TITAN IV Immunoelectrophoresis Procedure

Cat. No. 9050 and 9061

Helena Laboratories

Titan IV Immunoelectrophoresis (IEP) is intended for semiquantitative determinations by immunoelectrophoresis.

SUMMARY

Immunoelectrophoresis (IEP) combines two techniques, electrophoresis and immunodiffusion. In this two-part procedure, proteins in a serum or urine sample are first separated according to charge by electrophoresis. Then, antisera complimentary to the proteins under study are applied to the plate and allowed to diffuse. When a favorable antigen to antibody ratio exists, a precipitin arc will form on the plate.

IEP is used for the diagnosis and differential diagnosis of monoclonal gammopathies when using serum or urine specimens.[1-4] The method is also used for a number of other purposes, including screening for circulating immune complexes, characterization of cryoglobulinemia and pyroglobulinemia, recognition and characterization of antibody syndromes, and recognition and characterization of the various forms of dysgammaglobulinemias. IEP is a reliable and accurate method for routine protein evaluations, detecting both structural abnormalities and concentration changes.[1-6]

The most common application of IEP is the diagnosis of monoclonal gammopathies. A monoclonal gammopathy is a condition in which a single clone of plasma cells produces elevated levels of a single class and type of immunoglobulin. The elevated immunoglobulin is referred to as a monoclonal protein, M-protein, or paraprotein. Monoclonal gammopathies may indicate a malignancy such as multiple myeloma or macroglobulinemia. The class (heavy chain) and type (light chain) must be established since the patient's prognosis and treatment may differ depending on the immunoglobulin involved. Differentiation must also be made between monoclonal and polyclonal gammopathies.[1-6]

A polyclonal gammopathy is a secondary disease state caused by disorders such as liver disease, collagen disorders, rheumatoid arthritis, and chronic infection. It is characterized by the elevation of two or more (often all) immunoglobulins by several clones of plasma cells. Polyclonal increases are usually twice normal levels.[1-6]

PRINCIPLE

The patient's serum or urine sample and normal human serum control are electrophoresed on the agarose plate, separating the immunoglobulins according to their electrophoretic mobility. Antisera are then applied to troughs in the plate and allowed to diffuse into the agarose support medium. When a favorable antigen-to-antibody ratio exists, a precipitin arc will form on the plate. Proteins are thus differentiated not only by their electrophoretic mobility, but also by their diffusion coefficient and antibody specificity.

Diffusion is halted by rinsing the plate in 0.85% saline. Unbound protein is washed from the plate by the saline, and the antigen/antibody precipitin arcs are stained with a protein sensitive stain. The precipitin arcs formed by the patient sample and the control are compared for a semi-quantitative protein analysis.

REAGENTS

1. Titan IV IEPlate, Cat. No. 9050, 9061

Ingredients: Each IEP plate contains 1.5% agarose (w/v), in barbital-sodium barbital buffer with 0.1% sodium azide added as a preservative.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. This product contains sodium azide. Refer to the sodium azide warning.

Preparation for Use: The plates are ready for use as packaged.

Storage and Stability: Plates should be stored flat, in the protective packaging, at 2°C to 8°C and are stable until the expiration date indicated on the label. DO NOT FREEZE PLATES OR EXPOSE THEM TO EXCESSIVE HEAT.

Signs of Deterioration: The plates should have a smooth, clear agarose surface. Discard the plates if they appear cloudy, show bacterial growth, or if they have been exposed to freezing (a cracked or bubbled surface) or excessive heat (a dried, thin surface).

