IMMUNOFIXATION ELECTROPHORESIS
on agarose gels

IVD CE

Principle and Methodology

Hellabio Immunofixation Electrophoresis (IFEU) kits are intended for parallel in vitro diagnosis of monoclonal paraproteins in human both serum and urine of the same patient.
Urine analysis is an essential component in the investigation of patients with paraproteinaemia or with suspected B cell malignancies. An important feature used to help distinguishing malignant from non-malignant conditions is the finding of immunoglobulin fragments produced by tumour cells. The fragments, which may not be detectable on serum electrophoresis separations, are usually of lower molecular weight than intact immunoglobulin molecules. These fragments pass readily through the kidney, and may be clearly visible in the urine due to the concentration effect. The finding of Bence-Jones proteins (BJP) therefore provides a high index of suspicion for malignancy, although it does occur in apparently benign conditions. Even low concentrations (10mg/l) may be significant and therefore concentrated urine (at least 100-fold concentration of early-morning urine is preferable) must be run in the serum systems. Whatever system is used, a trace of albumin must be visible in all urine samples; if this is not the case, the sample should be re-run or further concentrated.
Where a BJP is present in significant concentrations (>100mg/l) with no accompanying glomerular or tubular proteinuria, detection is straightforward and the Immunofixation identification step is unequivocal. However, the renal damage associated with BJ proteinuria, frequently results in complex, non-standard patterns requiring Immunofixation to resolve the possible presence of BJP.
A low concentration of BJP may also accompany significant glomerular proteinuria in patients with light chain renal amyloidosis; the urine of any such patient should be investigated by Immunofixation even in the absence of a band suggestive of BJP. Patients with serum paraproteins may show a "leak" of the serum paraprotein into the urine. This may occur with or without Bence Jones protein and Immunofixation is essential to distinguish these.
A number of other proteins may appear as discrete bands on urine electrophoretic separations, particularly where there is an element of tubular proteinuria. These include the α- and β-microglobulins, lysozyme (migrating in the slow gamma region), degraded fragments of glomerular origin and rarely seminal fluid proteins. In some samples the β_2-microglobulin will be present in high concentrations and give a very prominent band.

Required Reagents and Equipment's included in each kit:
[Warning: All reagents from each kit must be used together]

Product	KIT IFEUD01 / 20 TESTS
Agarose Gels	**10**
Electrophoresis Buffer [50X concentrated]	**20 ml**
Staining Solution [5X concentrated]	**60 ml**
Diluent solution [ready to use]	**12 ml**
Destaining solution [500X concentrated]	**4 ml**
Gel Blotter Strips	**20**
Sample Templates	**10**
Antisera templates	**10**
Drying Blotter Sheets	**50**
Gel Blotter Sheets	**60**
Antisera [Anti IgG (γ), IgA (α), IgM (μ), κ- and λ-chain]	**Goat anti-Human antisera 0.6 ml / vial**
Protein fixation solution	**1.4 ml**
Instructions for use in English	

All reagents must be used according to the instructions and until the expiration date indicated on the kit

Preparation, storage and stability of the reagents included in the kit:

a) Agarose Gels: Agarose Gels are in non- barbital buffer and other non-reactive ingredients for long stability and optimum resolution of protein fractions. The Gels must be stored at **15 - 25 ^{0}C on horizontal position** until the expiration date indicated on the kit. Do not freeze the gels. Carefully discover the gel just before use and follow the instructions of the manual.
b) Diluent Solution: Working electrophoresis buffer + Bromphenol blue and other non-reactive ingredients. Store it at 15 - 25 ^{0}C until the expiration date indicated on the kit. **Ready to use**.
c) Electrophoresis Buffer: Non-barbital buffer and other non-reactive ingredients. It is in concentrated solution. It must be stored at 15 – 25 ^{0}C until the expiration date indicated on the kit. To prepare working solution dilute the content of the bottle according to the instructions on the bottle. The diluted solution is enough for electrophoresis of all gels of the kit. **The buffer solution is for one use only**. Store the diluted solution at room temperature for 2 months. If crystals appear, place the vial in warm water to dissolve the crystals.
d) Staining Solution: Concentrated Amido Black solution. Store the concentrated solution at 15 - 25 ^{0}C until the expiration date indicated on the kit. To prepare working solution dilute the content of the bottle according to the instructions on the bottle. The diluted solution is enough for the staining of all gels of the kit. It should be stored in a closed flask at room temperature until 3 months.
e) Gel Blotter strips: Thin filter paper strips to blot the gel in the application area. Avoid humidity. Blot just for 5 seconds. Avoid humidity.
f) Gel Blotter Sheets: Thin filter paper sheet to blot the entire surface of the gel.
g) Drying Blotter Sheets: Thick filter paper sheet which absorbs the excess humidity of the gel.
h) Destaining solution: 2% citric acid solution. To prepare working solution dilute the content of the bottle according to the instructions on the bottle. Store at room temperature until the expiration date indicated on the kit.
i) Washing solution: 0.9% Saline (NaCl).
j) Antisera kit: Each kit contains protein fixation solution, goat anti-Human heavy chain of IgG, IgA and IgM and free + bound anti light chain of kappa (κ) and lambda (λ). Store at -18 ^{0}C (long term condition) until the expiration date indicated on the kit.

