenic extracts prepared from different source materials or methods. Part II outlines a method for comparing IgE binding activity of sera from individual allergic patients. Part III describes a BRIEF inhibition procedure where an inhibitor competes with the membrane bound extract for IgE binding sites. At this time, this method remains a qualitative tool for the study of allergenic extracts, patient sera reactivity and inhibition of IgE binding. In all of these evaluations, the basic procedure involves the passive transfer of electrophoresed extract components from an non-fixed, non-stained acrylamide IEF gel. In the future, it may be possible to transfer untreated protein components from other types of IEF gels (i.e. agarose); it may also be possible to use other types of labels to reveal the bound allergen specific IgE.

Reagents:

1. Blocking Solution (RAST A): 0.1 M PO_4, pH 7.5, containing 1% Tween 20 and 1% Bovine Serum Albumin (BSA)

2. Washing Solution (RAST B): 0.1 M PO_4, pH 7.5, containing 1% Tween 20

3. I-125 labeled anti-human IgE

4. Nitrocellulose (NC), S&S Type BA85™ or Immobilon™, Millipore.

5. Plastic Support Film: Gel-Bond FMC or Plastic Support Fim for Agarose, Bio-Rad

6. Kodak X-OMat AR Film

**Note: Activity of some enzymes may be inhibited with the use of Phosphate Buffer; this should be examined if an alternate procedure is developed.

Procedures:

Part I. Comparison of Allergenic Extracts using BRIEF
DAY ONE

1. Separate extract components using IsoElectric Focusing (IEF) as described in the CBER method for IEF in polyacrylamide gels. The blotting procedure is initiated when the gel is removed from the apparatus; **do not fix or stain the portion of the gel to be blotted.** Since the physical procedure of blotting will destroy the gel, prepare a duplicate section of gel containing extract for staining. If possible, the sample to be stained and the sample to be blotted should be assayed on the same gel. It is helpful to use a template with marked sample application sites which will be visible when sample applicators are removed.

2. Upon completion of the IEF, remove the gel and template, placing both on a glass plate. Remove the sample applicators and electrode wicks; then remove the section to be fixed and stained, using a sharp blade or scissors to cut through the acrylamide gel and the plastic gel backing. Allow the portion of the gel to be blotted to remain stationary on the template, so the application sites are marked when the applicators are removed.

3. Cut a piece of NC to fit area of the gel to be blotted, handling the membrane with gloves to prevent the transfer of fingerprints. If using NC, do not pre-treat or pre-wet the membrane. Follow the manufacturer's instructions for any recommended pre-treatment of special membranes

4. Place the membrane on the focused gel; avoid trapping air bubbles between the membrane and the gel.

5. Label the sample application sites on the membrane with an appropriate marking device and mark the membrane for later identification.

6. Cover the membrane with several layers of absorbent towels and a 1-2 kg weight for 30 minutes at room temperature.

7. Remove weight and absorbent towels, leaving the membrane/gel/film complex on the glass plate.

8. Remove the plastic support film and IEF gel from the membrane by placing the gel and membrane in a shallow pan containing Blocking Solution for 2-3 minutes (see notes below for the appropriate blocking solution). If the gel fails to separate from the support film, soak the membrane/gel/film complex in the blocking solution for 1-2 more minutes; the plastic support film should then be easy to remove.

9. Once the plastic support film has been removed and the gel/membrane complex has been soaking in Blocking Solution for 2-3 minutes, the membrane becomes translucent. Remove the gel and membrane from the blocking solution and place the gel side on dry filter paper for approximately one minute. The filter paper will become wet as it absorbs the excess fluid from the gel.

10. Gently separate the membrane from the gel by peeling the membrane away from the gel/filter paper complex. Most of the gel will adhere to the filter paper; excess gel can be removed with a dry tissue. If the membrane appears to become brittle or dry, return the membrane to the blocking solution briefly before attempting to remove any remaining gel.

**Note: If major problems occur with the separation of the gel from the membrane, prepare a special formulation of Blocking Solution by diluting it with 50% glycerol and following steps 8 and 9 above. This use of this solution makes the membranes more flexible and more easy to manipulate until the researcher becomes accustomed to the tolerances of a particular membrane. Use of this solution will require more thorough washing and may inhibit drying of the membrane in later steps but will not affect the results of the assay.

11. When all of the gel has been removed from the membrane, place the membrane into fresh Blocking Solution for at least 1 hour at room temperature.

12. Place the blocked membrane into a screw cap test tube of appropriate size. The tube should be large enough to permit all areas of the membrane to be exposed to the added reagents. Generally, a 15 ml test tube is adequate to hold a membrane with two to three samples. For membranes containing four of more separated samples, a 50 ml test tube is appropriate.

13. Add 0.5 ml to 1.0 ml of the appropriate pooled allergic sera for each sample on the membrane.

14. Add a sufficient quantity of Blocking Solution to each tube so that the membrane is fully exposed to the reagent solution when mixing. The tube should be at least 75% full.

15. Cap tubes and mix gently overnight at room temperature.

DAY TWO: Perform the next steps under proper radioisotope conditions

1. Remove the membrane from the test tubes and place in a shallow pan with 300-400 ml of Washing Solution. Cover the pan and wash the membrane gently for 1 to 2 hours at room temperature.

2. Remove the membrane from buffer and place membrane in new test tubes or containers. Add a sufficient quantity of Blocking Solution to each container to fully immerse the membrane in reagent.

3. Add at least 300,000 CPM of I-125 labeled anti-IgE containing approximately 20-30 mg of anti-IgE antibody for each sample on the membrane.

4. Cap tubes tightly or cover the containers to prevent leakage and mix gently overnight.

DAY THREE

1. Wash the membrane in a pan containing 300-400 ml of Washing Solution for several hours making sure that membranes are not overlapping onto each other.

2. Place the membrane (curled side down) on plastic support film. Permit the membrane to air dry for 30 to 60 minutes at room temperature.

3. Cover the dried membrane with a second sheet of plastic film.

4. Expose radio-labeled membrane to Kodak X-O-Mat AR film for 24 to 48 hours or as long as necessary; develop the autoradiogram and compare the binding patterns to the stained gels.

Part II. Evaluation of Individual Patient Sera
DAY ONE

1. Run an IsoElectric Focusing (IEF) gel with multiple samples of the extract to be used for the screening. This extract should have been examining in earlier IEF, RAST inhibition and protein assays, and should be known to contain a full complement of potential allergens. A sufficient number of lanes should be loaded with the screening extract to examine the individual patient sera. Use a template similar to the one described earlier that will allow location of the samples when the sample applicators are removed. Permit one lane to be saved to provide a stained template.

2. After completion of the IEF procedure, place the gel and the template on a glass plate, remove sample applicators and electrode wicks: remove section to be fixed and stained in the normal IEF procedure. Do not fix or stain the portion of the gel to be blotted.

3. Place the membrane on the surface of gel as previously described.

4. Label the extracts application sites with an appropriate marker. Using the marked template as a guide and a sharp razor or scalpel blade, cut the membrane/gel/support film complex into strips that each contain a single samples of the focused extract. Do not completely separate the membrane/gel/support film assembly at this time. Number the strips for later identification.

5. Place absorbent paper and weight on membrane/gel/support film complex for 30 minutes.

6. Carefully separate plastic support film from the membrane/gel strips and soak membrane/gel strips in Blocking Solution for 2-3 minutes.

7. Remove membrane/gel complex strips from the blocking solution and carefully place them gel side down on filter paper for 2-3 minutes.

8. Separate the membrane strips from the gel as the gel adheres to the filter paper. Gently remove additional gel with a dry towel or tissue. If the membrane becomes brittle or appears to become dry, return the membrane to the blocking solution for several minutes before continuing.

9. Soak the membrane strips (free from gel residue) in Blocking Solution for 30 minutes.

10. Place each membrane strip into a separate test tube. Add 1.0 ml of an individual patient's serum to a test tube containing a membrane strip that represents a single sample of the focused extract. Repeat this procedure for each patient serum sample to be tested. Add a sufficient quantity of Blocking Solution to each test tube so that the membrane is fully immersed in solution.

11. Cap tubes and mix gently overnight at room temperature.

DAY TWO

1. Remove the membrane from the test tubes and place in a shallow pan with 300-400 ml of Washing Solution. Cover the pan and wash the membrane gently for 1 to 2 hours at room temperature.

   Perform the next two steps under proper radioisotope conditions.

2. Remove the membrane strips from buffer and place the strips in new test tubes or containers. Add a sufficient quantity of Blocking Solution to each container to fully immerse the membrane in reagent.

3. Add at least 300,000 CPM of I-125 labeled anti-IgE containing approximately 20-30 mg of anti IgE antibody to each membrane.

4. Cap tubes tightly or cover the containers to prevent leakage and mix gently overnight.

DAY THREE

1. Wash the membrane in a pan containing 300-400 ml of Washing Solution as previously described.

2. Place the membrane (curled side down) on plastic support film. Permit the membrane to air dry for 30 to 60 m nutes at room temperature.

3. Cover the dried membrane with a second sheet of plastic film.

4. Expose radio-labeled membrane to Kodak X-O-Mat AR film for 24 to 48 hours or longer if necessary. Develop the images and compare the images to the stained gel.

Part III. BRIEF Inhibition
DAY ONE

1. Run an IsoElectric Focusing (IEF) gel with multiple samples of the extract to be used for the support strips. This extract should have been examined in earlier IEF, RAST inhibition and protein assays, and should be known to contain a full complement of potential allergens. A sufficient number of lanes should be loaded with the support extract to examine the inhibitory effect of the support extract as well as a test preparation. Use a marked template similar to the one described earlier that will allow location of the samples when the sample applicators are removed. Permit one lane to be saved as a stained template.

2. After completion of the IEF procedure, place the gel and the template on a glass plate, remove sample applicators and electrode wicks; remove section to be fixed and stained in the normal IEF procedure. Do not fix or stain the portion of the gel to be blotted.

3. Place the membrane on the surface of the gel previously described.

4. Label the extracts application sites with an appropriate marker. Using the marked template as a guide and a sharp razor or scalpel blade, cut the membrane/gel/support film complex into strips that each contain a single sample of the focused extract. Do not completely separate the membrane/gel/support film assembly at this time. Number the strips for later identification.

5. Place absorbent paper and weight on membrane/gel/support film complex for 30 minutes.

6. Carefully separate plastic support film from the membrane/gel strips and soak membrane/gel strips in Blocking Solution for 2-3 minutes.

7. Remove membrane/gel complex strips from the blocking solution and carefully place them gel side down on filter paper for 2-3 minutes.

8. Separate the membrane strips from gel.

9. Soak the membrane strips in Blocking Solution for 30 minutes.

10. Place each membrane strip into a separate test tube. Add 1.0 ml of the appropriate pooled allergic serum to each test tube. Repeat this procedure for each strip that will contain inhibitor. In addition, add only allergic serum to one strip; this will be the positive control. Another strip must be reserved as the negative control; 5% Human Serum Albumin (HSA) has been routinely used as a negative control. Add a sufficient quantity of Blocking Solution to each test tube so that the membrane is fully immersed in solution.

11. Prepare serial three-fold dilutions of support extract and inhibitor extract. The quantity of inhibiting extract is computed from the amount of extract originally focused. For example, if the sample load is 20 ml of extract, then 60 ml of undiluted inhibitor should be added to the first membrane as the most concentrated inhibition. Following this approach, add 60 ml of each of the subsequent 3 three-fold dilutions to the individual membrane strips, creating a inhibition dose response curve similar to RAST inhibition.

12. Cap tubes and mix gently overnight at room temperature.

DAY TWO

1. Remove the membrane from the test tubes and place in a shallow pan with 300-400 ml of Washing Solution. Cover the pan and wash the membrane gently for 1 to 2 hours at room temperature.

Perform the next two steps under proper radioisotope conditions.

2. Remove the membrane strips from buffer and place the strips in new test tubes or containers. Add a sufficient quantity of Blocking Solution to each container to fully immerse the membrane in reagent.

3. Add at least 300,000 CPM of I-125 labeled anti-IgE containing approximately 20-30 mg of anti-IgE antibody for to each membrane.

4. Cap tubes tightly or cover the containers to prevent leakage and mix gently overnight.

DAY THREE

1. Wash the membrane in a pan containing 300-400 ml of Washing Solution as previously described.

2. Place the membrane (curled side down) on plastic support film. Permit the membrane to air dry for 30 to 60 minutes at room temperature.

3. Cover the dried membrane with a second sheet of plastic film.

4. Expose radio-labeled membrane to Kodak X-O-Mat AR film for 24 to 48 hours or as long as necessary. Develop the autoradiogram and compare the binding patterns to the stained gel.

Note for stained gel strips:

To analyze the autoradiograms, it is always necessary to retain a section of the focused gels that have been stained with Coomassie or silver stain. Depending on the staining method employed, preserve the gels in the best possible manner. If photographs of the stained gels are taken, always photograph the stained gels or the autoradiograms with clear ruler in view, to permit later sizing of the two images.

Revised 4/93 M. Braun

III. In vivo Test Methods

Revised: November 1994

Quantitative Intradermal Procedure to Determine Relative Potency and Compositional Differences of Allergenic Extracts using Parallel Line Bioassay: Screening and Proficiency Method

Paul C. Turkeltaub, M.D. and Suresh C. Rastogi, Ph.D.

Quantitative Intradermal Procedure for Estimating Relative Potency and Compositional Differences of Allergenic Extracts Using Parallel Line Bioassay: Screening and Proficiency Method

Screening Method

Purpose:

This screening method is proposed as a rapid method for the evaluation of the relative potency and compositional differences of allergenic extracts. It is intended to reduce subject time and discomfort. The "Screening Method" is also suitable for assessing the proficiency of the operator who is carrying out this technique and can be used to qualify personnel for submission of skin test data to CBER (See Proficiency Test Method, below) under an IND application.

Selection of Subjects:

1. Subjects should be selected from adults with a history of allergic disease, e.g., allergic rhinitis, related to exposure to the test allergen. To enhance safety, subjects with active asthma are excluded. Record information on the Product and Subject Information form.
2. Subjects should be sufficiently sensitive so that intradermal injection of concentrations of allergen less than dilution #5 are avoided. This can be achieved by selecting subjects with large puncture sum of erythema diameter responses (ΣE) to the allergen concentrate, e.g, ΣE >75mm. If subjects with this degree of response are not available, choose subjects with smaller responses, eg, ΣE> 60m followed by $\Sigma E \geq$ 50mm. For low potency extracts, subjects with progressively smaller puncture responses e.g.. ΣE> 40mm followed by ΣE> 30 mm will be required. For very low potency extracts, subjects with puncture ΣE=0 will be suitable for inclusion. Low potency products may require injection of intradermal concentrations less than dilution #5.

3. Subjects should not have received antihistamine decongestant medication within 48 hours of skin testing. Long-acting antihistamines require longer washout periods. If a long acting antihistamine has been used, date of last dose should be recorded. In subjects who have received antihistamines recently, puncture ΣE responses to Histamine base 1.8 and 0.1 of 35-65mm and 10-30mm respectively may demonstrate acceptable cutaneous reactivity.
4. Subject's skin coloring should permit evaluation of erythema.
5. Subjects, preferably, should not have received or be receiving immunotherapy with the test allergen. This requirement may be waived. Subjects should be specified as to immunotherapy status (past and present) and date of last dose.
6. At least 4 subjects are required per assay. One or more test extracts are to be compared against a reference in each subject.
7. For evaluating compositional differences, subjects selectively sensitive to heat stable or labile allergens or selectively sensitive to specific allergens in the crude mix should be selected. To select populations selectively sensitive to either heat stable or labile allergens, puncture test each subject with an unboiled and boiled preparation at equal concentrations. Subjects who have equivalent skin reactions to both extracts are reacting to heat stable allergens. Subjects who react to the unboiled extract, but have markedly diminished reactivity to the boiled extract may be reacting to heat labile allergens. Both types of subjects should be demonstrable in the population to verify the capability of the reagents to detect heat stable and labile allergens. The boiled extract may be prepared by placing the reference preparation diluted in 50% glycerin (or aqueous diluent) in a boiling water bath for 20 minutes. Alternatively, in order to enhance detection of compositional differences, subjects can be selected who differ widely (10 - 10,000 fold) in their allergen skin test sensitivity (D_{50}-see Proficiency Method) to the test or reference extracts .
8. Subjects should have no or minimal erythema response when tested with diluent (puncture and intradermal).

Materials:

1. Alcohol swabs

Preparation of Subject:

1. Using the skin test template, mark the subject's right and left arms (forearm in supination) according to the diagram in Figure 2A and the subject's back according to Figure 2B.
2 Skin test sites are placed on the glabrous skin of the volar surface of the arms, avoiding a one inch area above and below the antecubital fossa and a one inch area above the wrist.
3. On the arms, titrate with more dilute solutions distally. On the back randomize the sites for each product tested. For consistency, the back should be used for final titrations. The back is more sensisitive than the arms.
4. When the back is used, the top row (row 1) is placed at or above the level of the spine of the scapula. Columns C and D are placed approximately 3 cm to the left and right of the vertebral column. In smaller subjects a 36 site randomization scheme may be required (rows 1-6) to avoid placement of rows 7 and 8 below L-1 where smaller erythema responses are produced. (Figure 3).

Injection Technique:

Puncture Testing

1. For puncture testing, the bifurcated needle is inserted into the skin perpendicularly through the drop of extract, the flat surface of the device parallel to the long axis of the arm. Moisten the tines in the drop of extract prior to insertion. The puncture device tines are gently imbedded in the skin and the device is rocked once cephalad and once caudad and side to side four times without lifting the tines from the skin surface. Do not rotate the device. Do not gouge. Following removal of the tines, imprints should be easily seen at the puncture site without bleeding. Use the allergen concentrates for puncture testing.
2. When six subjects are tested the puncture test, if properly performed, should give a mean sum of erythema diameters of approximately 50 mm (35-65) following histamine base 1.8 mg/ml and 20 mm (10-30) following histamine base 0.1 mg/ml (see "Proficiency Test Method"). Consistency in puncture technique is essential.

3. A puncture test with the diluent found in the allergen concentrates used for puncture testing should beperformed and the response recorded.

Intradermal Testing

1. The volume of solution injected is 0.05 ml.
2. Insert the needle at a 30° angle, bevel down into the superficial skin layers until the bevel is covered. Inject the solution maintaining steady pressure on the plunger until the volume is completely injected and the needle withdrawn. A distinct injection bleb should be observed. Leakage may be avoided by gently advancing the needle tip during injection. Injections in which gross leakage of extract around the needle, an indistinct bleb or a subcutaneous injection occur should be repeated at a different site. A tiny drop of extract at the injection site is not uncommon.
3. The time of injection at each skin test site is recorded. THIS IS A TIME-DEPENDENT ASSAY.

Measurement of Skin Tests:

1. Exactly fifteen minutes following injection, the wheal and erythema margins are outlined using the skin marking pen (Figure 4). Always outline the erythema response before the wheal response and do not retrace outlines. This avoids artifacts from effects of the triple response.
2. In order to make a permanent record of the skin test reaction, transparent tape is placed on the skin over the skin test outline (Figure 5). Lifting of the tape from the skin results in transfer of the outline to the tape. The tape is then placed in a notebook for future reference (Figure 6).
3. The size of the skin response is obtained by measuring and recording the longest diameter of erythema and the orthogonal erythema diameter measured at one half the longest erythema diameter (Figure 7).
4. The sum of the longest and orthogonal erythema diameters (ΣE) [or wheal (ΣW)] constitutes the skin response at that site.
5. Measurements are made from the inner edge of the skin test outlines.

6. Erythema contained within ($\Sigma E < \Sigma W$) or at the margin ($\Sigma E \sim \Sigma W$) of the wheal also are measured and recorded.

Skin Test Procedure

1. The dose response line for each product is generated using 4 serial 3-fold dilutions with grade erythema responses which bracket ΣE=50mm and includes the end-point where ΣE=0 or $\Sigma E \sim \Sigma W$.
2. The skin response (ΣE) should fall within the limits of ≥ 0 to ≤ 125 mm. Each more concentrated dilution should produce a graded erythema response. This insures a high correlation coefficient. The acceptable lower limit for the correlation coefficient is 0.92. The four dilutions selected should span a wide range of ΣE *e.g.* from 0-20 mm to 80-125 mm and bracket ΣE of 50 mm. This insures a steep slope. The acceptable lower limit for slope is 13.
3. The above range of ΣE responses can usually be obtained by using the most concentrated dilution where ΣE is absent ($\Sigma E = 0$) or equal to the wheal ($\Sigma E \sim \Sigma$) and the next three more concentrated serial dilutions (Figure 8). For each extract, tested within the same subject, pick comparable dose response regions based on the smallest and largest ΣE responses. This increases the probability that the slope of each dose-response curve will be similar. Flat slopes will increase the error and decrease the reproducibility of this assay, thus avoid using as the end point dilution a dilution where erythema substantially exceeds the wheal. A graph of ΣE versus dilution # may be helpful in selecting suitable dose-response lines that are parallel.
4. Begin testing on the forearm. **Inject the test and reference extracts beginning with dilution #15.** The expected change in ΣE going from one dilution to the next higher or lower dilution is about 20 mm. Therefore, if dilution #15 is negative, proceeding to dilution #12 would not be expected to exceed a ΣE response of 60 mm. If dilution #12 is negative, a similar rationale explains proceeding to dilution #9. Do not inject reference extract dilutions more concentrated than #5 unless the extract is known to be of low potency *e.g.* concentrate fails to produce large puncture ΣE response in sensitive subject.
5. To reduce interactions on the arm between proximal and distal skin test sites, place the higher dilutions (*e.g.* end point dilutions) distally (near the wrist) and titrate with more concentrated

meet this requirement and computation for these test samples would continue.

5. For each sample using the combined data of both reference and sample, calculate the new best-fit parallel lines adjusted for the common slope and the two new intercepts.
6. Using the common slope and new intercepts, calculate the horizontal distance between these two lines by subtracting the intercept of the reference from the intercept of the test sample and dividing by the common slope. This distance is the $\log_3$ of the relative potency ($\log_3$ RP) of the test sample as compared to the reference.
7. Perform at least one independent valid assay on 4 different subjects for each test product, and calculate the mean and standard deviation of the $\log_3$ RP values. ALL VALID ASSAYS MUST BE INCLUDED. If the standard deviation of the mean of the $\log_3$ RP values is equal to or less than the upper limit shown in Table 1 for the corresponding number of valid assays, the variability of the $\log_3$ RP is acceptable, suggesting the test and reference extracts are compositionally similar in the subjects tested. If the standard deviation of the mean $\log_3$ RP values exceeds the upper limit shown in Table 1 for the corresponding number of valid assays, it suggests the test and reference extracts are compositionally different. To enhance detection of compositional differences, patients who differ widely in their skin sensitivity to the extract of interest should be selected. A method for determining skin sensitivity based on D_{50} (3-fold dilution for SE=50mm) can be found in The Proficiency Test Method, paragraph 4 and in the "Quantitative Intradermal Procedure for Evaluation of Subject Sensitivity to Standardized Allergenic Extracts and for Assignment of Bioequivalent Allergy Units to Reference Preparations Using the ID_{50}EAL Method (Intradermal Dilution for 50 mm Sum of Erythema Determines Bioequivalent Allergy Units, Revised October 1993)".
8. Determine the geometric mean (G.M.) of the relative potency by taking the antilog of the mean of the $\log_3$ RP values. If 100 x G.M. is between the limits shown in Table 2 for the corresponding number of tests, the test material is considered to be equal in potency to the reference extract.

PROFICIENCY TEST METHOD

To determine proficiency in the application of the parallel line bioassay described and to qualify personnel for submission of data to CBER, the following proficiency program has been developed.

1. Carry out the puncture testing and intradermal titrations as specified in at least 6 subjects using Histamine base 0.1 and 1.8 mg/ml and record on the Proficiency and Bioassay Data Forms. Begin intradermal histamine testing using Dilution # 8. Non-atopic patients are acceptable.
2. Determine the relative potency of Histamine base 1.8 mg/ml with respect to 0.1 mg/ml for each subject. The nominal reference relative potency (RP) of these two products is 18 ($\log_3$ RP = 2.63). Take the antilog to the base 3 of the geometric mean (GM) Log_3RP observed, divide by the nominal reference RP (18), and multiply by 100 to obtain the observed relative potency as a percent of the reference. When carried out in a multicenter study, the observed mean log3 RP was 2.43, GMRP = 14.4 (14.4/18*100) or 80% of the nominal reference RP.
3. Compare your Relative Potency results (as a percent of reference) and your standard deviation (SD) of mean $\log_3$ RP with the limits specified in Tables 1 and 2. If the observed RP and SD fall within the limits specified in Tables 1 and 2 for N assays, the investigator has demonstrated proficiency (accuracy and precision) in estimating relative potency and compositional differences of extracts from the same source using this assay.
4. In addition, compute the D_{50} (3-fold dilution for ΣE = 50 mm) from the best-fit line of each Histamine base concentration in each subject using the formula: D_{50} = (50 - Intercept)/slope (Figure 9). Do not use the common slope for these calculations. Compute the mean and SD of the D_{50}s for the subjects tested and compare with the limits specified in Tables 3 and 4. If the investigator's mean D_{50} and SD of mean D_{50} for histamine base 0.1 and 1.8 mg/ml fall within the limits specified in Tables 3 and 4 for N assays, the investigator has demonstrated proficiency in estimating D_{50}, an estimate of assay sensitivity.
5. Puncture test with 1.8 and 0.1 mg/ml histamine base using the bifurcated needle as described. Determine the mean ΣE of each histamine concentration. If the observed mean ΣE falls within 10-30 mm for 0.1mg Histamine base and 35-65 mm for 1.8mg

Histamine base, the investigator has demonstrated acceptable use of the bifurcated needle for patient selection for this assay.

6. Submit the Proficiency data (puncture and intradermal) to CBER for review of acceptability.

Acknowledgement: The authors wish to thank Jacqueline Matthews, R.N., M.S. for technical support and assistance in preparation of this manual.

Table 1.
One-Sided 99% Fiducial Upper Limit for SD of N Values of Mean $\log_3$ Relative Potency

N	99% Upper Limit*
2	1.00
3	0.84
4	0.76
5	0.71
6	0.68

*To convert these limits to Log_{10} multiply by 0.477

Table 2.
Two-Sided 95% Limits for Geometric Mean of N values of Relative Potency as a percent of Reference Relative Potency

	Percent of Reference	
N	Lower Limit	Upper Limit
1	43.2	231.6
2	55.2	181.1
3	61.6	162.4
4	65.7	152.2
5	68.7	145.6
6	71.0	140.9

Table 3.
One-Sided 99% Fiducial Upper Limit for SD of N Values of Mean D_{50} for Histamine Base 0.1 and 1.8 mg/ml

N	99% Upper Limit*
2	1.34
3	1.12
4	1.01
5	0.95
6	0.90

*To convert these limits to Log_{10} multiply by 0.477

Table 4.
Two-Sided 95% Limits of N values of Mean D_{50} for Histamine Base 0.1 and 1.8 mg/ml based on N assays

	95% Limits of Mean D_{50} Histamine Base (mg/ml)	
N	0.1*	1.8**
1	2.95 - 4.99	5.39 - 7.43
2	3.25 - 4.69	5.69 - 7.13
3	3.38 - 4.56	5.82- 7.00
4	3.46 - 4.48	5.90 - 6.92
5	3.51 - 4.43	5.95 - 6.87
6	3.55 - 4.39	5.99 - 6.83

*Mean D50 of Histamine base 0.1 mg/ml is 3.97, SD=0.52
**Mean D50 of Histamine base 1.8 mg/ml is 6.41, SD=0.52

Statistical Appendix

Calculations of relative potency of an allergenic extract with respect to a reference extract.

Suppose there are M doses for reference and N doses for sample.

I. Assume for reference we have:

$\log_3$ dose: $X_1, X_2, ..., X_M$
SE: $Y_1, Y_2, ..., Y_M$

Calculate:

1. $X = (SX_i)/M$; $Y = (SY_i)/M$

2. $A_1 = S(X_i-X)^2$; $A_2 = S(Y_i-Y)^2$; $A_3 = S(X_i-X)\ (Y_i-Y)$

3. Slope = $B + A_3/A_1$

4. Intercept = $I + Y-BX$

5. Cor. Coeff. = $R = A_3/\sqrt{(A_1 \cdot A_2)}$

6. Variance = $V = (A_2-B^2A_1)/(M-2)$

II. Repeat step I using sample data. Note that there are N doses in the sample.

III. Calculate: (Subscripts "r" and "s" stand for reference and sample respectively.)

1. Pooled variance = $V = [(M-2)V_r+(N-2)V_s]/(M+N-4)$

2. $C = 1/A_1)_r+1/(A_1)_s$

3. $E = \sqrt{(V \cdot C)}$

4. $T = (B_r - B_s)/E$ d.f. = M+N-4

IV. For a valid assay or test we must have:

1. Two correlation coefficients greater than or equal to 0.92

2. Two slopes greater than or equal to 13

3. T less than the tabulated t-value at P = 0.01

Statistical Appendix

V. Parallel Lines:

Common Slope = $B = [(A_3)_r + (A_3)_s]/[(A_1)_r+(A_1)_s]$

New Intercepts:

$I_r = Y_r - BX_r$

$I_s = Y_s - BX_s$

Log (Rel. Pot.) = $W = (I_s - I_r)/B$

Rel. Pot. = 3^W

Figure 1A.
Skin Test Template
Forearm

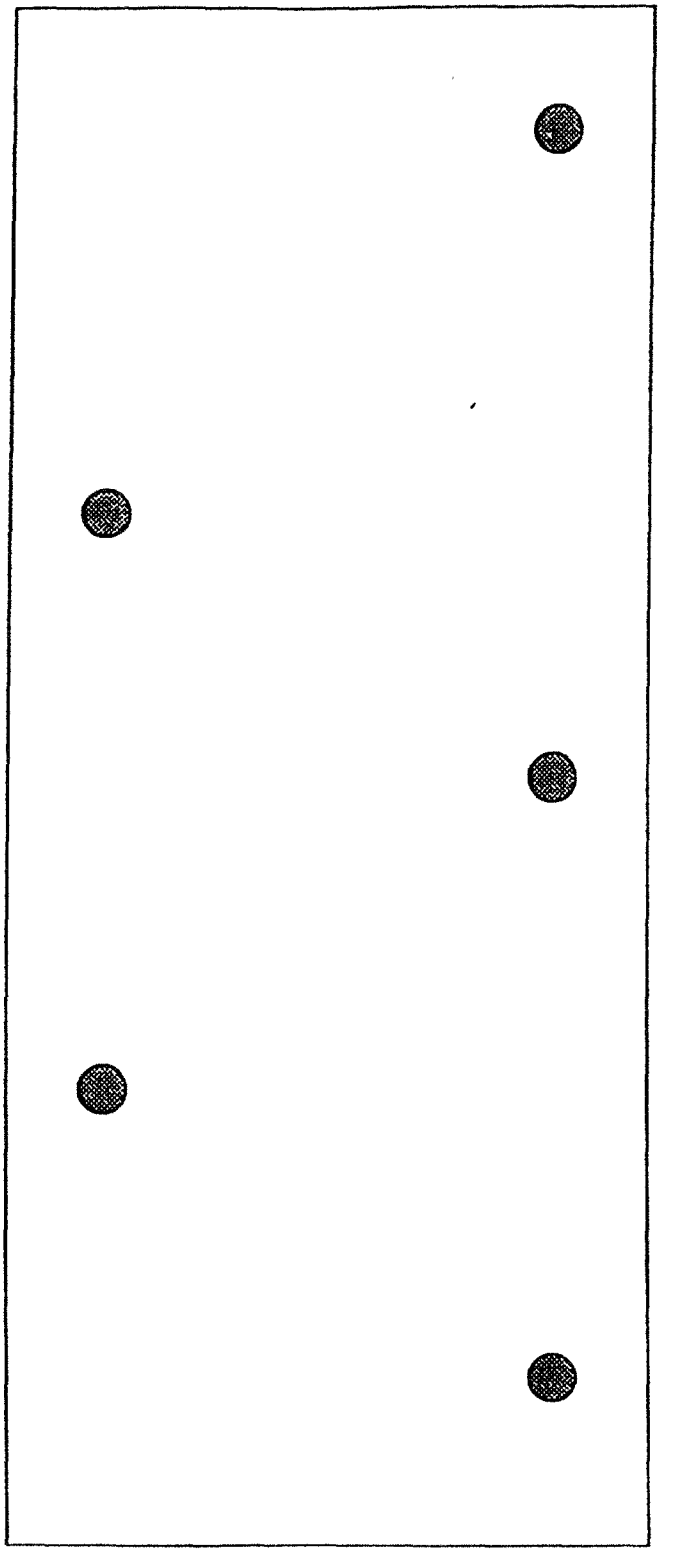
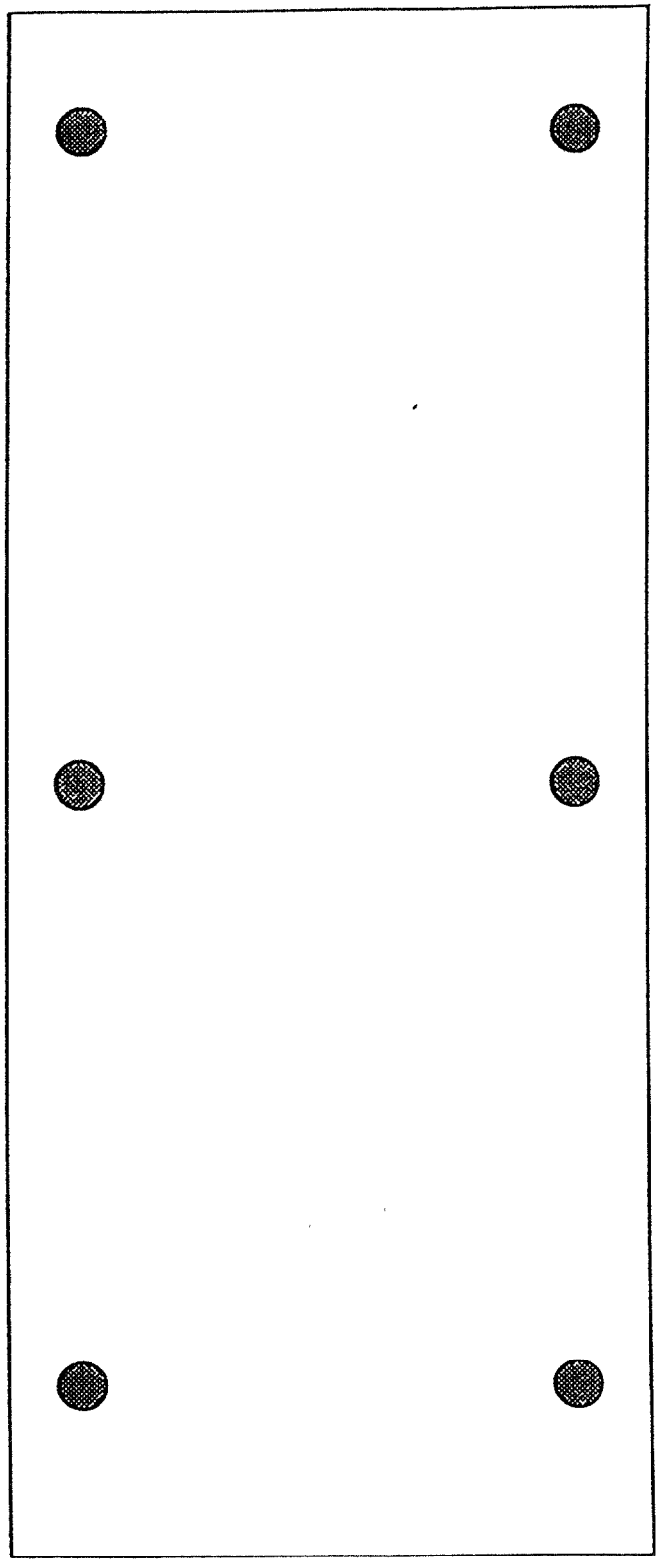


Figure 1B.
Skin Test Template
Back



Investigator: _____

Name: _____

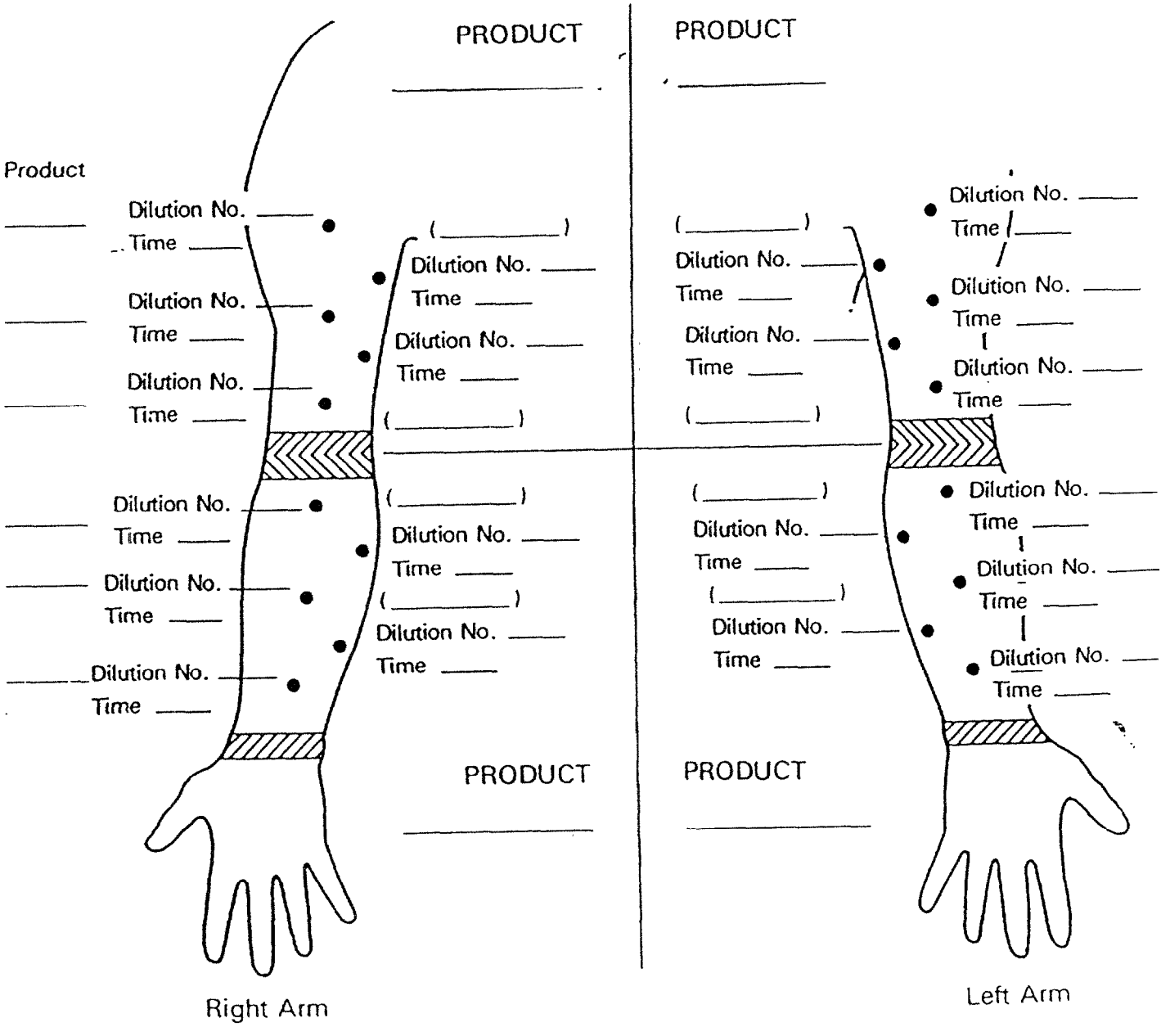


Figure 2A.

Arm Placement of Skin Test Sites for Determination of Allergen Dose Response

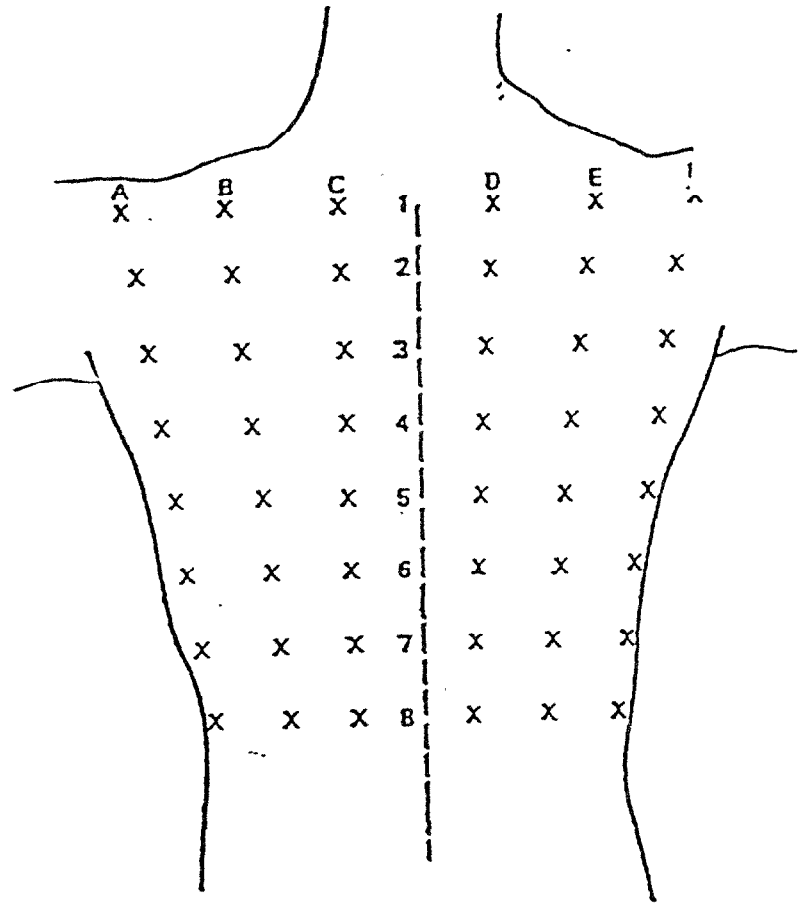
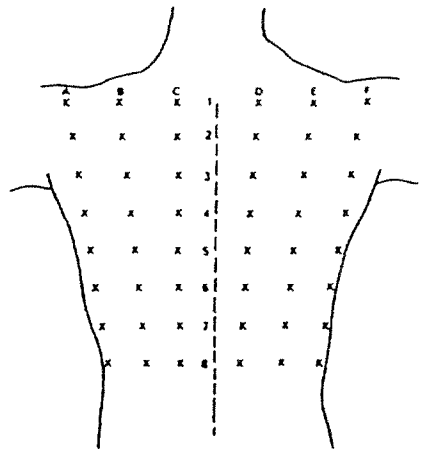


Figure 2B Schematic outline for placement of skin test

Figure 3.

Patient _____ Date _____

Product	Mfgr.	Lot #	Conc.	Exp. Date	Diluent



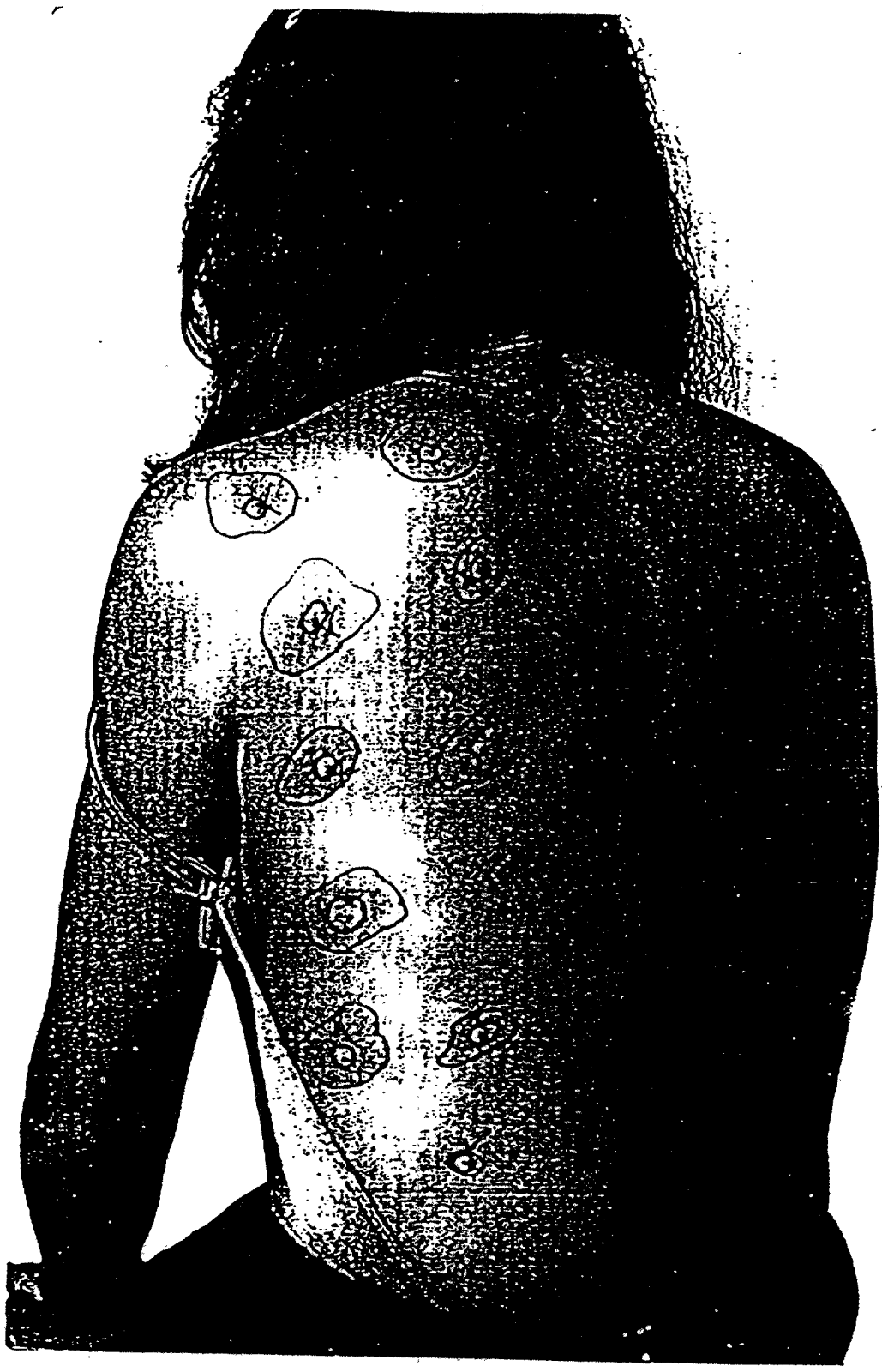
Dil.	Locus	Time

Dil.	Locus	Time

Dil.	Locus	Time	Dil.	Locus	Time	Dil.	Locus	Time	Dil.	Locus	Time

Dil.	Locus	Time	Dil.	Locus	Time	Dil.	Locus	Time	Dil.	Locus	Time

Figure 4.

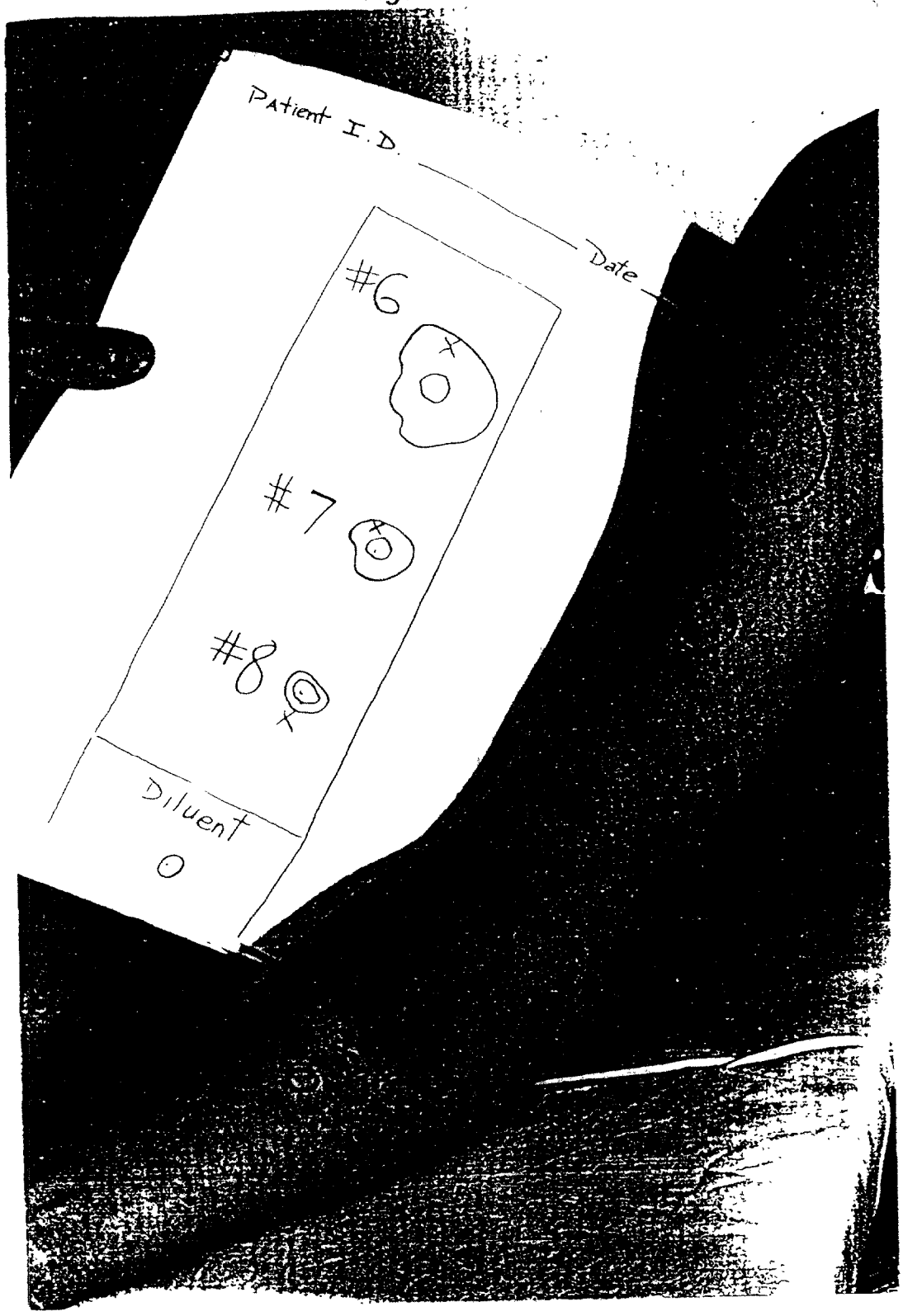


Example of tested subject

Figure 5.

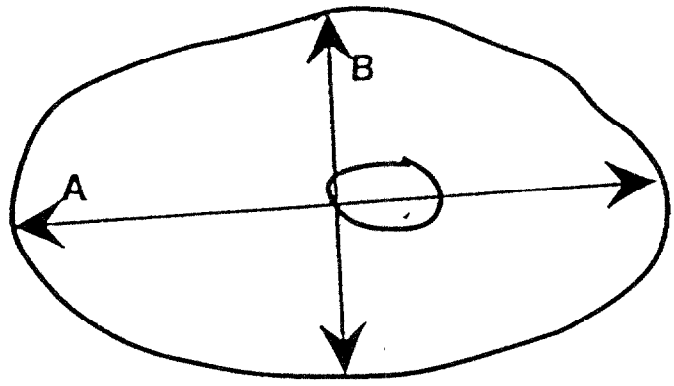
Outline wheal and flare reaction overlaid by transparent tape (sticky side down)

Figure 6.



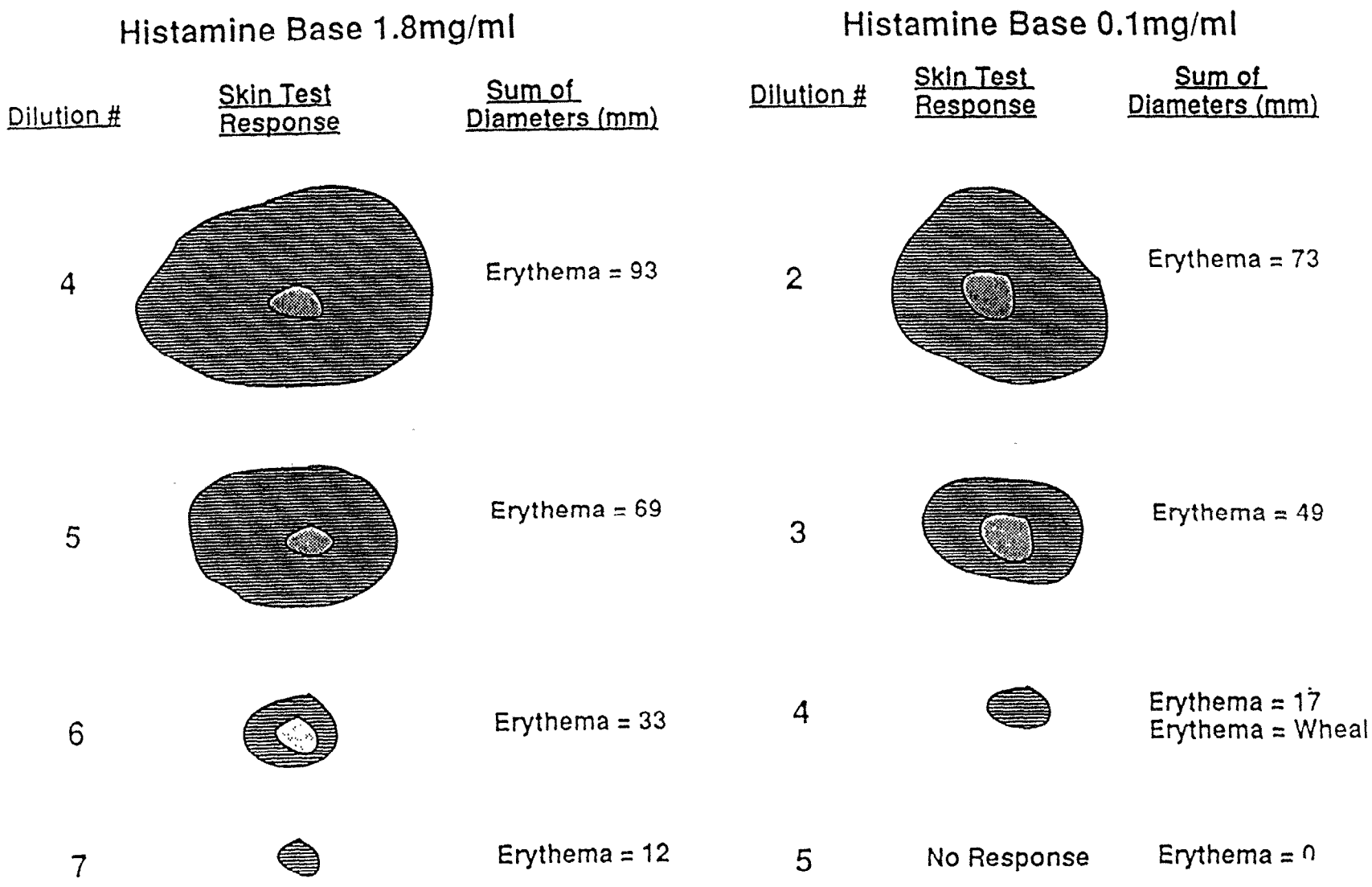
Permanent record of wheal and flare outlines made by lifting tape off penned outlines and placing tape (sticky side down) on white notebook paper.

Figure 7.



Diameters are measured from the inner margins of the penned outline. Longest (A) plus midpoint orthogonal diameters (B) are summed (A+B)

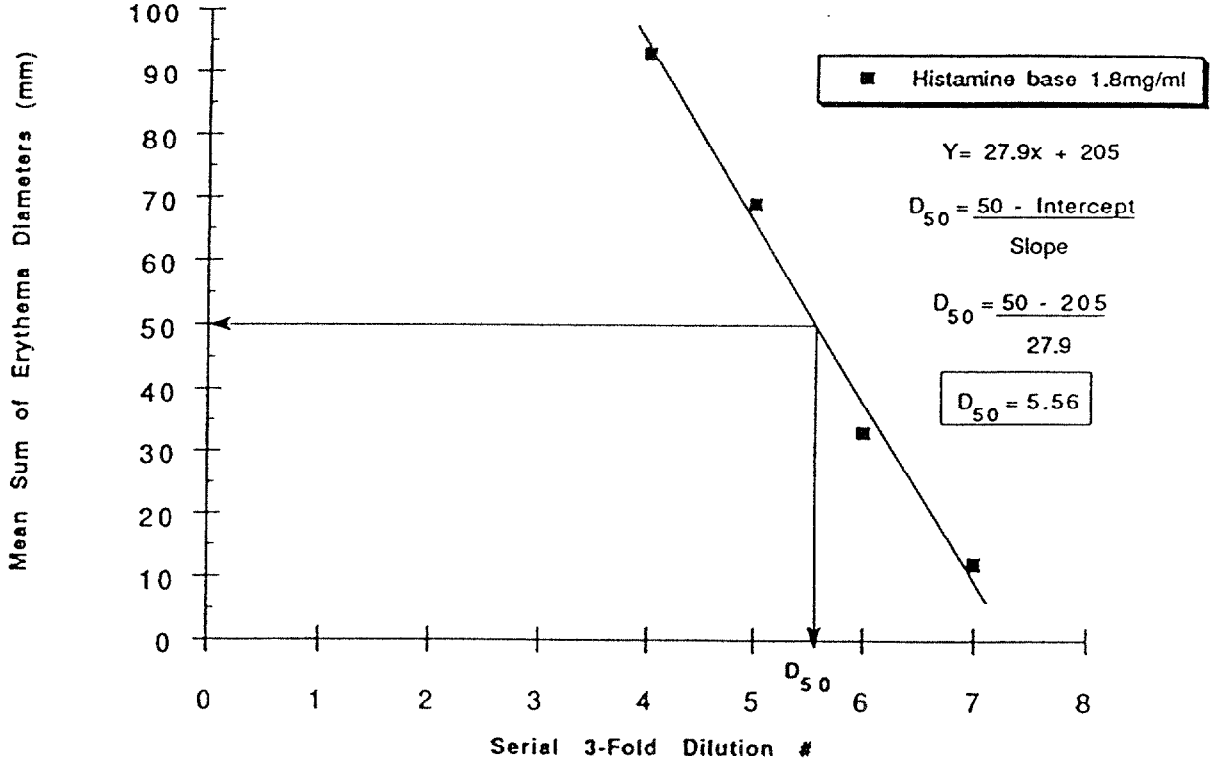
Figure 8.



Sample dose skin response for Histamine base 1.8mg/ml and Histamine base 0.1 mg/ml

FDA/CBER/DAPP

Figure 9.
Calculation of D_{50}



Product and Subject Information

Investigator_____ Date_____

A. Product Information

Product	Mfr.	Lot #	Conc.	A, G, FD(1)	Recon. Volume	Exp. Date
Histamine base 1.8 mg/ml			1.8			
Histamine base 0.1mg/ml			0.1			
Puncture Negative Control						
Intradermal Negative Control						
Diluent						
Allergens	**Mfr.**	**Lot #**	**Conc.**	**A, G, FD(1)**	**Recon. Volume**	**Exp. Date**

(1) A=Aqueous
G=50% Glycerin
FD=Freeze-dry

Product and Subject Information

B. Subject Information

N	ID	DOB	Age	Sex	Atopic Status(2)	Immunotherapy Status(3)	Date of Assay
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							

(2) N=None
A=Asthma
D=Atopic Derm
R=Allergic Rhinitis
U=Urticaria
F=Food allergy
I=Insect Allergy
P=Drug Allergy
Y=Anaphylaxis

(3) 1=On IT
2=Past IT (Last Inj)
3=Never IT

Bioassay Data Form

Date:_____

Investigator_____ Extract_____ Lot#____

Technician_____ Patient_____

☐ D_{50} format	ID:	ID:	ID:	ID:	ID:
☐ RP format	Ref:	Test:	Test:	Test:	Test:
Mfr.					
Lot #					
Dil. #	ΣE	ΣE	ΣE	ΣE	ΣE
Puncture: 0					
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
Correl Cof.					
Slope					
t-Test					
Intercept					
D50					
$\log_3$ RP					
RP					

Controls	I	P	PΣE
Histamine base 0.1 mg/ml	_____	_____	_____
Histamine base 1.8mg/ml	_____	_____	_____
Negative Control	_____	_____	_____
Other( )	_____	_____	_____

JLM April 28, 1993

Proficiency Form

Technician_____ Date of Proficiency_____

	Histamine 1.8 mg/ml Lot #:				Histamine 0.1 mg/ml Lot #				
N	PΣE	CC(1)	Slope(2)	D_{50}	PΣE	CC(1)	Slope(2)	D_{50}	LRP
1									
2									
3									
4									
5									
6									
Mean									
SD									
								RP	
								% Ref.	

(1) Acceptable Correlation Coefficient ≥ 0.92
(2) Acceptable Slope ≥ 13mm

Technician_____ Date of Proficiency_____

	Histamine 1.8 mg/ml Lot #:				Histamine 0.1 mg/ml Lot #				
N	PΣE	CC(1)	Slope(2)	D_{50}	PΣE	CC(1)	Slope(2)	D_{50}	LRP
1									
2									
3									
4									
5									
6									
Mean									
SD									
								RP	
								% Ref.	

(1) Acceptable Correlation Coefficient ≥ 0.92
(2) Acceptable Slope ≥ 13mm

Revised: November, 1994

Quantitative Intradermal Procedure for Evaluation of Subject Sensitivity to Standardized Allergenic Extracts and for Assignment of Bioequivalent Allergy Units to Reference Preparations Using the $ID_{50}EAL$ Method (Intradermal Dilution for 50 mm Sum of Erythema Determines Bioequivalent ALlergy Units)

Paul C. Turkeltaub, M.D., Suresh C. Rastogi, Ph.D.

Quantitative Intradermal Procedure for Evaluation of Subject Sensitivity to Standardized Allergenic Extracts and for Assignment of Bioequivalent Allergy Units to Reference Preparations Using the $ID_{50}EAL$ Method (Intradermal Dilution for 50 mm Sum of Erythema Determines Bioequivalent ALlergy Units)

Purpose:

Bioequivalent Allergy Units (BAU) are assigned to reference preparations based on the $ID_{50}EAL$ method. Once BAUs are assigned to a reference, BAUs of other products with respect to the Reference are determined by an in vitro potency assay acceptable to CBER. The $ID_{50}EAL$ method also permits determination of subject sensitivity to allergenic extracts based on the D_{50} (3-fold Dilution # for sum of erythema of 50 mm). The D_{50} can then be converted into BAU_{50} for inclusion in the labeling specifying the mean dose and dose range in BAU found for each standardized extract for sum of erythema diameters equal to 50mm. If Bioequivalent Allergy Units (AU) have not been assigned to the extract, subject sensitivity to the extract can be defined using the D_{50}. Investigators should be familiar with the Proficiency Method (see Quantitative Intradermal Procedure for Estimating Relative Potency and Compositional Differences of Allergenic Extracts Using Parallel Line Bioassay: Screening and Proficiency Method, Revised October 1993) and have submitted proficiency data qualifying their use of this method.

Selection of Subjects:

1. Subjects should be selected from adults with a history of allergic disease, e.g., allergic rhinitis, related to exposure to the allergen of intertest. To enhance safety, subjects with active asthma are excluded. Record information on the Protocol Sheet.
2. Fifteen subjects with large puncture sum of erythema diameter responses (ΣE) of $\geq$75mm to the allergen concentrate are to be selected. If subjects with this reactivity are not obtained, document the size of the population sampled. If subjects with the larger responses are not available, choose from subjects with smaller responses in descending order, eg. $\Sigma E > 60$m followed by $\Sigma E \geq 50$mm followed by $\Sigma E > 40$mm followed by $\Sigma E > 30$ mm. For very low potency extracts, subjects with puncture $\Sigma E=0$ will be suitable for inclusion. Although, subjects should be sufficiently sensitive so that intradermal injection of concentrations of allergen less than dilution #5 are avoided, low potency products may require injection of intradermal concentrations less than dilution #5.
3. Subjects should not have received antihistamine decongestant medication within 48 hours of skin testing. Long-acting antihistamines require

3. Serial 3-fold dilutions from the undiluted extract (#0) are labeled #1, #2, #3, up to #17. Therefore, the $\log_3$ doses are 0, -1, -2, -3, up to -17. Some highly sensitive patients may require dilutions up to #21.
4. USE A NEW SYRINGE AND NEEDLE FOR EACH 3 FOLD DILUTION TO AVOID AN INFINITE DILUTION SERIES. Eliminate air bubbles from the syringe barrel. DO NOT REASPIRATE EXTRACT ALREADY INJECTED INTO A VIAL.
5. Dilutions should be kept continuously refrigerated and discarded after 2-3 weeks, unless stability data indicates a longer expiration date.

Preparation of Subject:

1. Using the skin test template, mark the subject's right and left arms (forearm in supination) according to the diagram in Figure 2A and the subject's back according to Figure 2B.
2 Skin test sites are placed on the glabrous skin of the volar surface of the arms, avoiding a one inch area above and below the antecubital fossa and a one inch area above the wrist.
3. On the arms, titrate with more dilute solutions distally. On the back randomize the sites for each product tested. For consistency, the back should be used for final titrations. The back is more sensisitive than the arms.
4. When the back is used, the top row (row 1) is placed at or above the level of the spine of the scapula. Columns C and D are placed approximately 3 cm to the left and right of the vertebral column. In smaller subjects a 36 site randomization scheme may be required (rows 1-6) to avoid placement of rows 7 and 8 below L-1 where smaller erythema responses are produced (Fig. 3).

Injection Technique:

Puncture Testing

1. For puncture testing, the bifurcated needle is inserted into the skin perpendicularly through the drop of extract, the flat surface of the device parallel to the long axis of the arm. Moisten the tines in the drop of extract prior to insertion. The puncture device tines are gently imbedded in the skin and the device is rocked once cephalad and once caudad and side to side four times without lifting the tines from the skin surface. Do not rotate the device. Do not gouge. Following removal of the tines, imprints should be easily seen at the puncture site without bleeding. Use the concentrate for puncture testing.
2. When six subjects are tested the puncture test, if properly performed, should give a mean sum of erythema diameters of approximately 50 mm (35-65) following histamine base 1.8 mg/ml and 20 mm (10-30) following histamine

base 0.1 mg/ml (see "Proficiency Test Method"). Consistency in puncture technique is essential.

3. A puncture test with the diluent found in the allergen concentrates used for puncture testing should be performed and the response recorded.

Intradermal Testing

1. The volume of solution injected is 0.05 ml.
2. Insert the needle at a 30° angle, bevel down into the superficial skin layers until the bevel is covered. Inject the solution maintaining steady pressure on the plunger until the volume is completely injected and the needle withdrawn. A distinct injection bleb should be observed. Leakage may be avoided by gently advancing the needle tip during injection. Injections in which gross leakage of extract around the needle, an indistinct bleb or a subcutaneous injection occur should be repeated at a different site. A tiny drop of extract at the injection site is not uncommon.
3. The time of injection at each skin test site is recorded. THIS IS A TIME-DEPENDENT ASSAY.

Measurement of Skin Tests:

1. Exactly fifteen minutes following injection, the wheal and erythema margins are outlined using the skin marking pen (Figure 4). Always outline the erythema response before the wheal response and do not retrace outlines. This avoids artifacts from effects of the triple response.
2. In order to make a permanent record of the skin test reaction, transparent tape is placed on the skin over the skin test outline (Figure 5). Lifting of the tape from the skin results in transfer of the outline to the tape. The tape is then placed in a notebook for future reference (Figure 6).
3. The size of the skin response is obtained by measuring and recording the longest diameter of erythema and the orthogonal erythema diameter measured at one half the longest erythema diameter (Figure 7).
4. The sum of the longest and orthogonal erythema diameters (ΣE) [or wheal (ΣW)] constitutes the skin response at that site.
5. Measurements are made from the inner edge of the skin test outlines.
6. Erythema contained within ($\Sigma E < \Sigma W$) or at the margin ($\Sigma E \sim \Sigma W$) of the wheal also are measured and recorded.

Skin Test Procedure

1. The dose-response line for each product is generated using 4 serial 3-fold dilutions with graded erythema responses which bracket ΣE=50mm and includes the end-point where ΣE=0 or $\Sigma E \sim \Sigma W$.

2. The skin response (ΣE) should fall within the limits of ≥ 0 to ≤ 125 mm. Each more concentrated dilution should produce a graded erythema response. This insures a high correlation coefficient. The acceptable lower limit for the correlation coefficient is 0.92. The four dilutions selected should span a wide range of ΣE *e.g.* from 0-20 mm to 80-125 mm and bracket ΣE of 50 mm. This insures a steep slope. The acceptable lower limit for slope is 13.
3. The above range of ΣE responses can usually be obtained by using the most concentrated dilution where ΣE is absent (ΣE = 0) or equal to the wheal (ΣE ~ ΣW) and the next three more concentrated serial dilutions (Figure 8). For each extract, tested within the same subject, pick comparable dose response regions based on the smallest and largest ΣE responses. This increases the probability that the slope of each dose-response curve will be similar. Flat slopes will increase the error and decrease the reproducibility of this assay, thus avoid using as the end point dilution a dilution where erythema substantially exceeds the wheal. A graph of ΣE versus dilution # may be helpful in selecting suitable dose-response lines that are parallel.
4. Begin testing on the forearm. **Inject the test and reference extracts beginning with dilution #15**. The expected change in ΣE going from one dilution to the next higher or lower dilution is about 20 mm. Therefore, if dilution #15 is negative, proceeding to dilution #12 would not be expected to exceed a ΣE response of 60 mm. If dilution #12 is negative, a similar rationale explains proceeding to dilution #9. Do not inject reference extract dilutions more concentrated than #5 unless the extract is known to be of low potency *e.g.* concentrate fails to produce large puncture ΣE response in sensitive subject.
5. To reduce interactions on the arm between proximal and distal skin test sites, place the higher dilutions (*e.g.* end point dilutions) distally (near the wrist) and titrate with more concentrated dilutions proximally. For final titrations use the back for consistency. All titrations may be carried out on the back.
6. Apply each dilution in singlicate for each product and reference.
7. If the forearms are used for titrations, to avoid bias in skin reaction size (right versus left), each product should be tested using a randomized block design with two subjects in each block. Within each block, the right arm of the first subject will randomly get one of the two products and the left arm the other product. The second subject will get products in reverse order.
8. Always include an intradermal diluent control test.

Computation of Subject Sensitivity and Assignment of Bioequivalent Allergy Units

A. Calculation of D_{50}

1. Calculate the ΣE diameter for each dilution in each subject. The dose-response line consists of four serial 3-fold dilutions with graded ΣE responses which bracket ΣE=50mm and includes the end-point dilution where ΣE=0 or ΣE~ΣW. If not, repeat the assay (Fig. 8).
2. Calculate individual best-fit regression lines for each subject, using the formula:

   Y = I + BX

   where:
   Y = Sum of erythema diameters at each dose
   I = Y axis intercept
   B = Slope
   X = Logarithm to the base 3 of dose

3. For each test subject, the correlation coefficient (R) for the best-fit line should be ≥ 0.92. If R of the test product is less than 0.92, the assay is considered invalid. For each subject the slope should be ≥ 13 mm. If less than 13 mm, the line is discarded.
4. Calculate Subject Sensitivity (D_{50}) using the formula: **D_{50} = (50-I) / B** (Fig. 9).

B. Assignment of Bioequivalent Allergy Units (BAU)

1. In the 15 subjects with acceptable puncture reactivity and D_{50}, calculate the mean D_{50} and standard deviation (SD) of the mean. The 99% upper limit of the SD of the mean D_{50} when N=15 is 2.74. If the standard deviation exceeds the upper limit, the sensitivty of the subjects selected was more variable than in the reference population usually due to inclusion of subjects with puncture responses <75mm. Attempt to select additional subjects with target puncture reactivity ≥ 75mm.
2. Use the following Table to estimate BAU/ml:

Estimated BAU/ml based on Mean D_{50}

Target Mean D_{50}	95% CI of Mean D_{50}	BAU/ml
14	13-15	100,000
11.9	10.9-12.9	10,000
9.8	8.8-10.8	1,000
7.7	6.7-8.7	100

3. Calculate actual BAU/ml in concentrate (Dilution#0) using the formula: **BAU/ml= $3^{-(14-\text{Mean } D_{50})} \cdot 100{,}000$.**

4. In each subject determine BAU/ml for 50mm sum of erythema diameters (BAU_{50}) using formula: $\mathbf{BAU_{50}=3^{-D_{50}} \bullet BAU/ml}$ **in concentrate.** Calculate mean, SD, and range of BAU_{50} for 15 subjects.
5. Calculate the dilution factor to equal the Target Mean D_{50} closest to the observed Mean D_{50}. Calculate a mean BAU_{50} and BAU_{50} range adjusted to equal the Target Mean D_{50}.
6. Once BAU have been assigned, studies of site to site replicability of BAU will need to be based on selection of subjects similar to that used in the reference study. The puncture ΣE response criteria of the subjects selected in the replicability study will need to target that observed in the reference population. All other aspects of the procedure will need to be the same as that used in evaluating the reference population.

Intradermal Titration

Allergen: _____ Lot #_____

Date of intradermal test ___/___/___
M D Y

Use serial __3__ fold dilutions (See Intradermal Titration Technique, paragraph 1).

Dilution #0 contains _____ (Allergy Units/ml, w/v, protein, etc.).

Dilution #	ΣE	ΣW
_____	____	____
_____	____	____
_____	____	____
_____	____	____
Intradermal Diluent	____	____

MAKE SURE THAT THE ENDPOINT DILUTION #, WHERE ΣE = 0 or ~ΣW, IS INCLUDED IN TEST SERIES AND ΣE BRACKETS 50 mm.

Compute allergen best fit line where Y = ΣE and X = Dilution #.

Slope = _____ mm. Is slope ≥10 mm? _____ (Y=yes;N=No) If no, repeat titration.

Do ΣE values bracket 50 mm? _____ (Y=yes;N=No) If no, repeat titration.

Correlation coefficient (r) = _____

Is correlation coefficient (r) ≥0.85? _____ (Y=yes; N=No) If no, repeat titration.

Y Intercept (when X = 0) = _____mm.

Compute dilution # (D_{50}) when ΣE is 50 mm

$$D_{50} = \frac{50 - \text{intercept}}{\text{slope}}$$

$$D_{50} = \frac{(50 - \qquad)}{(\qquad)}$$

$$D_{50} =$$

Did subject experience an adverse Reaction? _____ (Y=yes; N=No). If yes, describe _____.

Intradermal Titration

Histamine:

Use serial 3 fold dilutions (See Intradermal Titration Technique, Paragraph 1).

Dilution #0 contains _____ mg/ml histamine base.

Dilution #	ΣE	ΣW	
_____	____	____	MAKE SURE THAT THE ENDPOINT
_____	____	____	DILUTION #, WHERE ΣE = 0 or ~ΣW,
_____	____	____	IS INCLUDED IN TEST SERIES AND
_____	____	____	ΣE BRACKETS 50 mm.

Compute histamine best fit line where Y = ΣE and X = Dilution #.

Slope = _____ mm. Is slope ≥10 mm? _____ (Y=yes;N=No) If no, repeat titration.

Correlation coefficient (r) = _____

Is correlation coefficient (r) ≥0.85? _____ (Y=yes; N=No) If no, repeat titration.

Y Intercept (when X = 0) = _____mm.

Compute dilution # (D_{50}) when ΣE is 50 mm

$$D_{50} = \frac{50 - \text{intercept}}{\text{slope}}$$

$$D_{50} = \frac{(50 - \qquad)}{(\qquad)}$$

$$D_{50} =$$

ID_{50}EAL Method Work Sheet

Investigator _____ Date _____

Technician _____ Extract _____ Mfr. _____

Lot # _____ Conc. _____

	Patient ID ____	Patient ID ____	Patient ID ____	Patient ID ____	Patient ID ____
(3-fold) Dilution #	ΣE	ΣE	ΣE	ΣE	ΣE
*0					
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
Correl. Coeff:					
Slope:					
Intercept					
D_{50}					
*Histamine base 1.8 mg/ml					
*Histamine base 0.1 mg/ml					
Intradermal Negative Control					

*Puncture test