TITAN GEL® ImmunoFix Procedure

Cat. No. 3046

TITAN GEL ImmunoFix is intended for the identification of monoclonal gammopathies in serum, urine or cerebrospinal fluid using high resolution protein electrophoresis and immunofixation.

SUMMARY

Immunofixation electrophoresis (IFE) is a two stage procedure using agarose gel high resolution protein electrophoresis in the first stage and immunoprecipitation in the second. The specimen may be serum, urine or cerebrospinal fluid. There are numerous applications for IFE in research, forensic medicine, genetic studies and clinical laboratory procedures. The greatest demand for IFE is in the clinical laboratory where it is primarily used for the detection and identification of monoclonal immunoglobulin gammopathies. A monoclonal gammopathy is a primary disease state in which a single clone of plasma cells produces elevated levels of an immunoglobulin of a single class and type. Such immunoglobulins are referred to as monoclonal proteins, M-proteins, or paraproteins. In most cases they are indicative of a malignancy such as multiple myeloma or Waldenstrom' s macroglobulinemia. Differentiation must be made between polyclonal and monoclonal gammopathies because polyclonal gammopathies are only a secondary disease state due to clinical disorders such as chronic liver diseases, collagen disorders, rheumatoid arthritis, and chronic infections. Alfonso first described immunofixation in the literature in 1964.[1] Alper and Johnson published a more practical procedure in 1969 as a result of their work devoted to the detection of genetic polymorphisms of ceruloplasmin and Gc-globulin and the conversion of C3 during activation.[2] They later extended their studies to genetic polymorphisms of complement components and the identification of alpha$_1$ antitrypsin.[3, 4] Immunofixation has been used as a procedure for the study of immunoglobulins since 1976.[5, 6]
Immunofixation offers a number of advantages over immunoelectrophoresis for the detection and identification of monoclonal immunoglobulins. It is more sensitive, requires less time and is easier to intepret. Because of one or all of these factors, many laboratories have chosen to switch from using IEP to using immunofixation. Helena Laboratories has made revisions in the TITAN GEL ImmunoFix Procedure that streamlines handling steps and reduces the time required to perform the test to 50 minutes.

PRINCIPLE

Proteins are first resolved by electrophoresis. In the second stage, the soluble antigen and its antibody are allowed to react. The resultant antigen-antibody complex(es) may become insoluble (as long as the antibody is in slight excess or near equivalency) and precipitate.The precipitation rate depends on the proportions of the reactants, temperature, salt concentration and the pH of the solution. The unreacted proteins are removed by a washing step and the antigen-antibody complex (which might be visible as a white cloudy band in unstained gel against a dark background), is visualized by staining. The bands in the protein separation are compared with the precipitin bands obtained with immunofixation.

REAGENTS

1. **TITAN GEL IFE Gel**
   Ingredients: Each gel contains agarose in tris-barbital/aspartate buffer with 0.1% sodium azide and thimerosal added as preservatives.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. CAUTION: The gel contains barbital which, in sufficient quantity, can be toxic. Refer to the Sodium Azide Warning.
   Preparation for Use: The gels are ready for use as packaged
   Storage and Stability: The gels should be stored at room temperature (15 to 30°C) and are stable until the expiration date indicated on the package. The gels must be stored in the protective packaging in which they are shipped. DO NOT REFRIGERATE OR FREEZE
   Signs of Deterioration: Any of the following conditions may indicate deterioration of the gel: (1) crystalline appearance indicating the agarose has been frozen, (2) cracking and peeling indicating drying of the agarose, (3) bacterial growth indicating contamination.
2. **TITAN GEL IFE Buffer**
   Ingredients: The buffer contains barbital and sodium barbital with 0.1% sodium azide and thimerosal as preservatives.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. TOXIC - DO NOT INGEST. CAUTION: The buffer contains barbital which, in sufficient quantity, can be toxic. Refer to the Sodium Azide Warning.
   Preparation for Use: Dissolve one package of buffer in 1500 mL deionized or distilled water. The buffer is ready for use when all material is completely dissolved.
   Storage and Stability: The packaged buffer should be stored at 15 to 30°C and is stable until the expiration date indicated on the package. Diluted buffer is stable two months stored at 15 to 30°C or 2 to 8°C.
   Signs of Deterioration: Discard packaged buffer if the material shows signs of dampness or discoloration. Discard diluted buffer if it becomes turbid.
3. **Acid Blue Stain**
   Ingredients: The stain is comprised of acid blue stain.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.
   Preparation for Use: Dissolve the dry stain in 1000 mL 5% acetic acid.
   Storage and Stability: The dry stain should be stored at 15 to 30°C and is stable until the expiration date indicated on the package. The stain solution is stable for six months when stored at 15 to 30°C in a closed container. Used stain may be returned to the container and reused.
   Signs of Deterioration: The diluted stain should be a homogeneous mixture free of precipitate.
4. **TITAN GEL IFE Protein Fixative**
   Ingredients: The fixative contains 10% sulfosalicylic acid and 10% acetic acid.
   WARNING: CORROSIVE - FOR IN-VITRO DIAGNOSTIC USE ONLY. NEVER PIPETTE BY MOUTH. DO NOT INGEST.
   Storage and Stability: The fixative should be stored at 2 to 8°C and is stable until the expiration date indicated on the vial.
   Signs of Deterioration: The fixative should be a clear yellow solution.
5. **Antisera to Human IgG, IgA, IgM, Kappa Light Chain and Lambda Light Chain**
   Ingredients: Antisera vials in the kit contain monospecific antisera to human immunoglobulin heavy chains, IgG, IgM, and IgA, and to human light chains, Kappa and Lambda. The antisera have been prepared in sheep and goat. Each vial of antiserum contains a stabilizer and sodium azide as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. Refer to the Sodium Azide Warning.
   Preparation for Use: The antisera are ready for use as packaged.
   Storage and Stability: The antisera should be stored at 2 to 8°C and are stable until the expiration date indicated on the vial.
   Signs of Deterioration: Extremely cloudy antisera may be indicative of bacterial contamination.

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: The specimen may be serum, cerebrospinal fluid or urine.
Serum Specimen Preparation:

1. Dilute all serum samples with 0.85% saline. Sample dilutions should be freshly prepared on day of use.
   a. Dilute serum 1:2 for the SPE reference pattern.
   b. Dilute serum 1:10 for the immunofixation electrophoresis patterns.
   c. When typing minimonoclonal specimens, if the sample IgG level exceeds 1500 mg/dL, the sample should be diluted 1:20 for the IgG slot only.
   d. When typing IgM or Lambda proteins in specimens containing mini monoclonal bands, a sample dilution of 1:5 is recommended for the IgM and Lambda patterns.

Urine Specimen Preparation:
Detection of Bence Jones proteins (free kappa and lambda light chains): If necessary concentrate urine sample to 100 mg/dL of total protein.

Cerebrospinal Fluid Specimen Preparation:
Concentrate CSF to an IgG level of 100-200 mg/dL for typing oligoclonal bands in CSF. Use concentrated specimen for all patterns.

Interfering Factors:
1. Evaporation of uncovered specimens may cause inaccurate results.
2. Plasma should not be used because the fibrinogen may adhere to the gel matrix resulting in a band in all patterns across the gel.

Storage and Stability: Fresh serum, CSF or urine is the specimen of choice. If storage is necessary, samples may be stored covered at 2 to 8°C for up to 72 hours.

PROCEDURE

Materials Provided: The following materials are contained in the TITAN GEL ImmunoFix Kit, (Cat. No. 3046).

TITAN GEL IFE Gels (10)
TITAN GEL IFE Buffer (1 pkg)
Acid Blue Stain (1 vial)
TITAN GEL IFE Sample Templates (10)
TITAN GEL IFE Antisera Templates (10)
TITAN GEL Protein Fixative (1 x 0.75 mL)
TITAN GEL IFE Antiserum to Human IgG (1 x 0.75 mL)
TITAN GEL IFE Antiserum to Human IgA (1 x 0.75 mL)
TITAN GEL IFE Antiserum to Human IgM (1 x 0.75 mL)
TITAN GEL IFE Antiserum to Human Lambda Light Chain (1 x 0.75 mL)
TITAN GEL IFE Antiserum to Human Kappa Light Chain (1 x 0.75 mL)
TITAN GEL Blotter A (30)
TITAN GEL Blotter C (30)
TITAN GEL Blotter D (10)
TITAN GEL Blotter X (10)

Materials provided by Helena Laboratories but not contained in the kit above:	Cat. No.
ImmunoFix Controls (3 x 0.5 mL)	9400
TITAN GEL IgD (1 x 1.0 mL)	9409
TITAN GEL IgE (1 x 1.0 mL)	9410
Dialamatic Microdispenser and Tubes (1-10 uL)	6210
TITAN GEL Electrophoresis Chamber	4063
Titan Plus Power Supply	1504
TITAN GEL Isoenzyme Incubation Chamber	4062
1000 Staining Set and Rack	5122
Titan Carrying Rack	5110

Materials and Supplies Needed but not Supplied:

Glacial Acetic Acid
Destaining Solution: 5% acetic acid. Store at 15 to 30°C.
Saline (0.85%)
Laboratory Rotator

SUMMARY OF CONDITIONS

Gel	TITAN GEL IFE Gel
Buffer	1500 mL
Buffer Volume	40 mL each side
Serum Dilution	1:2 (0.85% Saline) for SP Pattern 1:10 (0.85% Saline) Immunoglobulin identification
Blotter C	Blot gel
Sample Volume	3 µL
Sample Absorption Time	5 minutes
Application Point	Cathode
Electrophoresis Time/Voltage	20 minutes/120 volts
Fixative Volume	1 drop (approx. 50-75 µL)
Antisera Volume	1 drop (approx. 50-75 µL)
Incubation Time	10 minutes
Press Conditions	Press 1 - 1 Blotter C & 1 Blotter X Press 2 - 1 Blotter C & 1 Blotter D
Press Time	5 minutes and 1 minute
Wash Time	4 minutes
Drying Time/Temp	1-2 minutes/56°C
Staining Time	4 minutes
Destaining Time	2 x 2 minutes
Drying Time/Temp	2-4 minutes/56°C

STEP-BY-STEP METHOD

PART I: PROTEIN ELECTROPHORESIS

A. Patient Sample Preparation

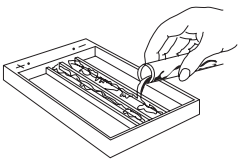
1. Dilute the patient serum samples with 0.85% Saline as follows: 1:2 (1 part serum + 1 part saline) for the serum protein pattern 1:10 (1 part serum + 9 parts saline) for identification of all immunoglobulins

Identification	Dilution for Serum
SP	1:2
IgG, IgA, IgM Kappa, Lambda	1:10

2. If necessary, concentrate urine and spinal fluids according to instructions provided in SPECIMEN COLLECTION AND HANDLING.

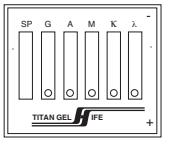
B. Preparation of Electrophoresis Chamber

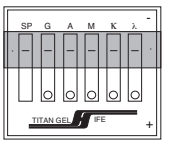
1. Dissolve one package of TITAN GEL IFE Buffer in 1500 mL deionized or distilled water. Mix well for complete dissolution.
2. Pour 40 mL buffer into each of the inner sections of the TITAN GEL Chamber. Total buffer volume = 80 mL
3. Cover the chamber until ready to use to prevent evaporation.



C. Sample Application

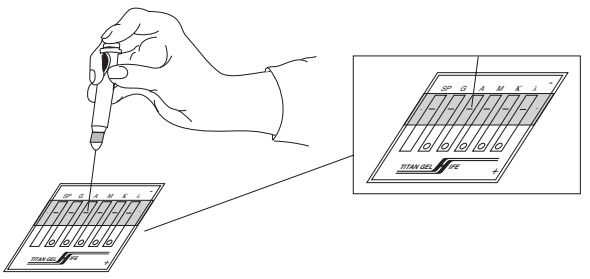
1. Remove the TITAN GEL IFE gel from the protective packaging and discard the paper overlay.
2. Gently blot the surface of the gel with TITAN GEL Blotter C.
3. Place the sample template on the gel so that the small hole, in the corner of the template, is positioned at the lower left, and the application slits align with the arrows on the gel edges. Proper placement of the template is with the slightly rough side of the slits away from the gel ensuring uniform absorption of of the sample. Apply slight fingertip pressure to the template making sure there are no air bubbles between it and the gel.



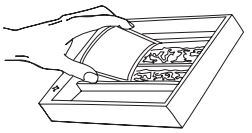


NOTE: When wearing rubber gloves to perform this step, place a Blotter A over the template and then apply fingertip pressure. The powder from the gloves can produce artifacts on the gel.

