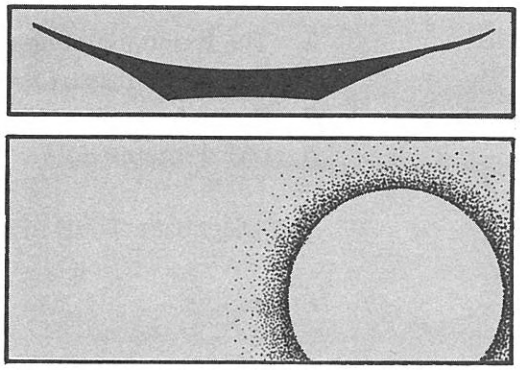
HELENA DIAGNOSTIC IMMUNOLOGY HANDBOOK



Helena Laboratories

HELENA DIAGNOSTIC IMMUNOLOGY HANDBOOK

Immunochemical Assay – Theory, Technique, Application

Helena Laboratories

Helena Laboratories, the world's largest electrophoresis manufacturer, is pleased to introduce the latest in its continuing education series, the Helena Diagnostic Immunology Handbook. As always, our Immunology technical staff, our regional technical representatives, and I stand ready to provide assistance to you in the use of our Immunology products or any of our electrophoresis products.

Best personal regards,

Tipton Golias

Tipton Golias
President

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I. INTRODUCTION

The immune system assumes more importance daily as biomedical research increases our knowledge of the pathogenesis of many diseases. Immune mechanisms are significant in diagnostic medicine for two important reasons. First, many diseases involve changes in a person's immune response mechanisms, i.e. immuno-deficiency states, auto-immune disorders, graft rejection phenomena, gammopathies, and a variety of others. Secondly, these basic immune mechanisms of man and animals can be used to produce laboratory reagents with diversified applications to the research and clinical laboratory. Qualitative and quantitative tests specific for proteins, drugs, hormones, bacteria, viruses, and many other substances have been devised. It is the intent of this Diagnostic Immunology Handbook to provide an understanding of the immune response mechanisms, specifically those of the humoral nature, as they function in-vivo and their powerful application to in-vitro diagnostic test systems for the detection of human protein abnormalities. Emphasis will be placed on those systems most widely used in the research and clinical fields and which are available from Helena Laboratories.

II. THE DEVELOPMENT OF IMMUNOCHEMICAL ASSAY

Historically, the immune system has been cast in a defensive role. In the 1600's, the Chinese practiced variolation (Gr. variola: smallpox). This was a prophylactic measure which involved grinding exudate from smallpox sores into powder which was then inhaled. This produced a local infection and provided protection against smallpox in some cases. Edward Jenner (1749-1823) was the first physician to use the technique of vaccination. He observed that milkmaids possessed immunity to smallpox following infections with the agent of cowpox. Jenner extended his observation by injecting his nephew James Phipps with fluid from a cowpox lesion. Several weeks later he challenged the boy by inoculating him with exudate from active smallpox sores. The boy failed to develop smallpox and Jenner concluded that he was immune to the disease. Pasteur (1822-1895) injected animals with what is now known as attenuated strains of several microorganisms. Following what Pasteur termed vaccination (after Jenner) he showed the animals to be immune to challenge with the virulent organisms.

As evidence accumulated to show the existence of the immune system, data describing the mechanisms of its action began to appear in the 1890's. The experiments of Von Behring, Pfeiffer, Ehrlich, Calmette and others indicated that soluble factors in the fluids of the body (serum, urine) defended against microorganisms and other toxic agents. These soluble serum factors were found to be proteins and were termed "antibodies." The substances used to stimulate antibody formation were termed "antigens." The combination of antibody with antigen in vitro was observed and the serological reactions of bacteriolysis, precipitation, agglutination and later complement fixation were described. Antibodies were produced in animals to toxins such as the tetanus toxin and these antitoxins were administered to animals and humans as prophylactic agents. This process was termed "passive immunity" as compared to "active immunity" produced following vaccination.

The experiments of Ehrlich, Landsteiner and others demonstrated that most substances could serve as antigens. Molecules which themselves initiate antibody formation after injection into an animal are "immunogens." Those molecules, usually small molecules of molecular weight under 10,000, which must be chemically coupled to a carrier such as albumin to initiate antibody formation are termed "haptens." The early work from the laboratories of Ehrlich and Landsteiner showed that antibodies formed following immunization were very specific in their ability to bind with the injected immunogen or hapten. Molecular differences such as that between meta-and para-azobenzene-sulfonate could be detected with antibodies directed against these small organic molecules.

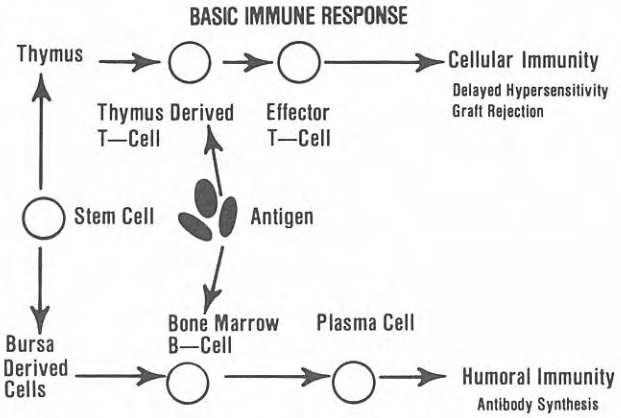
Kabat used a polydextran as an immunogen to produce antidextran antibodies. Using this antisera and subunits of the dextran, he showed that the antibodies bound completely with polydextrans of 6-8 dextrose units. Antibodies, then, do not combine with the entire immunogen but react with small portions of the antigen called "antigenic determinants." Large molecules such as proteins may have several antigenic determinants and initiate specific antibodies to each determinant.

At the time antibodies were being accepted as mediators of immunity, Metchnikoff observed that some cells had defensive capabilities and described the process of "phagacytosis." Phagocytic cells include polymorphonuclear leukocytes and macrophages. The so called "experiments of nature," namely Bruton's type agammaglobulinemia and DiGeorge's syndrome (thymic aplasia), also indicated a cellular and humoral division of the immune system. Later in studies of the reaction to tuberculin, the graft rejection process, and other reactions involving delayed immunological reactions, lymphoid cells were shown to participate as mediators of immunity.

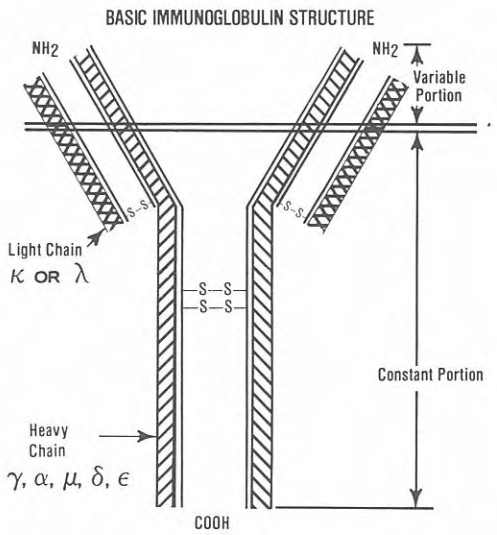
In the early 1950's, Glick and others were able to more clearly divide the immune system into humoral and cellular components. The unique anatomy of the chicken allowed an experimental model for testing this division. The Bursa of Fabricius is an out-pouching of lymphoid tissue near the terminal end of the chicken's intestine. Neonatal bursectomy greatly reduced serum gamma globulin levels and reduced or abolished the hormoral response (antibody formation) to many immunogens. Bursectomy had no apparent effect upon cellular

immunity. Experiments involving neonatal thymectomy showed reduction of cellular immunity while immunoglobulins remained essentially at normal levels with the humoral response to most immunogens unaffected. Cellular immunity, then, involves lymphocytes known as "T-cells", those cells originating or maturing in the thymus, and humoral immunity involves "B-cells", those cells originating or maturing in the bursa or mammalian bursal equivalents. The bursal equivalent in the mammal is believed to be the bone marrow.

The two components of the immune system are well documented today. The diagram shown below outlines the basics of the immune response mechanisms.

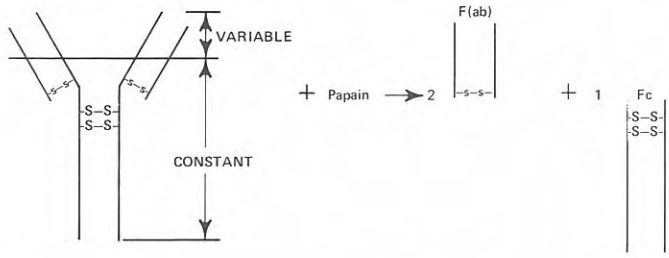


As protein fractionation techniques improved, it became evident that the gamma globulins, (antibodies), were a very heterogeneous population of proteins. To facilitate the interpretation of a rapidly growing body of literature, a new terminology was employed to describe the gamma globulins and their functions. These gamma globulins and other globulins with antibody activity were termed immunoglobulins (Ig's). In the late 1950's and during the 1960's, the molecular structure of Ig's emerged from the laboratories of Porter, Edelman, Nisonoff, and others. The basic structure of the Ig's is shown below:



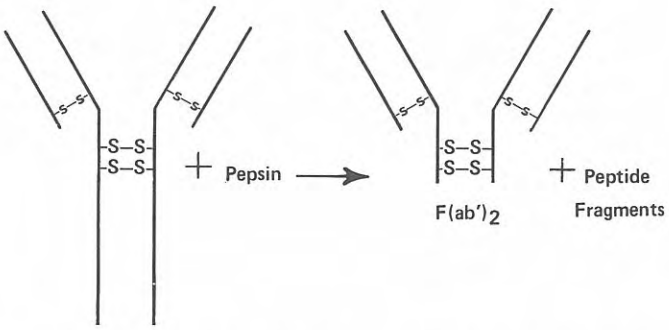
Immunoglobulins are composed of light chains and heavy chains. The N-Terminal ends of the light and heavy chains show remarkable heterogeneity in their amino acid sequences. The Carboxyl-Terminal portions of the chains show only slight amino acid sequence heterogeneity.

The reasons for this heterogeneity became evident following the work of Porter, Nisonoff, and other investigators. Porter digested rabbit Immunoglobulin G(IgG) with the enzyme papain and observed that the molecule was split as follows:



The variable portion of the IgG molecule was found to contain the antigen binding site and was labeled F(ab) for "fragment binding antigen." The non-antibody binding fragment had a homogeneous structure and could be crystallized. It was termed the Fc portion for "fragment crystallizable." The Fc portion was shown also to be responsible for complement fixation, fixing to cell surfaces in some cases, and other non-antigen binding functions of immunoglobulins.

Further definition of antibody structure and function resulted from the digestion of IgG with the enzyme pepsin.



Each molecule of IgG antibody can bind two molecules of specific antigen. This divalency along with multiple antigenic determinants on the antigen allows the formation of an antigen-antibody lattice and precipitation or agglutination occurs.

To date, five major classes of Ig's have been described. These classes are designated IgG, IgA, IgM, IgD, and IgE. The classes are based on the fact that the heavy chains have specific antigenic determinants on the constant portions of the molecule that are not present on other Ig classes. Similarily, two light chain types, kappa and lambda, have been described. Each Ig

molecule has either two kappa chains or two lambda chains attached to it but not one of each type. Subclasses of IgG, IgM, and IgA have been described based on variability in amino acid sequence within a given Ig class.

These discoveries of Ig class, subclass, and light chain types is the point at which humoral immunity ceases its strictly defensive role and assumes an offensive role. Due to the remarkable specificity and sensitivity of the immune response, antibodies can be produced in man and animals to most any substance including proteins, drugs, hormones, and toxins. Using specific antisera produced in animals, many problems in diagnostic medicine can be approached with ease. Utilizing the fact that antibodies bind a specific antigen, and in cases of di- or polyvalent antigens form complexes, many immunochemical tests have been designed. The techniques of agar gel diffusion, electrophoresis, RIA, and light diffraction using specific antisera are being used to qualitatively and quantitatively assay many proteins, drugs, hormones, and a spectrum of other substances.

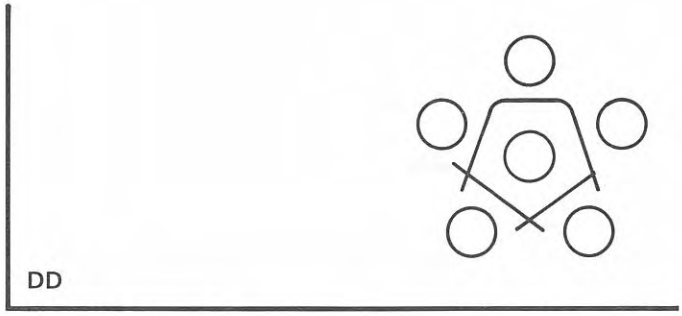
The past history of the immune response as a defensive system has been most exciting. Current research on this amazing defense mechanism may provide answers to the control of diseases including cancer, auto-immune conditions and even the process of aging. As an offensive weapon, the field of immunology is equally exciting. Immunochemical techniques offer a virtually unlimited, accurate, and economical approach to problems encountered in diagnostic medicine.

III. IMMUNOLOGICAL ANALYSIS TECHNIQUES

Immunological test mechanisms are based on the precipitation reaction which occurs when a specific antigen meets its specific antibody. An antigen is a substance which elicits a specific immune response when introduced into the tissues of an animal. An antibody is a protein with the molecular properties of an immunoglobulin and is capable of specific combination with antigen. The protein carries antibody combining sites that link non-covalently with the corresponding antigenic determinant. Antibodies are produced in the body by the cells of the lymphoid cell series, especially plasma cells, in response to stimulus by an antigen. Because the binding of a particular antigen with its specific antibody produces a visible precipitate, it has been possible to design several immunological diagnostic test systems. These various test mechanisms which can be qualitative or quantitative have been utilized in a variety of ways by the clinical investigator as well as the research investigator. A description of these immunochemical assay mechanisms follows.

Ouchterlony Double-Diffusion (DD)

The Ouchterlony double-diffusion method is based upon the simultaneous application of antigen and antibodies in separate but adjacent wells of an agar or agarose plate. As the materials diffuse toward one another, precipitin lines form resulting from the antigen-antibody interactions. This qualitative technique can be used to reveal the immunological identity, partial identity, or non-identity of proteins. For example, it can be used as a semiquantitative assay by placing antibody in the center wells and serial dilutions of the antigen in surrounding wells. In the clinical laboratory, it is applied often to the identification and semiquantitation of Bence Jones proteins, secretory IgA, $alpha_1$ antitrypsin, and myoglobin.

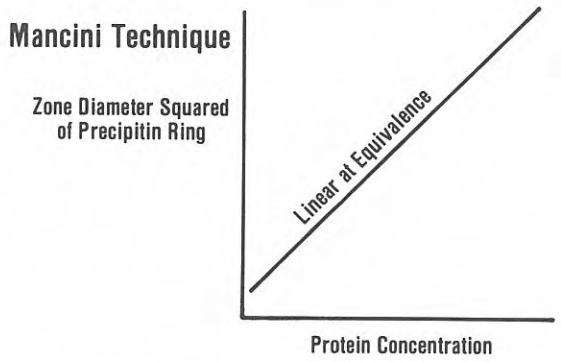


Radial Immunodiffusion (RID)

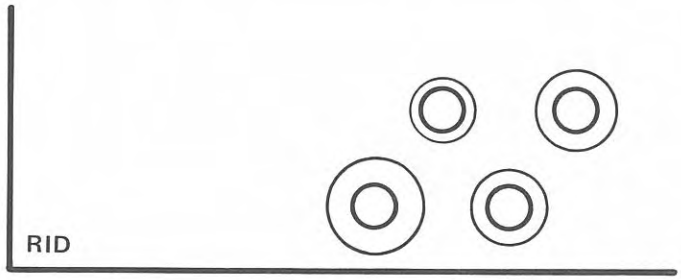
The technique of quantitative radial immunodiffusion is based upon the reaction between an antigen, a human protein for example, and a specific antibody directed toward that protein during a diffusion period. In the test mechanism, antigen placed in a well diffuses into an agarose matrix containing the antibody. As the two entities meet, an antigen-antibody reaction occurs which produces a visible opaque precipitin ring. Fahey and McKelvey's work showed that a linear relationship exists between the log of the protein concentration and the diameter of the precipitin ring prior to equivalence, i.e., the completion of the diffusion. Mancini showed that a linear relationship exists between the area or diameter squared of the ring and the protein concentration at equivalence or endpoint of the diffusion phase.

The Mancini methodology is the most accurate and reproducible of the two techniques. A reference curve is established by plotting the diameter squared against protein concentration of a set of standards of known protein content. Diameter squared in mm^2 is plotted on the Y axis and protein concentration, usually in mg/dl, is plotted on the X axis. Unknown protein concentrations in patient or research samples are determined off the reference curve.

QUANTITATIVE RADIAL IMMUNODIFFUSION: THEORY



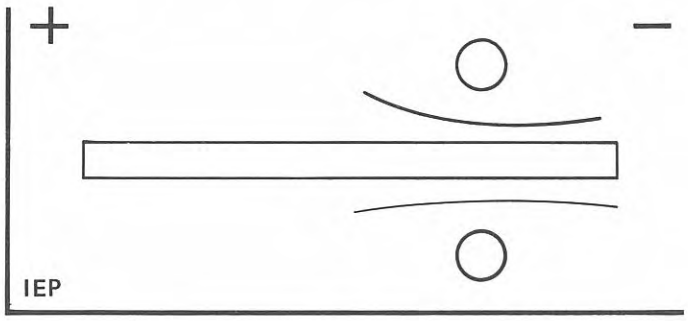
Quantitative sample application plus the incorporation of high affinity, monospecific antisera in the agarose matrix of the plates are required to achieve CV's of 5% or less. The RID technique combines rapid and easy sample application and data collection with a degree of accuracy and reproducibility superior to most laboratory techniques. It is currently the most reliable and widely used protein quantitation technique.



Immunoelectrophoresis (IEP)

Immunoelectrophoresis as first described by Grabar and Williams (1953) is a powerful diagnostic tool. The procedure combines the techniques of electrophoresis and immunodiffusion. In practice, a serum sample is electrophoresed through an agarose matrix. Antisera containing specific antibodies are applied parallel to the migration and allowed to diffuse toward the separated protein, or antigens. When an antigen meets its specific antibody, a precipitin arc forms. Proteins, therefore, are separated by their electrophoretic mobilities and by their diffusion coefficients. Immunoelectrophoresis is a very sensitive qualitative technique for detection of various protein abnormalities. It is the logical step in protein assay following serum protein electrophoresis.

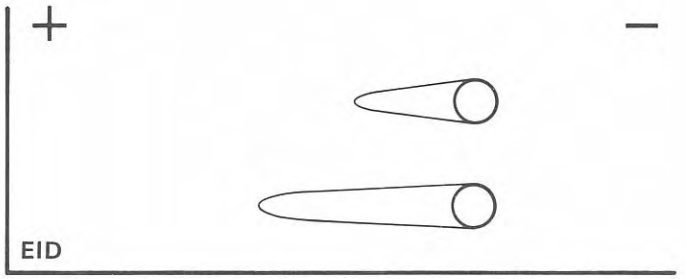
Antisera of sufficient titer, affinity, and monospecificity must be employed in IEP for definitive results. Antisera are produced chiefly in rabbits, goats, and horses. By adjusting antigen boosting schedules and animal bleeding schedules, antisera of high affinity and titer can be achieved. Affinity and titer of the antisera provide the crispness and clarity of precipitin arcs. ***The use of the technique of solid phase immunoadsorption during the antisera purification step by the commercial antisera producer insures the investigator of monospecificity and the elimination of materials causing non-specific precipitation lines.***



In practice, IEP is completed in 18 hours and the results may be viewed without staining of the precipitin arcs. For observation of trace amounts of proteins, the plates should be stained with a protein stain such as Amido Black. Immunoelectrophoresis is a powerful qualitative diagnostic test with multiple applications to the research and clinical fields. It is the major clinical diagnostic tool for the detection of serum protein abnormalities.

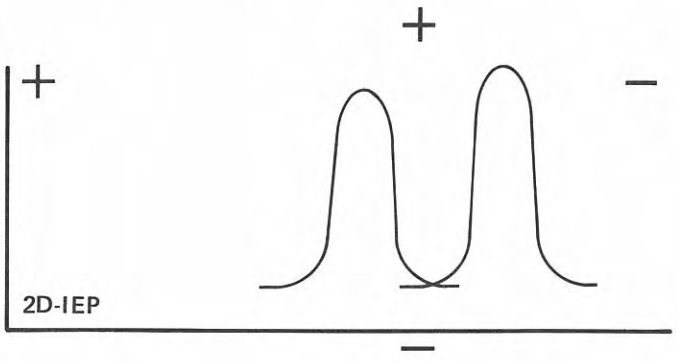
Electroimmunodiffusion (EID)

Electroimmunodiffusion involves the electrophoresis of protein through an agarose matrix containing monospecific antibody. The precipitation reaction which results is a rocket-shaped precipitin formation. The height of this "rocket" is proportional to the concentration of the antigen. EID is applied to any protein whose electrophoretic mobility differs from that of the antibody incorporated in the agarose. It is not routinely used for analysis of the immunoglobulins unless they are modified by special treatments to increase their anodic migration. As an alternate to agar or agarose support media, cellulose acetate may be used as the support medium. Results may be achieved rapidly through the technique of EID but sample dilution and other handling techniques require more technician time than does RID.



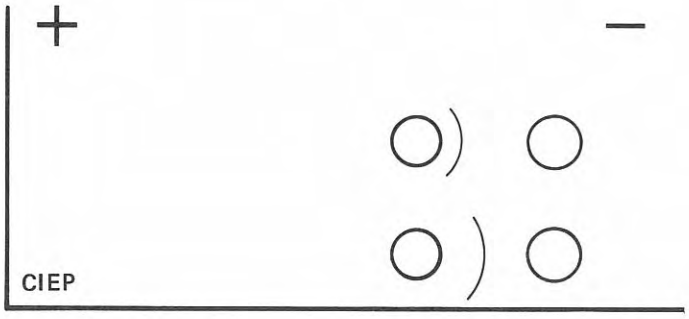
Two-Dimensional Immunoelectrophoresis (2D IEP)

The combination of IEP and EID is referred to as the technique of two-directional immunoelectrophoresis. The first step is the electrophoretic separation of a protein mixture in agarose. The second step involves the electrophoresis of the separated proteins through an agarose gel containing, for example, polyvalent antisera. This second electrophoresis step occurs at a right angle to the first step. The latter step results in rocket-like immunoprecipitates. This technique can be used for the quantitation of several individual proteins simultaneously. As it is presently applied, 2D IEP is a research tool rather than a clinical tool for protein identification and quantitation.



Cross-Immunoelectrophoresis (CIEP)

Cross-immunoelectrophoresis is an immunochemical technique involving reactions occuring between anodically migrating antigens and cathodically migrating antibodies during electrophoresis. Pairs of wells are punched in agarose plates in which antigen is placed in one well of each pair and antibody in the other. Following electrophoresis, precipitin lines will be visible between the wells of a pair of wells of matching specificity. CIEP has most frequently been utilized in the clinical laboratory as a qualitative assay for hepatitis-associated antigens.



Fluorescent Antibody Assay (FA)

The immunofluorescence technique involves the conjugation of a specific antibody with a dye of high quantum yield. The technique has been used to identify bacterial, viral, and fungal antigens. In the clinical area, the technique has been employed for the localization of serum antibodies in tissues. The technique utilizes two main fluorochromes, fluorescein isothiocyanate (FITC) or lissamine rhodamine B (RB 200). They are used separately or together and the various techniques require sophisticated instrumentation. Recent systems have been devised for the quantitation of the immunoglobulins in serum and other body fluids. The FA technique has been applied also to the detection of autoantibodies and the confirmation of immune-complex diseases.

Automated Immunoprecipitation (AIP)

This technique for the quantitation of various serum proteins involves the photometric determination of the degree of turbidity produced by a given antibody-antigen reaction. The reaction is allowed to occur in highly dilute solutions of antigen and of antibody. The resultant antigen-antibody complexes may be read at 436-450 nm to determine the degree of turbidity of the reaction. Alternately, a measurement of the light scatter of the complex, or the nephelometric technique, may be made. When the amount and potency of the reacting antiserum is held constant and the antigen concentration is varied, a standard curve can be established of optical density or of light scatter versus protein concentration. The technique is rapid, allowing the determination of 50-60 samples per hour. The main limitation of the system at present rests in the variability of instrumentation currently available. However, AIP of one methodology or another promises to become the routine technique for high volume protein quantitation in the future.

Radioimmunoassay (RIA)

Radioimmunoassay is a quantitative method of protein analysis which offers extreme accuracy and sensitivity (picogram range). The technique involves the mixing of three basic reagents – unlabeled antigen, radiolabled antigen, and specific antibody. Following incubation of the reagents, bound antigen is separated from free antigen and the bound antigen is counted in a gamma counter or liquid scintillation counter. The amount of bound labeled antigen in a test sample is compared with a standard curve to determine the antigen concentration of the test sample. The RIA technique for Ig quantitation is used chiefly today in the clinical laboratory for the quantitation of IgE. Because it is such a sensitive technique and as more antisera become available to certain drugs, for example, RIA is becoming a routine method for testing for drugs, hormones, toxins, and other substances existing in trace amounts in tissues and body fluids.

IV. A SYNOPSIS OF THE HUMAN PROTEINS

A. The Immunoglobulins

IgG

IgG is the immunoglobulin containing the majority of antibacterial, antiviral, and antitoxic antibodies. It affords the body the main antibody response. It is the chief protein migrating in the gamma region and on IEP the IgG precipitin arc stretches from the cathodic side of the antigen well to the anodic side. The normal range of IgG in the human is 710–1540 mg/dl. Increased levels of IgG are seen in monoclonal and polyclonal IgG gammopathies, IgG myeloma, chronic infections, and liver disease. Decreased levels of IgG are seen in agammaglobulinemia, hypogammaglobulinemia, protein losing conditions, and myelomas of other Ig classes.

IgA

IgA is the primary immunoglobulin of secretions. It offers a first line of defense for the body as it coats mucous surfaces. IgA possesses numerous antibody functions including isoagglutination, antibacterial activity, poliovirus neutralization, and other antiviral activities. IgA migrates in the beta area and forms a long thin precipitin arc on IEP beginning at the well and stretching toward the anode. The normal human serum level of IgA is 60–490 mg/dl. Increased levels of IgA are observed in IgA myeloma, cirrhosis, and chronic infections. Decreased levels are seen in myeloma conditions of the other Ig classes, protein losing conditions, and selective IgA deficiency.

IgM

Upon exposure to an antigen, the first immunoglobulin produced by the immune response is IgM. Upon second and subsequent exposure to the antigen, the secondary immune response produces IgG type antibodies. IgM provides the natural antibodies which include, for example, the ABO blood group isohemagglutinins. IgM molecules are responsible for Wassermann antibodies, heterophil antibodies, anti-IgG auto-antibodies, and saline Rh antibodies. IgM migrates in the gamma region and is visible on IEP forming a symmetrical arc positioned equidistant between the well and the trough, the center of the arc in line with the center of the well. IgM exists in a range of 37–204 mg/dl in the male and 42–261 mg/dl in the female. Increased levels of IgM occur in Waldenstrom's macroglobulinemia, chronic infections, and liver disease. Decreases in serum levels occur in myelomas of other Ig classes, protein losing conditions, and immune deficiency states.

IgD

At the present time, the antibody functions of IgD are not known. It appears that IgD may function as a receptor molecule located on the surface of B lymphocytes. The very low normal serum concentration of IgD, 0.3 to 30 mg/dl, precludes its precipitin arc's being visible on immunoelectrophoresis. When increased levels occur as seen in IgD myeloma, the precipitin arc is visible as a dense, wide arc in the beta region.

IgE

The immunoglobulin existing in normal serum in the smallest levels, 6–780 ng/ml, is IgE. IgE antibodies are allergic, homocytotropic, anaphylactic, reaginic, atopic, and skinsensitizing antibodies. IgE antibodies are involved also with antiparasitic activity. Increased levels of IgE occur in approximately 65% of allergy patients, in certain immuno-deficiency states, and in the rare IgE myeloma condition. Due to the extremely low normal concentration of IgE in serum, it is not routinely assayed by the technique of IEP. In the case of IgE myeloma, IgE forms a distinctive precipitin arc in the gamma region positioned around the well area.

Molecular configurations of immunoglobulins vary with the Ig type. Normal IgG, IgD, and IgE molecules and the majority of serum IgA molecules consist of monomers. Less than 10% of serum IgA exists in polymeric forms which may be dimers, trimers, tetramers, and possibly pentamers. In addition to serum IgA, secretory IgA also exists. It is composed of two IgA molecules linked by a polypeptide chain called the secretory piece or transport piece as well as another joining "J" chain. Normal IgM (19S) molecules are present in serum as pentamers. In addition to this high molecular weight IgM, a low molecular weight 7S IgM has been shown in trace amounts in normal serum. 7S IgM exists as a monomer.

B. The Major Plasma Proteins

Albumin

Albumin is the major plasma protein representing over 50% of the total protein present in serum. It is a polypeptide chain consisting of 610 amino acids and has a molecular weight of 65,000. It is the major protein produced by the liver and has two primary functions. The first function is that of a transport molecule, primarily of fatty acids, hormones, calcium, metal ions, drugs, vitamins, and bilirubin. The second major biological function is that of providing colloid osmotic pressure. It is possible also that albumin is an endogenous source of amino acids. On immunoelectrophoresis, albumin is visible at the extreme anodal side of the plate forming a symmetrical arc. The normal serum concentration is 3500-5000 mg/dl. Clinical syndromes associated with decreased or increased levels of albumin include: congenital analbuminemia, hepatic damage, acute phase conditions, protein losing conditions, and malnutrition.

Alpha$_1$ antitrypsin

Alpha$_1$ antitrypsin is a human plasma glycoprotein having a molecular weight of 45,000 It consists of 381 amino acids and contains 12.4% carbohydrate. The biological function of this protein

is to inhibit the actions of trypsin and chymotrypsin. It migrates in the alpha$_1$ region on IEP overlapping albumin.

Several clinical abnormalities are associated with changes in the serum levels, normal range being 210-500 mg/dl, of alpha$_1$ antitrypsin. Since it is one of the acute phase reactants, acquired increased levels are seen in acute and chronic inflammatory diseases, stress syndrome, malignant tumors, and hematological abnormalities. Acquired decreases in A_1AT are seen in pulmonary disorders, especially emphysema, severe hepatic damage, severe nephrotic syndrome, and malnutrition.

A_1AT levels are genetically controlled. After the description of A_1AT deficiency by Laurell and Eriksson (1963), it was thought that only individuals homozygous (ZZ) for the deficiency gene developed clinical manifestations of chronic obstructive pulmonary disease (COPD). Later studies have shown that heterozygotes (MZ) are also at risk for COPD. Serum levels of persons homozygous for the deficiency gene are roughly 10% of the normal; heterozygotes have intermediate levels, approximately 60% of normal. Kueppers (1967) found the normal range to be 212 ± 32, ZZ to be 25 ± 6, and MZ to be 120 ± 46 mg/dl. Because A_1AT deficiency, both homozygous and heterozygous, is probably only a predisposing factor of COPD, it is believed that environmental factors such as smoking and air pollution are necessary to produce a disease state.

Alpha$_2$ macroglobulin

Alpha$_2$ macroglobulin is a large molecular weight plasma protein comprising nearly 80% of the alpha$_2$ region. It is the most non-specific of the protease inhibitors. In addition to its major protease inhibitor functions, it acts as a transport protein for certain hormones. Because its concentration in serum varies with age, it is believed this protein may be closely related to growth. It is a strongly immunogenic molecule and thereby produces an antibody readily. On IEP, it may be observed clearly and distinctly forming an asymmetrical arc between alpha$_1$ antitrypsin and transferrin in the alpha$_2$ region. Alpha$_2$ macroglobulin exists in normal human serum in a concentration of 200-350 mg/dl. Decreased levels are seen in rheumatoid arthritis while increased levels are seen during pregnancy, in nephrotic syndrome, and certain chronic liver diseases.

Beta lipoprotein (LDL)

Beta lipoprotein, low density lipoprotein, is one of the four main classes of lipoproteins in human serum. It is a macromolecule in which lipids are transported in serum. These lipoproteins contain 46% cholesterol, 20% peptide, 2% carbohydrate, 10% triglyceride, and 22% phospholipid. The beta lipoprotein precipitin arc occurs over a wide range from the alpha$_2$ to the beta zone on IEP. It is nearly a straight line having a milky appearance due to the high lipid content. The normal serum range for males is 200-740 mg/dl and for females is 190–600 mg/dl. Deficiencies of these low density lipoproteins may be divided into abetalipoproteinemia and hypobetalipoproteinemia. In the former condition, there is virtually no detectable beta lipoprotein. In the latter, the low density lipoproteins decrease to 10–50% of the normal level. Increased levels of beta lipoprotein are seen in hyperlipoproteinemia.

C_3

C_3 is the principal component of the complement series. Its molecular weight is 180,000 and the protein can be cleaved by convertase into two fragments, C_3b and C_3a. C_3 exists in fresh serum as C_3 B_{1C}–globulin. As serum ages B_{1C}–globulin gradually transforms to inactive B_{1A}–globulin. In fresh serum, B_{1C}–globulin is seen as a symmetrical arc bridging between transferrin and IgG. In stored serum, B_{1A}–globulin is located anodal to transferrin due to B_{1A}'s higher mobility. C_3 is routinely quantitated as the B_{1A} form and as such exists in adult serum in a range of 55-120 mg/dl. Since this protein is involved in activation of the inflammatory response, increases in C_3 levels are seen in acute stress syndromes, acute hepatitis, and connective tissue disease. Decreased levels are seen in a variety of disorders including serum sickness, acute glomerulonephritis, systemic lupus erythematosus, and other autoimmune diseases.

Fibrinogen

Fibrinogen and its degradation products are involved with many other protein constituents in plasma in blood coagulation and fibrinolysis. Fibrinogen is a large molecule of 3 polypeptide chains having a total molecular weight of 341,000. It may be assayed only in plasma, not serum. The arc formed on IEP is visualized in the Beta region. Fibrinogen is present in normal plasma in a range of 200-450 mg/dl. Fibrinogen is an acute phase reactant and is valuable in the detection, diagnosis, and prognosis of diseases involving tissue damage and inflammation.

Transferrin

Transferrin is a β-globulin having a molecular weight of 80,000. Each molecule of transferrin binds 2 atoms of free iron - hence its function as the iron transport protein. Transferrin forms the most distinctive precipitin are in the Beta area positioned near the trough. The normal level of transferrin in serum is 200-300 mg/dl. The determination of increased levels of transferrin is critical in identifying iron deficiency anemia. The transferrin concentration reflects the total iron-binding capacity (TIBC) and is used as an indicator of iron metabolism. Quantitation of serum transferrin also aids in the detection of congenital atransferrinemia, nephrotic disease, protein losing conditions, and hypoproteinemia.

Ceruloplasmin

Ceruloplasmin is an alpha$_2$ globulin of 150,000 molecular weight. It is capable of binding 8 atoms of copper per molecule. It appears blue-green in color owing to its high copper content. It carries over 95% of all copper present in the body. On IEP, it is observed in the alpha$_2$ region between alpha$_2$ macroglobulin and haptoglobin. Ceruloplasmin degenerates on storage so frequently is not seen on immunoelectrophoresis of old serum. The serum concentration in normal adults covers a range of 27–63 mg/dl. Decreased levels are seen in the congenital hypoceruloplasminemia state of Wilson's Disease and in nephrotic syndrome, hypoproteinemia, anemia, malabsorption syndrome, acute hepatitis, severe liver damage, and multiple sclerosis. Increased levels are seen in pregnancy, acute and chronic inflammatory diseases, and malignant tumor.

Haptoglobin

Haptoglobin is one of the alpha$_2$ globulins possessing several unusual properties. Structurally, haptoglobin ressembles the immunoglobulins in that its basic unit consists of two identical heavy polypeptides, beta chains, and two identical light polypeptides, called alpha chains. The beta chain is common to any haptoglobin molecule but there are three different types of alpha chains. Therefore, 3 main electrophoretic types of haptoglobin exist, Hp 1-1, Hp 2-1, and Hp 2-2. Haptoglobin combines with hemoglobin thereby providing the major determinant of the renal threshold for hemoglobin. It is also the chief component of the acute phase proteins. On IEP, the haptoglobin arc is seen as a symmetrical arc with a small curvature in the alpha$_2$ region. The serum concentration of haptoglobin in normal adult varies considerably, 30-290 mg/dl, and is apparently dependent on hereditary factors. Variations in serum levels of haptoglobin are seen in hemolytic anemias, acute hepatitis, severe liver damage, inflammatory diseases, and tissue necrosis.

C. Bence Jones Proteins

Free light chains of either the Kappa or Lambda type are referred to as Bence Jones Proteins. They may occur in excess in serum or urine in a variety of clinical disorders. They are thermoreactive proteins precipitating on heating to the 40° to 60°C range but redissolving at higher or lower temperatures. They may exist as monomers, dimers, or tetramers. The presence of pathological amounts of Bence Jones proteins is usually associated with multiple myeloma, Waldenstrom's macroglobulinemia, lymphatic leukemias, and Bence Jones myeloma or "light chain disease." Free light chains may be detected on immunoelectrophoresis by testing serum and urine samples against heavy chain specific IgG antisera and antisera specific for kappa chain and for lambda chain. Free light chains, or Bence Jones proteins, demonstrate different electrophoretic mobilities than when bound to heavy chain molecules. The quantity of Bence Jones proteins excreted is usually correlated with the increasing severity and cause of a renal disease state. Urine concentrations in patients with benign monoclonal gammopathies range from 0.25 to 6 mg/dl and in Waldenstrom's from [42–440 mg/dl]. Levels as high as 20 gm/l have been detected in patients with multiple myeloma.

D. CSF Proteins

Elevations of the immunoglobulins IgG, IgA, and IgM in CSF occur in several diseases which affect the blood-brain barrier. The quantitation of total cerebrospinal fluid (CSF) proteins or CSF albumin in addition to IgG, IgA, and IgM quantitation enables the clinician to distinguish the various central nervous system disorders. Increased levels of IgG occuring with normal levels of the other immunoglobulins is often seen in patients with active multiple sclerosis. Normal levels of CSF=IgG, 0 to 5.5 mg/dl; IgA, 0-0.6 mg/dl; and IgM, 0 to 1.3 mg/dl. Normal albumin levels=8.4 to 34.4 mg/dl.

V. THE DIAGNOSTIC TOOLS OF APPLIED CLINICAL IMMUNOLOGY

The most widely used immunochemical techniques for the assay of human proteins are immunoelectrophoresis (IEP) and radial immunodiffusion (RID). Both techniques lend themselves to a multitude of applications in the research laboratory and/or the clinical laboratory. The Helena IEPlate System for IEP and the Helena Quiplate System for RID complete the protein assay steps which begin with the Helena Zip Zone System for serum protein electrophoresis (SPE).

A. The Helena IEPlate System

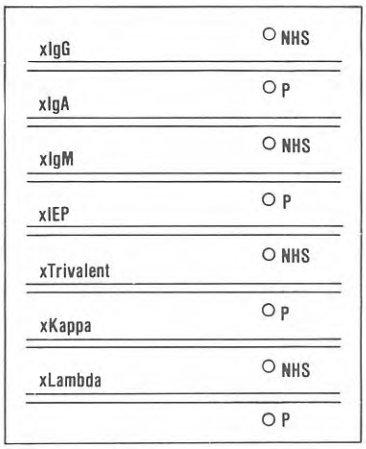
When an abnormality is observed in the albumin, alpha$_1$, alpha$_2$, beta, or gamma regions on serum protein electrophoresis, the patient sample may be further assayed for identification by immunoelectrophoresis. The Helena IEPlate System is totally compatible with the basic components of the Zip Zone System for SPE. All plate sizes and configurations may be electrophoresed using the Zip Zone Chamber (cat. no. 1283) and Titan Power Supply (cat. no. 1500). The IEPlates consist of an agarose film secured on a rigid plastic gel tray. The gel trays are stored easily and are stackable. Plates are available in a variety of configurations for the small and/or large volume laboratory. Microdispensers (cat. no. 6008) or Ziptrols (cat. no. 6009) may be used for sample applications.

Helena offers a complete line of antisera for use with the IEPlates. Each antisera is a high titered, high affinity material which has been purified by solid phase immunoadsorption. Sharp, well-defined arcs are visible in 18 hours and the IEP patterns thus formed may be interpreted and photographed at that time. The Immuno Vubox (cat. no. 9025) and Immuno

Cameras (cat. no. 9026) may be purchased for viewing and recording IEP results. If preferred, the plates may be stained for interpretation and photographed stained for a permanent record or dried down and kept indefinitely.

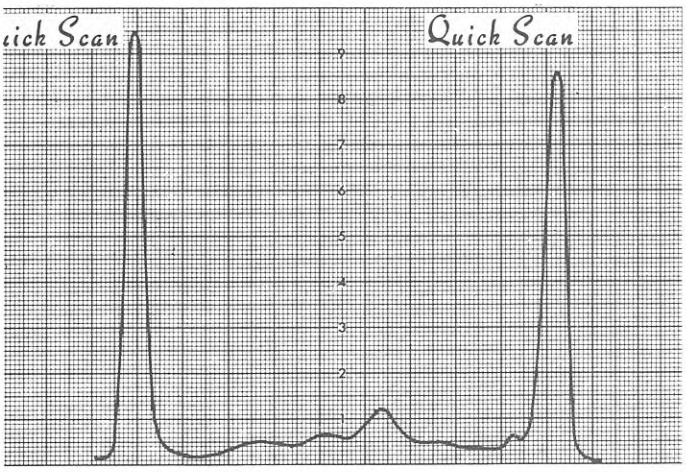
Titan IV IEPlates are used for a variety of diagnostic purposes. In routine IEP patient profiling, a patient's serum sample is compared to a normal human serum (NHS) sample in alternate wells of a (cat. no. 9050) 8 well, 7 trough IEPlate. Each serum sample is tested against antisera to human IgG, IgA, IgM; Trivalent and Polyvalent antisera; and to Kappa and Lambda Light Chain antisera. The profiling pattern may be diagrammed as follows:

BASIC IMMUNOGLOBULIN PROFILING

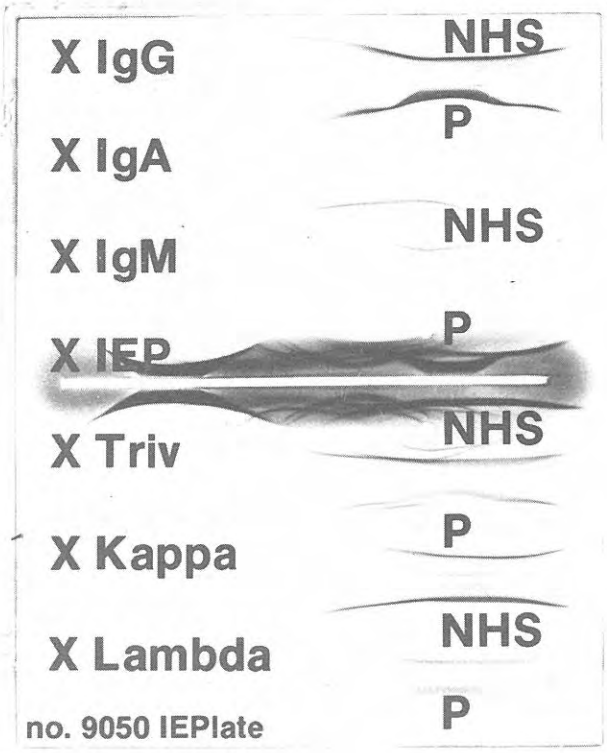


When a spike in the gamma region is seen on a scan of an SPE pattern of a patient serum sample, the IEP technique is used to determine if the abnormality is polyclonal or monoclonal in nature. Further, the technique differentiates the various monoclonal gammopathy types. To the experienced IEPlate pattern interpretor, subtle differences in the ratio of kappa light chains to lambda light chains become clear.

A patient serum sample producing the SPE scan shown produced the IEPlate pattern also shown below:



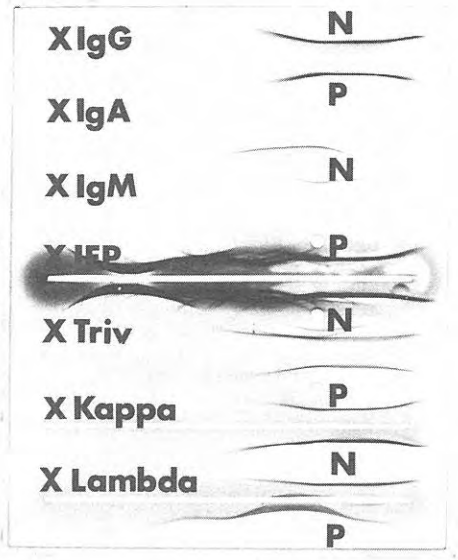
IgG Monoclonal Gammopathy, Lambda



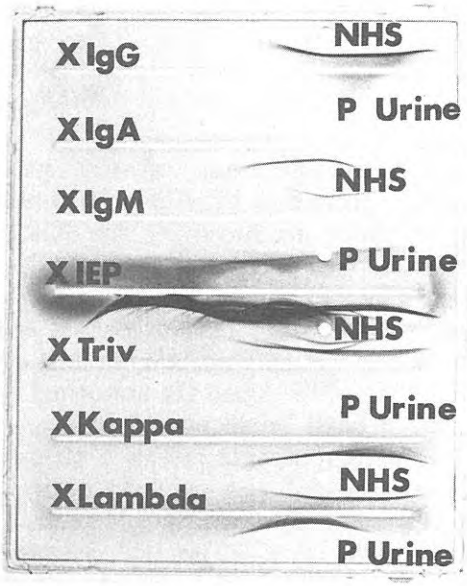
Careful interpretation of the pattern of precipitin arcs produced by the patient sample as compared to those produced by the NHS control leads the clinician to a tentative diagnosis. The patient serum forms a dense, bowed arc against IgG antisera. There appears to be a diminished level of IgA in the patient serum and virtually no IgM content compared to IgA and IgM in NHS. Again the abnormal IgG band is visible against both Trivalent and Polyvalent (IEP) antisera. The patient sample reveals a normal Kappa arc but a very bowed abnormal Lambda arc. The composite is indicative of an "IgG Monoclonal Gammopathy, Lambda type." The IEP System can be used for the detection of a variety of disorders associated with the immunoglobulins IgG, IgA, IgM, IgD, and IgE.

Additionally, the components of the Titan IV IEPlate System may be used for the detection of Light Chain Disease. For confirmation of this disorder, the investigator should test both patient serum and patient urine against the immunoglobulin profiling antisera. It is necessary to compare the precipitin arcs formed by the patients material against heavy chain specific IgG, IgA, and IgM to those arcs formed against Kappa and Lambda Light Chain antisera. A patient whose serum produced an elevation in the beta region on SPE produced the following patterns when serum and urine were tested by immunoelectrophoresis:

IEPlate – Patient Serum vs NHS

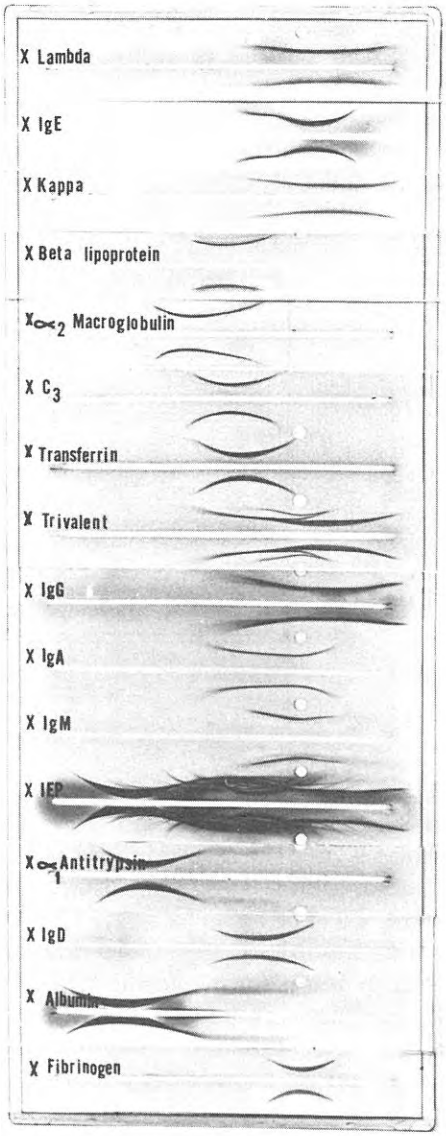


IEPlate – Patient Urine vs NHS



Precipitin arcs formed with heavy chain specific antisera reveal no apparent abnormality. However, patient serum and patient urine against lambda light chain antisera produce a very bowed arc with an abnormal configuration. Occasionally the migration of the light chain protein in the serum differs from that of the light chain protein in the urine. This electrophoretic migration difference is probably a result of the dimerization of the Bence Jones protein in the urine.

The Helena antisera produced to all the major plasma proteins may be used for the detection of a variety of protein disorders. Confirmation of a albuminemia, alpha$_1$ antitrypsin deficiency, alpha$_2$ macroglobulin increases with nephrotic syndrome, and several other clinical syndromes is possible through the technique of IEP. On the research level, the IEPlate System affords unlimited applications to the investigator including antisera quality control, identification of unknown antigens, monitoring of protein isolation procedure steps, and identification of plant and animal proteins.



B. The Helena Quiplate System

Following immunoelectrophoresis or directly from serum protein electrophoresis, it is frequently necessary to quantitate one or more of the serum proteins. Quantitation of protein may be accomplished quickly and easily through the technique of radial immunodiffusion. RID analysis is based upon the reaction between an antigen (specific serum protein) and a specific antibody directed to that antigen. In the test mechanism, antigen diffuses into an agarose matrix containing antibody. As the two entities meet, an antigen antibody reaction occurs which is visible as a precipitin ring. In the Mancini method of RID, the zone diameter squared of that ring is directly proportional to the antigen, or protein, concentration. Unknown protein

concentrations may be determined off a reference curve established by testing serial dilutions of a standard of known protein concentration. Through the Helena QUIPlate System for radial immunodiffusion, the clinician can quantitate accurately and reproducibly all the immunoglobulins plus all the major plasma proteins.

All plates in the QUIPlate System consist of an agarose film on a sturdy plastic gel tray. The wells of the 19 well QUIPlates are numbered and the plate specificity is connotated in the upper left-hand corner of each plate. All plates in the system are capable of achieving definitive endpoint within 18-24 hours of incubation. Precipitin ring diameters may be read to the nearest tenth of a millimeter using a QUIPmeter or a Helena Vubox with ocular. Each QUIP Kit contains graph paper for plotting the reference curve, complete procedural information, and a set of reference standards. Accompanying each kit is the Quality Control Data Sheet for the lot of plate within the kit. Ziptrol or Microdispensers are available for standard and sample applications. Protein quantitation may be completed with a minimum amount of equipment and technician time using the QUIPlate System components.

In the case of the IgG Monoclonal Gammopathy, Lambda Type discussed earlier, it was evident from the IEP pattern that a greatly increased level of IgG was present in the serum plus diminished levels of IgA and IgM. For exact quantitation of the immunoglobulins involved, QUIPlates for assay of IgG, IgA, and IgM were used. The following protein levels were determined for the patient:

IgG = 6000 mg/dl
IgA = 140 mg/dl
IgM = 50 mg/dl

In addition to plates for the quantitation of adult normal levels of IgG, IgA, IgM, and IgD, Helena offers Low Level QUIPlates for quantitation of low levels of immunoglobulins and of albumin in pediatric serum, CSF, and other body fluids.

Increases or decreases in the levels of various plasma proteins are clinically significant in several disorders. Helena QUIPlates are available for quantitation of C_3, C_4, alpha$_1$ antitrypsin, and transferrin.

The protein ranges covered by the various plates of the QUIPlate System are as follows:

Specificity	Range
IgG	422, 845, 1690 mg/dl
IgA	100, 200, 400 mg/dl
IgM	50, 100, 200 mg/dl
LL-IgG	1.5, 3, 6, 12 mg/dl†
LL-IgA	5, 10, 20, 40 mg/dl††
LL-IgM	6, 12, 24, 48 mg/dl††
LL-Alb.	5, 10, 20, 40 mg/dl†
Alpha$_1$ AT.	15, 31, 62 mg/dl*
Trans.	110, 220, 440 mg/dl
IgD	2.5, 5, 10, 20 mg/dl
C_4	15, 30, 60 mg/dl
C_3	43, 86, 172 mg/dl

† For use in CSF quantitation
†† For use in pediatric serum quantitation
* Dilute patient samples 1:10 in 0.85% Saline

Several of the Quiplates for radial immunodiffusion have very special applications.

–The LL-IgM Quiplates for quantitation of IgM in a range of 6 to 48 mg/dl can be utilized for the screening of all newborns for infections. The technique is rapid plus economical which makes the establishment of such a screening program possible for any hospital.

–Recent research and clinical studies have demonstrated a relationship between many pulmonary disorders and decresed serum levels of alpha$_1$ antitrypsin. Quantitation of alpha$_1$ antitrypsin, therefore, is becoming increasingly important both to the medical diagnostician and to the industrial personnel working with materials potentially hazardous to the respiratory system. The AT-Quiplate serves as an excellent screening plate for suseptibility to COPD because it detects levels as low as 15 mg/dl. This is important to the clinician in detection of homozygous and heterozygous states in families having a history of emphysema or other chronic pulmonary disorders. It is an equally invaluable screening tool for alpha$_1$ antitrypsin deficiency for industries dealing with potentially hazardous inhalants. Employees may be evaluated for A_1AT levels with minimal cost, materials, and time involved.

–The LL-IgA Quiplates for quantitation of IgA in the range of 5-40 mg/dl can be used to quantitate IgA levels in potential blood transfusion recipients. Approximately 1 in 700 persons is IgA deficient. If not detected before transfusion, this deficiency can result in anaphylactic reactions in the recipient.

–The Quiplates for quantitation of low levels of IgG, IgA, and IgM can be used collectively for the confirmation of the various types of humoral immuno-deficiency in children.

The Helena Quiplate System product line has been designed to offer the investigator a simple, accurate, and economical technique for protein quantitation with multiple and varied applications.

Immunochemical assay is a rapidly expanding field in diagnostic medicine. Presently, Helena offers the IEPlate System for immunoelectrophoresis and the Helena Quiplate System for quantitative radial immunodiffusion for analysis of human proteins. The techniques are widely used and easily adapted to the laboratory. Helena product quality assures the accuracy and reproducibility demanded in both the clinical and research fields. In the future, Helena Laboratories will be offering additional immunochemical assay techniques of equal quality and ease of usage.

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Published by Helena Laboratories Corporation
PO BOX 752, BEAUMONT TX 77704-0752
Toll free 800-231-5663

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9/91(2)