2. Antisera for Assay of Immunoglobulins

Antiserum to Human IgG, Cat. No. 9232
Antiserum to Human IgA, Cat. No. 9231
Antiserum to Human IgM, Cat. No. 9234
Antiserum to Human IgD, Cat. No. 9249
Antiserum to Human IgE, Cat. No. 9250
Antiserum to Human Kappa Light Chain, Cat. No. 9262
Antiserum to Human Lambda Light Chain, Cat. No. 9257
Trivalent Antiserum to Human Immunoglobulins (Heavy Chain Specific for IgG, IgA, IgM), Cat. No. 9236
Antiserum to Human Serum, Cat. No. 9233
Pentavalent antiserum to Human Immunoglobulins (Specific for IgG, IgA, IgM, IgD, IgE), Cat. No. 9251

Ingredients: Each vial of antiserum contains the specificity as indicated on the vial. The antisera are prepared in horse, sheep, goat, donkey or rabbit. Each vial contains 0.1% sodium azide as a preservative.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. Refer to the sodium azide warning.

Preparation for Use: The antisera are in liquid form and are ready for use as packaged.

Storage and Stability: The antisera should be stored at 2°C to 8°C and are stable until the expiration date indicated on the vial.

Signs of Deterioration: The antisera should be colorless to light yellow.

3. IEP Normal Human Serum Control, Cat. No. 9010

Ingredients: The control contains pooled normal human serum with 0.1% sodium azide as a preservative.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. Refer to the sodium azide warning. This material has been determined negative for Hepatitis B Antigen (HBsAg), HIV I/II and HCV antibodies; however, it should be handled with the same precautions as those observed when handling any human serum.

Preparation for Use: The control is in liquid form and is ready for use as packaged. Before using, add two drops of albumin marker to the control vial.

Storage and Stability: The control should be stored at 2°C to 8°C and is stable until the expiration date indicated on the vial. Stability is not affected by the addition of albumin marker.

Signs of Deterioration: The control should be light yellow and slightly hazy before the addition of marker.

4. Electra® B_1 Buffer, Cat. No. 5016

Ingredients: Contains barbital-sodium barbital, pH 8.3-8.7.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. The buffer contains barbital which, in sufficient quantity, can be toxic.

Preparation for Use: Dissolve one package of buffer in one liter of deionized water. The buffer is ready for use when it is completely dissolved.

Storage and Stability: The packaged, dry buffer should be stored at room temperature (15 to 30°C) and is stable until the expiration date on the package. Buffer solution is stable for two months at 15 to 30°C.

Signs of Deterioration: Discard packaged buffer if the material shows signs of dampness or discoloration. Discard buffer solution if it becomes turbid.

5. Albumin Marker, Cat. No. 9011

Ingredients: The albumin marker is 0.5% bromophenol blue in aqueous solution.

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.

Preparation for Use: Add two drops of Albumin Marker to each vial of IEP Normal Human Serum Control. The bromophenol blue binds with the albumin in the control. The tagged albumin allows verification of protein mobility during electrophoresis.

Storage and Stability: The marker should be stored at 15 to 30°C and is stable until the expiration date indicated on the vial.

Signs of Deterioration: Discard if the solution color changes from the yellow-brown color.

SODIUM AZIDE WARNING

To prevent the formation of toxic vapors, do not mix with acidic solutions. When discarding, always flush sink with copious amounts of water. This will prevent the formation of metallic azides which, when highly concentrated in metal plumbing, are potentially explosive. In addition to purging pipes with water, plumbing should occasionally be decontaminated with 10% NaOH.

SPECIMEN COLLECTION AND HANDLING

Specimen: Fresh human serum or urine are the specimens of choice. Urine samples should be tested unconcentrated as well as concentrated (10X to 50X) due to the wide range of light chain concentrations.

Patient Preparation: No special patient preparation is necessary.

Interfering Factors:

1. Samples showing evidence of hemolysis should not be used.
2. Inaccurate results may be obtained on specimens left uncovered, due to evaporation.
3. Microbial contamination of samples will cause protein denaturation affecting results.
4. Patient age, sex, history and clinical presentation will affect immunoglobulin levels and must be considered.

Storage and Stability: Serum and urine samples should be assayed fresh if possible. Samples may be stored at 2 to 8°C for up to 5 days after collection. Serum and urine refrigerated samples should be warmed to room temperature and mixed well prior to testing.