Additional Reagents and Equipments required and which can be provided by Hellabio:

Power supply, Electrophoresis tank, Staining-distaining baths, HellabioScan (Gel Analyzer).

Collection and handling of specimens:

The samples should be collected according to the standard hospital methods.
a) Blood: Analysis is preferably performed on sera in order to avoid the fibrinogen band (in plasma), which migrates in the gamma zone and could lead to a false interpretation. Venous blood is drawn into tube. After centrifugation the supernant fluid (serum) can be stored at 4-8°C for 48-72 hours. Storage at -20°C may disturb some of the electrophoretic patterns. Care should be taken to prevent haemolysis in the serum because it will cause false elevation in the α_2 and β-fractions. Samples with much precipitate that will not dissolve on warming to 37°C should be either centrifuged or allowed to settle before the clear supernant fluid is applied to the gel surface.
***b) Cerebrospinal fluid:** Normally, some 80% of the proteins of cerebrospinal fluid originate from plasma, and the rest have been synthesised locally. The filtration resistance for plasma protein rapidly increases with molecular size and therefore the ratio of large proteins normally is much lower in CSF than in plasma. The protein content of CSF is in the range of 15-40 mg/dl. Therefore it must be concentrated at least 150-fold before electrophoresis.*
***c) Urine:** Analysis of the urinary proteins shows that normal urine contains more kind of proteins which are much diluted and therefore it must be concentrated. For the detection of Bence-Jones proteins, free Kappa and Lambda light chains, the sample has to be concentrated to ≥ 100 mg/dl of total protein and to about 80 mg/dl of total proteins for immunoglobulins.*

Procedure of Immunofixation electrophoresis

Extreme care must be taken in choosing the appropriate dilution of the serum sample (100 – 200 mg/dl). The incorrect choice of serum dilution may result in either inability to detect a minor monoclonal protein or a prozone effect. When the protein concentration is low (cerebrospinal fluid, urine), concentrate the sample to get a protein concentration at least 100 mg/dl.

I. Prepare (with proteins diluent solution) freshly dilution of serum sample, so that the concentration of each globulin in corresponded dilution is near 100-200 mg %. *For example: when in a case the concentration of IgG globulin's is 3000 mg %, then the sample should be diluted 1:16 for IgG (=187mg%). In case of sample with globulin concentration near the normal level, or with unknown concentration, dilute the sample 1:6 (1 vol. serum + 5 vol. protein diluent) for PE position and 1:9 for all other positions (1 volume serum +8 volume diluent).*
II. Fill the electrophoresis chamber with adequate volume (it depends on the chamber volume) of electrophoresis buffer.
III. Take the agarose gel out of its packaging, uncover it from the plastic plate and put it on the backside of the plate in horizontal position.
IV. Blot the gel for 5'' with a gel blotter strip in the zone of sample - application,
V. Place the sample template on the application zone carefully. Rub the template with forefinger gently to eliminate trapped air bubbles.
VI. Using a 5-µl pipette, apply 5 µl of serum dilution and 5-µl from concentrated urine across each corresponding slit [*The application of the samples should be done as quickly as possible. The application slits should not be allowed to dry].*
VII. Let the samples absorb into the gel for 2 minutes and then blot the superfluous sample with a gel blotter strip.
VIII. Remove both the sample template gently and gel blotter strip and discard them.
IX. Place the gel into the electrophoresis chamber with the samples on the cathodic side, and run electrophoresis for 20 minutes in 100 volts (the time depends on the kind of the power supply).
X. After electrophoresis switch off the power supply and place the gel film in a strictly horizontal position.
XI. Blot the gel surface with a gel blotter sheet and apply on the gel surface the antisera template. Rub the template with forefinger gently to eliminate air bubbles (very important).
XII. Apply into the corresponding troughs of the antisera template:

Position	Reagents / Volume
PE	PFS / 80µl
IgG. IgA, IgM	Anti-IgG. -IgA, -IgM / 50µl
Kappa, lambda	Anti κ, -λ / 25µl

XIII. Incubate the gel film for 10 minutes at room temperature in a moist chamber in strictly horizontal position.
XIV. Remove the antisera template and discard it.
XV. Put on the gel one gel blotter sheet and one drying blotter sheet; place a development weight (about 2 kg) for 2 minutes.
XVI. Soak the gel in saline solution for 10 minutes.
XVII. Repeat step (**XV**) and (**XVI**) three more times.
XVIII. Clean with a soft paper the back site of the film and dry the gel with hot air (less than 85°C), and stain it for 3 minutes with protein staining solution.
XIX. Decolonise the gel for 5 minutes in three baths of destaining solutions, subsequently.
XX. Dry again the gel and evaluate visually the results (see conclusion/ troubleshooting).

Results:

***The identification and the determination of the monoclonal band should be done by comparing the location of the band(s) with the band(s) with the same location in the PE position (see* conclusion /troubleshooting).**

In case of monoclonal paraproteinemia monoclonal band(s) will be appear with one or more anti heavy and /or light chain globulin. If you get monoclonal band(s) in PE position, the conclusion / troubleshooting should be as follow**:**

PE	Anti IgG	Anti IgA	Anti IgM	Anti κ	Anti λ	Conclusion/ Troubleshooting
	■	-	-	-	-	Monoclonal IgG heavy chain/-
	■	-	-	■	-	Monoclonal IgG κ- chain/-
	-	-	-	■	-	Monoclonal light κ- chain/ Think about IgD and IgE too.
	■	-	-	■	-	Monoclonal IgG λ-chain
	-	■	-	■	-	Monoclonal IgA κ- chain
	-	■	-	-	-	Monoclonal IgA heavy chain
	-	■	-	-	■	Monoclonal IgA λ- chain
	■	■	-	-	■	Biclonal IgG, IgA IgA λ- chain
	-	-	■	-	-	Monoclonal IgM heavy chain
	-	-	■	-	■	Monoclonal IgM λ- chain
	-	-	-	-	-	Fibrinogen or Heavy chain of IgD or IgE. Do not use plasma for electrophoresis! Examine the case with Anti IgD and IgE antisera too.
	■	■	■	■	■	If cryoglobulins, rheumatoid factor, or immuno complexes are present in sample monoclonal bands appear with more than one or with all antisera.

Sensitivity: The sensitivity of fixation solution (on PE position) is 10X higher than with Amido Black. The antisera can detect the specific antigen in a concentration of 80-400mg%.

Specificity: The antisera are monospecific and they do not react with fibrinogen or other human proteins.

Limitation / Caution: Do not use the agarose gel film if it seems to be dried. Do not freeze the agarose gel. Store the Kit in horizontal position. Avoid using hemolytic sera or plasma.

REFERENCES:

Brigden M.L.: Lab Medical International July/August 2000.Merlini G. et al: Biotech Lab International May-June/98.
Dimitriadis F. et al : Hema 2003 (under press).
Farhangi, M. and Merlini G.: Seminars in Ongology, 13: 366-79 (1986).
Kohn J.: J Clin Path, 28: 77-82 (1975).
Merlini, G.: The Cancer J,8: 173-80 (1995).
Ritchie,R.Fand Smith,R.: Clin Chem 22,a) 497-99, b) 1735-37 and c) 1982-85 (1976).
Sun T et al: Amer J Clin Path 72: 5-11 (1979).
Whicher J.T.et al: Ann Clin Biochem 24: 119-32 (Review).

DIMITRIADIS IOANNIS AND SON PC
Production of electrophoresis products
Business Incubator Thermi
Steliou Kazantzidi 47, 57001, Thermi, Greece
E-mail: hellabio@hellabio.com
Phone: + 30 2311 999911 Fax: +30 2311 999912
www.hellabio.com

Expected values and Evaluation of Results: