SPIFE® 4000 Immunofix Procedure

Cat. No. 2300

INTENDED USE

SPIFE 4000 ImmunoFix is intended for the qualitative identification of monoclonal gammopathies in serum or urine using protein electrophoresis and immunofixation on the SPIFE 4000 system.

SUMMARY

Immunofixation electrophoresis (IFE) is a two stage procedure using agarose gel high resolution electrophoresis in the first stage and immunoprecipitation in the second. 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 of monoclonal 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. Their presence may be of a benign nature or of uncertain significance. In some 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] The SPIFE 4000 IFE method offers many advantages including hands-free operation, ease of interpretation, excellent resolution, reagent conservation and rapid turnaround.

PRINCIPLE

Proteins are first resolved by electrophoresis. In the second stage, the soluble antigen and 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 the 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.

REAGENT

1. **SPIFE 4000 IFE Gel**
   Ingredients: Each gel contains agarose in tris-barbital/MOPS buffer with a stabilizer and a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. –TOXIC-CAUTION: DO NOT INGEST. The gel contains barbital which, in sufficient quantity, can be toxic.
   Preparation for Use: The gels are ready for use as packaged.
   Storage and Stability: The gels should be stored horizontally 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, (4) thinning of gel blocks.
2. **Acid Violet Stain**
   Ingredients: The stain is comprised of Acid Violet stain.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST.
   Preparation for Use: Dissolve the dry stain in 1 liter of 10% acetic acid and mix thoroughly. Fill the stain container as needed.
   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.
   Signs of Deterioration: The diluted stain should be a homogeneous mixture free of precipitate. The stain must be replaced after processing ten gels to avoid contamination.
3. **Citric Acid Destain**
   Ingredients: After dissolution, the destain contains 0.3% (w/v) citric acid.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST - IRRITANT.
   Preparation for Use: Pour 11 L of deionized water into the Destain vat. Add the entire package of Destain and mix until completely dissolved. Pour the entire contents of the Destain Additive bottle into the prepared Destain and mix.
   Storage and Stability: Store the Destain at 15 to 30°C. It is stable until the expiration date on the package.
   Signs of Deterioration: Discard if solution becomes cloudy.
4. **Tris-Buffered Saline**
   Ingredients: The powder contains a Tris base with Tris HCl and sodium chloride.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE
   Preparation for Use: Dissolve the powder in 8 L of deionized water and mix thoroughly.
   Storage and Stability: Store the dry powder at 15 to 30°C until the expiration date indicated on the label. The buffer solution should be stored at 15 to 30°C.
   Signs of Deterioration: The buffer solution should be discarded if it shows signs of bacterial contamination.
5. **Destain Additive**
   Ingredients: The product is a wetting agent.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. IRRITANT--DO NOT INGEST.
   Preparation for Use: Pour the entire contents of the Destain Additive bottle into the prepared Citric Acid Destain.
   Storage and Stability: The Additive should be stored at 15 to 30°C and is stable until the expiration date indicated on the package.
   Signs of Deterioration: The product should be free of precipitate.
6. **SPIFE IFE Protein Fixative**
   Ingredients: The fixative contains 2.5% sulfosalicylic acid, 1.0% trichloroacetic acid, and 0.25% glutaraldehyde.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE. CORROSIVE - NEVER PIPETTE BY MOUTH. DO NOT INGEST.
   Preparation for Use: The fixative is ready for use as packaged.
   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 solution.
7. **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, IgA and to human light chains, Kappa and Lambda. The antisera have been prepared in goat. Each vial of antiserum contains a stabilizer and sodium azide as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. 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.
   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.

INSTRUMENT

A SPIFE 4000 analyzer must be used to apply samples, electrophorese, apply antisera and fixative, wash, stain, destain and then dry the gels. Refer to the Operator's Manual for detailed instructions.

SPECIMEN COLLECTION AND HANDLING

Specimen: Fresh serum or urine is the specimen of choice.

Storage and Stability: If storage is necessary, samples may be stored covered at 2 to 8°C for up to 72 hours.

Urine Sample Preparation: Urine samples may be run neat, diluted or concentrated. If concentration is desired, swirl samples to homogenize. Centrifuge desired volume at 2000 x g for 5 minutes. Remove supernatant and concentrate as follows:

Total Protein (mg/dL)	Conc. Factor
< 50	100x
50-100	50x
100-300	25x
300-600	10x
> 600	5x

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.

PROCEDURE

Materials provided: The following materials needed for the procedure are contained in the SPIFE 4000 IFE Kit (Cat. No. 2300).

SPIFE 4000 IFE Gels (10)	Fixative	1 vial
Acid Violet Stain (1 vial)	IgG	1 vial
Tris-Buffered Saline (1 pkg)	IgA	1 vial
Citric Acid Destain (1 pkg)	IgM	1 vial
SPIFE 4000 IFE Applicator Blades (20)	Kappa	1 vial
SPIFE 4000 Blotter C (10)	Lambda	1 vial
SPIFE 4000 Blotter J (10)		
SPIFE 4000 Blotter Combs(10)		
Destain Additive (28 mL)		

Material provided by Helena Laboratories but not contained in the kit:

	CAT. NO.
SPIFE 4000 Analyzer	1620, 1621
SPIFE 4000 IFE Sample Trays	2305
SPIFE 4000 Cassettes (10)	1630
SPIFE 4000 Rigid Antisera Template	1635
SPIFE 4000 Maintenance Blotters	2307
SPIFE 4000 Gel Staging Lid	2308
Gel Block Remover	1115
SPIFE 4000 IFE Antisera Pipette Tips	2304
SPIFE 4000 Replacement Electrodes	1625

Materials and Supplies Needed but not Supplied:

10% Glacial acetic acid
0.85% saline

STEP-BY-STEP METHOD

I. Sample Preparation

A. Serum

The SPIFE 4000 automatically samples and dilutes the specimens as follows.