4. Apply 3.0 µL of the appropriate serum sample dilution or concentrated urine or cerebrospinal fluid to the template slits. When using a concentrated sample, apply the concentrate to every position across the gel.



5. Wait five (5) minutes after the last sample has been applied to allow the samples to diffuse into the agarose.
6. After allowing the samples to absorb into the agarose, gently blot the template with TITAN GEL Blotter A to remove unabsorbed sample. Then carefully remove the template.



D. Electrophoresis of the Sample Gel

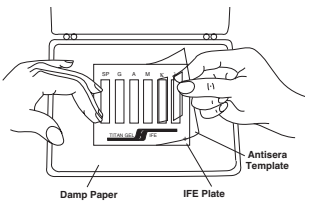
1. Place the TITAN GEL IFE Gel in the inner sections of the electrophoresis chamber, agarose side up, with the edges of the gel in the buffer. The application point should be on the cathodic (-) side. Two gels may be run per TITAN GEL Chamber. A maximum of 4 gels can be run on a Titan Plus Power Supply.
2. Place the cover on the TITAN GEL Chamber.
3. Electrophorese the gel at 120 volts for 20 minutes.

Alternate Electrophoresis Procedure

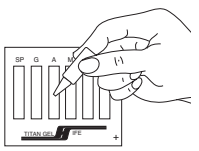
1. While the sample is absorbing into the gel, pour 125 mL buffer into each of the outer sections of the TITAN GEL Chamber. Total Volume = 250 mL. Remove the TITAN GEL Chamber Cooling Device from the refrigerator and place it in the center of the TITAN GEL Chamber. Wet the entire surface of the cooling device with a few drops of buffer.
2. Quickly place the TITAN GEL IFE Gel in the electrophoresis chamber, agarose side up, on top of the TITAN GEL Chamber Cooling Device. The application point should be on the cathodic (-) side. Avoid trapping air bubbles between the agarose gel and glass of the Chamber Cooling Device. Run one gel per chamber.
3. Prepare a wick for each side of the gel by placing three TITAN GEL IFE Wicks together in two sets making two thick wicks. Evenly align the edge of each set of wicks, and dip them into the chamber buffer. Then attach the wicks to each side of the gel parallel to the edge of the TITAN GEL Chamber Cooling Device. Gently rub one finger across the gel at the wick contact area to insure good contact and to displace trapped bubbles.
4. Place the cover on the TITAN GEL Chamber.
5. Electrophorese the gel at 250 volts for 15 to 18 minutes.

PART II: IMMUNOFIXATION

1. Remove the electrophoresed gel from the chamber.
2. Place the gel in the TITAN GEL Isoenzyme Incubation Chamber which has been lined with a damp blotter or filter paper or return the gel to the protective plastic package. Be sure the gel is laying flat against the wet blotter. Should the gel maintain a bowed shape after removal from the electrophoresis chamber, moisten the blotter in the incubation chamber sufficiently to hold it flat.
3. Apply the IFE Controls
   a. Since the control wells are very small and may be filled with buffer after electrophoresis, blot them very carefully with a Blotter A to ensure that the wells will hold all control material applied.
   b. Align the Antisera Template on the gel so that the slits in the template are aligned over the antisera application areas on the gel. Make sure the template makes good contact with the agarose, using gentle fingertip pressure along the edges and over the channel dividers.



   c. Apply 2 µL of the controls to the appropriate wells. The IgG Kappa control is applied to both the “G” and “Kappa” wells. The IgA Lambda control is applied to the “A” and “Lambda” wells and the IgM control applied to the “M” well only.
   d. Close the incubation chamber and allow the controls to absorb into the agarose for 2.5 minutes.
   e. Open the incubation chamber and blot the wells with a Blotter A to ensure that the excess unabsorbed control material does not float out of the well during antisera application resulting in a poorly defined control ring.
4. Apply TITAN GEL Antisera and Protein Fixative. The antisera and fixative are packaged in dropper vials and can be applied directly to the gel from the vial. No pipetting is required. The IFE Protein Fixative is applied to the “SP” position of the gel to develop a complete protein pattern. To apply, squeeze the vial until a drop of antiserum or Fixative is “hanging” on the tip of the vial and touching the agarose. Maintain fingertip pressure on the vial, but do not continue to squeeze more antiserum from the vial. Pull this drop down the immunofix channel. The antisera (or Fixative) will be quickly and evenly applied in this manner.