PROCEDURE

Materials Provided by Helena:	**Cat. No.**
Titan IV IEPlates, 8 wells, 7 troughs	9050
Titan IV IEPlates, 7 wells, 6 troughs	9061
Electra® B_1 Buffer	5016
IEP Normal Human Serum Control	9010
Albumin Marker	9011
Antiserum to Human IgG	9232
Antiserum to Human IgA	9231
Antiserum to Human IgM	9234
Antiserum to Human IgD	9249
Antiserum to Human IgE	9250
Antiserum to Human Kappa Chain	9262
Antiserum to Human Lambda Chain	9257
Trivalent Antiserum to Human Immunoglobulins (IgG, IgA, IgM)	9236
Pentavalent antiserum to Human Immunoglobulins (Specific for IgG, IgA, IgM, IgD, IgE), Cat. No. 9251	
Antiserum to Human Serum	9233
Microdispenser and Tubes	6210
TITAN GEL Chamber (Zip Zone® Chamber, Cat. No. 1283, may be used)	4063
IEP Sponge Wicks, Long	9015

Materials Needed but Not Supplied:

Saline Solution (0.85%)
5% acetic acid

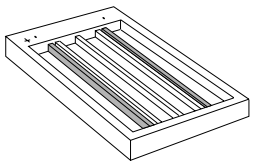
SUMMARY OF CONDITIONS

Plate	Titan IV IEPlate
Buffer	B_1 Buffer dissolved in 1.0 L deionized water
Sample Volume	4 µL
Migration Distance	Cat. No. 9061, 20 mm Cat. No. 9050, 35-40 mm
Electrophoresis Time	40-50 minutes
Voltage	100 V
Antisera Volume	Cat. No. 9050, 75-100 µL Cat. No. 9061, 150 µL
Incubation Time	18-24 hours
Incubation Temperature	15 to 30°C
Wash Solution	0.85% Saline
Saline Wash Time	48-72 hours
Staining Time	30 min.
Destaining Time	90-180 minutes

STEP-BY-STEP METHOD

A. Preparation of TITAN GEL Chamber

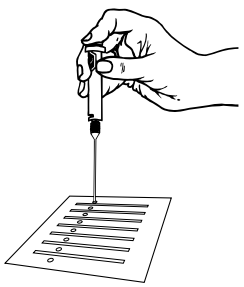
1. Dissolve 1 package of Electra B_1 Buffer in one liter of deionized water.
2. Pour 100 mL of buffer into each outer section of the chamber (total of 200 mL of buffer used). The buffer can be reused one time by reversing the polarity of the chamber. The IEPlates must then be placed with the wells on the left side (formerly the anodic side).



3. Place a long IEP Sponge Wick in each buffer-filled compartment. Allow the sponges to become saturated with buffer. Place the sponges against the chamber walls as shown.
4. Cover the chamber until ready to use.

B. Sample Application

1. Remove the Titan IV IEPlate(s) from the refrigerator. Allow the plate(s) to come to room temperature prior to use (requires 20-30 minutes).
2. Add two drops of Albumin Marker to a vial of Normal Human Serum Control.
3. Remove the lid from the plate.
4. Apply 4 µL of the control to every other well in the plate



using a Microdispenser. Take care not to damage the wells during sample application.

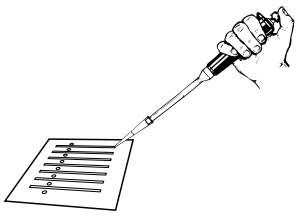
5. Apply 4 µL of the patient sample to every other well.