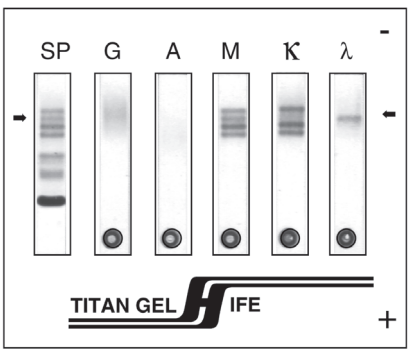


5. Incubate the gel for 10 minutes at room temperature (15 to 30°C) in the closed incubation chamber.
6. Wash and press the gels to clear unprecipitated protein.
   NOTE: The kit contains 3 different types of blotters used in this step. Note that Blotter X is used in the first pressing step and Blotter D is used in the second pressing step. Blotter C is used in both pressing steps and is always placed directly on the agarose.
   a. Remove the gel from the incubation chamber and rinse the gel in 0.85% saline before removing the antisera template. This can be accomplished by quickly dipping the gel in and out of a small container of saline. The antisera template will wash off in the process. This will wash excess antisera from the surface and thoroughly wet the surface of the gel.
   b. Remove the antisera template (usually washes off in the saline).
   c. Place 1 Blotter C, wetted in saline, on the surface of the gel followed by 1 Blotter X.
   d. Place the gel with blotters on top in the Immuno SuperPress, tighten it and press the gels for 5 minutes. Approximately 12-15 gels with blotters can be stacked in the press.
   e. Remove the gels from the press, discard the blotters and place the gels in 0.85% saline wash for 4 minutes. A single gel can be washed by laying it in a shallow dish and covering it with 50 mL saline. Up to 12 gels may be placed in the Titan Carrying Rack and washed in one dish. Place the gels in the wash dish so that they are in a horizontal position (the rack must be rotated so that the gels change from vertical to horizontal positioning).
   f. Remove the gel(s) from the saline wash. Place 1 Blotter C, wetted in saline, followed by 1 Blotter D on each gel. Place the gels with blotters in the SuperPress for 1 minute.
   g. Remove the gels from the press and discard the blotters. Dry the gel in a drying oven at 56-60°C for 1 minute or until the agarose is completely dry.
7. Stain the gel 4 minutes in Acid Blue Stain. Again a single gel can be placed in a shallow staining dish or 12 gels can be stained together in a 1000 Staining Dish and Titan Rack.
8. Place the gels in 2 washes of Destain Solution for 2 minutes each or until the background is clear. (See Materials and Supplies Needed but not Supplied for destain formulation.)
9. Place the gels in the drying oven at 56-60°C until destain has evaporated and gels are completely dry (about 2-4 minutes).
10. Observe the control wells for the presence of precipitin rings indicating appropriate reactivity in the antisera, and interpret results.

Stability of End Product: The completed, stained and dried immunofixation gel is stable for an indefinite period of time.

Quality Control: The ImmunoFix Controls (Cat. No. 9400) are recommended for use as qualitative controls for verification of the appropriate reactivity of the antisera. The set contains three monoclonal proteins: IgG Kappa, IgA Lambda and IgM.

Figure 1: A TITAN GEL ImmunoFix gel showing the protein electrophoresis pattern and immunofixation results and precipitin rings obtained with the controls.



INTEPRETATION OF RESULTS

The majority of monoclonal proteins migrate in the cathodic (gamma) region of the protein pattern. But, due to their abnormality, they may migrate anywhere within the globulin region on protein electrophoresis. The monoclonal protein band on the immunofixation pattern will occupy the same migration position and shape as the monoclonal band on the reference protein electrophoresis pattern. The abnormal protein is identified by the corresponding antiserum used.

Figure 1 illustrates the results obtained with immunofixation. When low concentrations of M-protein are present, the immunofixation band may appear on the stained background of the polyclonal immunoglobulin. A stained background may also appear when the M-protein is present along with a large polyclonal increase. For an in depth discussion of IFE interpretation, call Helena Laboratories toll free and request the free publication,“ImmunoFixation for the Identification of Monoclonal Gammopathies” Form R5.

LIMITATIONS

1. Antigen excess will occur if there is not a slight antibody excess or antigen antibody equivalency at the site of precipitation. Antigen excess in IFE is usually due to a very high level of the immunoglobulin in the patient sample. The dissolution of immunoprecipitation is manifested by a loss of protein at the point of highest antigen concentration resulting in staining in the margins and

leaving the central area with little demonstrable protein stain. In this case it may be necessary to adjust the protein content of the sample by preparing a greater dilution than originally used. Electrophoresing excessive amounts of antigen decreases resolution and requires higher concentrations of antibody. For optimum separation and sufficient intensity for visual detection, care must be taken in adjusting antibody content, sample concentration, time and voltage. The TITAN GEL ImmunoFix Method has been optimally developed to minimize the antigen excess phenomenon.

2. Monoclonal proteins may occasionally adhere to the gel matrix, especially IgM. These bands will appear in all five antisera reaction areas of the gel. However, where the band reacts with the specific antisera for its heavy chain and light chain, there will be a marked increase in size and staining activity, allowing the band to be identified
3. The level of monoclonal protein in urine may not correlate well with the total protein quantitation.

Further Testing Required:

Specimens containing a band on serum protein electrophoresis suggestive of a monoclonal protein, but which do not react with IgG, IgA or IgM antisersa, may require further testing as follows:

1. Serum samples which have a precipitin band with Kappa or Lambda Light Chain antisera but no corresponding band with IgG, IgA or IgM antisera may have a free light chain or they may have an IgD or IgE monoclonal protein. Such sera should be tested with TITAN GEL ImmunoFix IgD and IgE antisera.
2. Cerebrospinal fluid may contain a non-immunoglobulin band, referred to as gamma-trace, which migrates in the gamma region. Because gamma-trace is non-immunoglobulin in nature, it will not react with antisera against human immunoglobulins. Gamma-trace is often detected in normal cerebrospinal fluid.[7, 8]
3. A CRP band may be detected in patients with acute inflammatory response.[9, 10] CRP appears as a narrow band on the most cathodic end of the high resolution agarose protein electrophoresis pattern. Elevated alpha1 antitrypsin and haptoglobin (acute phase proteins) are supportive evidence for the presence of a CRP band. Patients with a CRP band will have a positive CRP by latex agglutination or an elevated quantitative CRP.

PERFORMANCE CHARACTERISTICS

1. Specimens containing monoclonal proteins: Serum samples were tested by the Helena TITAN GEL ImmunoFix Procedure, high resolution electrophoresis, immunoelectrophoresis and other appropriate procedures to identify abnormals and to classify the M-proteins by class and type in serum, with 100% agreement.
2. Specimens negative for protein abnormalities: In a study, ten patients, determined free of protein abnormalities, were tested by immunofixation. All normal specimens gave negative results on the TITAN GEL ImmunoFix Method.

BIBLIOGRAPHY

1. Alfonso, E., Quantitation Immunoelectrophoresis of Serum Proteins, Clin Chem Acta, 10:114-122, 1964.
2. Alper, C.A. and Johnson, A.M., Immunofixation Electrophoresis: A Technique for the Study of Protein Polymorphism. Vo Sang 17:445-452, 1969.
3. Alper, C.A., Genetic Polymorphism of Complement Components as a Probe of Structure and Function. Progress in Immunology, First International Congress of Immunology. Edited by Amos, B., New York, Academic Press, 609-624, 1971.
4. Johnson, A.M., Genetic Typing of Alpha$_1$-Antitrypsin in Immunofixation Electrophoresis. Identification of Subtypes of P.M., J Lab Clin Med, 87:152-163, 1976.
5. Cawley, L.P. et al., Immunofixation Electrophoretic Technique Applied to Identification of Proteins in Serum and Cerebrospinal Fluid, Clin Chem, 22:1262-1268, 1976.
6. Ritchie, R.F. and Smith, R., Immunofixation III, Application to the Study of Monoclonal Proteins, Clin Chem, 22:1982-1985, 1976
7. Keshgegian, A.A., Oligoclonal Immunoglobulins in Cerebrospinal Fluid Multiple Sclerosis, Clin Chem, 26(9):1340-1345, 1980.
8. Papadopoulos, N.M., et al., A Unique Protein in Normal Human Cerebrospinal Fluid, Clin Chem, 29(10):1842-1844, 1983.
9. Jeppsson, J.E. et al, Agarose Gel Electrophoresis, Clin Chem, 25(4):629-638, 1979.
10. Killingsworth, L.M. et al., Protein Analysis, Diagnostic Medicine, 3-15, Jan/Feb, 1980.

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