C. Electrophoresis of the IEPlate(s)

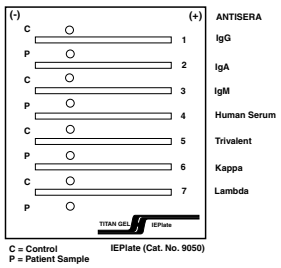
1. Quickly put the plate(s) in the electrophoresis chamber, agarose side down, with the wells toward the cathode (-) side of the chamber.
   Make sure that the agarose makes good contact with the sponge wicks. Two plates may be electrophoresed in one chamber.
2. Put the cover on the electrophoresis chamber and wait 30 to 60 seconds before applying current. This allows the plate(s) to equilibrate with the buffer.
3. Electrophorese the plate(s) at 100 volts for the appropriate migration distance. This requires approximately 40 to 50 minutes. Migration distance can be verified visually by observing the position of the Albumin Marker.
   The migration distances for the plates are as follows:
   Cat. No. 9061: 20 mm
   Cat. No. 9050: 35-40 mm

D. Antisera Application

1. Remove the plate(s) from the chamber and put them on a flat surface, agarose side up.
2. Apply the appropriate antiserum to each trough in the plate using the appropriate volume of antisera as follows:
   Cat. No. 9061, 150 µL/trough
   Cat. No. 9050, 75-100 µL/trough
   Fill the troughs by placing the tip of a pipette in the end of the trough farthest from the sample well. Holding the pipette in place, slowly depress the plunger and dispense the antiserum into the trough. The antiserum will flow down the trough by capillary action. Severe overfilling may cause antisera to contaminate other troughs, yielding erroneous results.



3. Before moving the plates, allow the antisera to absorb for approximately 3 to 5 minutes.
4. Stack the plates in an enclosed container with a moist paper wick.
5. Incubate the plate(s) for 18 to 24 hours at room temperature (15 to 30°C). The minimum incubation time is 18 hours. After incubation, place the plates in a 0.85% saline wash to stop the precipitin reaction.



E. Interpretation of Unstained Plates

1. Place the plate on a light box for viewing. The precipitin arcs will appear as dense opaque white arcs in the agarose layer.
2. Compare the patient arcs with the Normal Control in order to determine the presence or absence of an abnormal immunoglobulin.
3. For a permanent record, photograph the IEP plate.

Stability of End Product: The dried Titan IV IEPlate is stable for an indefinite period of time.

Quality Control: IEP Normal Human Serum Control with Albumin Marker added should be used as a control for each antiserum specificity used.

RESULTS

The formation of a precipitin arc between a well containing test specimen and a trough containing antiserum indicates the presence of the protein specific to the antiserum. The lack of a precipitin arc indicates that a detectable amount of the protein is not present in the test specimen or that prozoning has occurred. The size, location, and shape of the precipitin arc, as compared to the control, are indications of the amount of protein in the test sample. IEP is a semiquantitative technique.

In general, when protein concentrations are below normal, precipitin arcs are shortened and located farther from the antiserum trough compared to the corresponding arc in the control. When protein concentrations are above normal, precipitin arcs are thicker and located closer to the antiserum trough compared to the control.

Further Testing Required:

A. IgM Typing (IgA)

Interpretation of Light Chain reactions associated with monoclonal IgM is often difficult due to the umbrella effect of IgG. The following method may be used to depolymerize IgM (19S) into single molecular units (7S), which diffuse through the agarose more rapidly. In some instances, this may be necessary for IgA typing.

Perform this procedure under a fume hood. The vapor from 2-mercaptoethanol (2-ME) may cause skin irritation. Avoid contact with skin, eyes and clothing.

1. Materials:
   2-mercaptoethanol
   10 µL disposable pipettes
   100 µL disposable pipettes
   Fume hood
2. Make 1:10 dilution, 2-ME to deionized or distilled water.
3. Add 10 µL of diluted 2-MI to 100 µL of patient sample.
4. Assay immediately, running the plate(s) with treated and untreated patient sample in alternate wells.

B. Correction for Antigen Excess

Antigen excess (prozoning) is an incomplete precipitin reaction caused by antigen excess (too high an antigen-to-antibody ratio). Prozoning should be suspected if a precipitin arc appears to “run” into a trough or if a light chain appears fuzzy when a heavy chain is increased or if an arc appears to be incomplete. To correct for antigen excess, use one of the following three methods.

1. After incubation, but before washing in saline, add an additional 75-100 µL of antisera to the troughs in question and incubate an additional 18 to 24 hours.
2. Retest the sample using twice the volume of originally specified antiserum in the trough(s) in question. Add half the volume and allow the antiserum to diffuse into the plate, then add the additional half volume.
3. Make a 1:2 dilution of the patient sample with saline and repeat the entire procedure.

INTERPRETATION OF RESULTS

A sample immunoglobulin profile is illustrated below. The pattern of precipitin arcs is interpreted comparing the patient sample to the control. In the figure, the patient serum forms a dense, bowed arc against IgG antiserum. There appears to be a diminished IgA level and virtually no IgM in the patient serum when compared to the IgA and IgM in the control. The abnormal IgG band is also visible against both Human Serum and Trivalent antisera. The patient sample reveals a bowed, abnormal kappa arc and a decreased lambda arc. The

composite is indicative of an "IgG Monoclonal Gammopathy Kappa type".

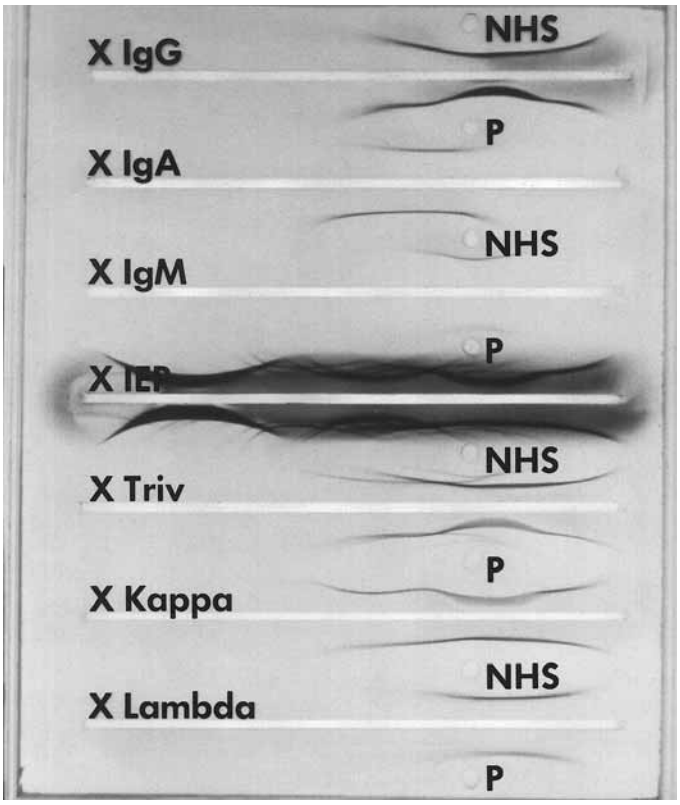


Fig. 1: Titan IV IEPlate illustration indicative of an IgG monoclonal gammopathy, kappa type.

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Additional References

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4. Ritzmann, S.E. et al., Bence Jones Proteins: Another Look, Lab Management, 17-21, September 1981.

Titan IV IEPlate System

	Cat. No.
The following items are available individually:	
Titan IV IEPlates, 8 wells, 7 troughs (5 plates)	9050
Titan IV IEPlates, 7 wells, 6 troughs (5 plates)	9061
Electra® B_1 Buffer (10 pkg/box)	5016
IEP Normal Human Serum Control (1 x 2.0 mL)	9010
Albumin Marker (1 x 2.0 mL)	9011
Antiserum to Human IgG (1 x 2.0 mL)	9232
Antiserum to Human IgA (1 x 2.0 mL)	9231
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Antiserum to Human Serum (1 x 2.0 mL)	9233
Microdispenser and Tubes (1 to 10 µL)	6210
TITAN GEL Chamber (Zip Zone® Chamber, Cat. No. 1283, may be used)	4063
IEP Sponge Wicks, Long (2/pkg)	9015

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