SP = 1:3
IgG = 1:5
IgA = 1:5
IgM = 1:5
κ = 1:5
λ = 1:5

Desired dilutions are operator programmable. The more concentrated samples are more likely to prozone while the more diluted samples may not exhibit desired sensitivity.

B. Urine

Urine specimens cannot be tested on the same gel with serum specimens because the running parameters are different. For urine specimen volumes measuring less than 500 µL, 20 µL must be hand-pipetted into the Sample Tray for application. If there is 500 µL or more, the specimen can be transferred into a tube and automatically dispensed into the Sample Tray.

II. SPIFE 4000 Preparation

1. Stack the appropriate number of Disposable Sample Trays into the Sample Tray Holder (one tray per gel).
2. Place IFE Applicator Blades (two per gel) in the Applicator Tray.
3. Fill the designated bottles with 0.85% saline, deionized water, destain and Tris-Buffered Saline.
4. Add prepared acid violet stain to the stain bottle. The stain must be replaced after processing ten gels to avoid contamination.
5. Fill the DI Water Surfactant jar with deionized water, and replace the lid and tubing. Ensure that the ends of the tubing are below the water level.
6. Remove the antisera/water reservoir from the antisera station.
7. Place the Protein Fixative and antisera vials into the appropriately labeled wells of the antisera tray. Fill the "H_2O" well with deionized water. Recap Fixative and antisera vials after use and store at 2 to 8°C.
8. Place the lid on the reservoir and insert one IFE Antisera Pipette Tip into each hole in the lid. Carefully place the reservoir in the antisera station.
9. One Blotter J and one Blotter Comb are needed for each gel being run.
10. Turn on the SPIFE 4000. Wait about 3 minutes after turning on the lower unit. Click the SPIFE 4000 icon on the screen for the instrument to initialize.
11. Prime the sample handler and the surfactant delivery system according to the instructions in the Operator's Manual.
12. Carefully open one end of the pouch, remove the gel from the protective packaging and discard the overlay. Use lint-free tissue to wipe around the edges of the plastic gel backing.
13. Using a SPIFE 4000 Blotter C, gently blot the entire gel. Discard the blotter.
14. Hold the gel so that the barcode is at the top. Place the gel into the cassette by holding the gel backing in one hand and gently bending the gel. Slide each end of the gel backing into the slots of the cassette to hold it in place. Align the cut out in the gel backing with the alignment pin in the cassette.
15. Ensure the gel blocks make good contact with the electrodes to prevent skewed patterns.
16. Place the cassette(s) with the gel(s) into the humidor and cover the topmost cassette with the Gel Staging Lid. Close the humidor lid to minimize gel dehydration.
17. Repeat Steps 12-16 for each gel needed.
18. Load patient sample test tubes into the carousel counter-clockwise starting at the space numbered 1.

III. Electrophoresis Parameters

A. Serum

1. Using the instructions provided in the Operator's Manual, select
   -- **Test Type: Serum Immunofixation Electrophoresis (IFE)**
   Test Name: Serum Immunofixation Electrophoresis (IFE)

Check the programmed parameters for each of the following processes.

Sample Preparation
Sample Application
Electrophoresis
Fixative Application
Pre-Dry
Wash
Stain
Destain
Dry
Scan

Sample Preparation	Volume (µL)	19
	Primary Wash Time (mm:ss)	00:06
	Primary Wash Cycles	4
	Secondary Wash Time (mm:ss)	00:03
	Secondary Wash Cycles	0
	Samples per Gel	4
	Application per sample	6

Dilutions:

Sample	SP	G	A	M	K	L
1-4	1:3	1:5	1:5	1:5	1:5	1:5

Sample Application	Applicator Load Time (mm:ss)	00:30
	Applicator Load Speed	115

	Application Rows	2
	Row 1 Location (mm from gel edge):	47.0
	Apply Time (mm:ss)	01:00
	Apply Cycles	1
	Absorption Time (mm:ss)	00:00
	Inter-Gel Start Delay (mm:ss)	08:00
Electrophoresis	Voltage	350
	Minimum Current (mA)	10
	Maximum Current (mA)	100
	Temperature (°C)	20
	Time (hh:mm:ss)	00:06:30
Fixative Application	Absorption (hh:mm:ss)	00:02:00
	Blot Time-Comb Blotter(hh:mm:ss)	00:02:00
	Blot Time-Flat Blotter(hh:mm:ss)	00:00:10
Pre-Dry	Temperature (°C)	53
	Time (hh:mm:ss)	00:13:00
Wash	Cycles	1
	Time (hh:mm:ss)	00:10:00
Stain	Type	Acid Violet
	Time	00:02:00
Destain	Cycles	4
	Time (hh:mm:ss)	00:02:00
	Agitate	No
Dry	Temperature (°C)	60
	Time (hh:mm:ss)	00:12:00
Scan	Sequence	1-4
	Aperture	5
	Gain Mode	Auto
	Smooth	No
	Auto Interpretation	No
	Image Contrast	0.0
	Fraction Detection	
	Sensitivity	5.0
	Force Fractions	No
	Combine Split Beta	No

2. Proceed to Section IV. Electrophoresis

B. Urine

1. Volume of 500 µL or Greater

a. Pipette 500 µL or more into a test tube and place it in the sample carousel.

b. Using the instructions provided in the Operator's Manual, select

-- **Test Type: Urine Immunofixation Electrophoresis(IFE)**
Test Name: Urine Immunofixation Electrophoresis(IFE)

Check the programmed parameters for each of the following processes.

Sample Preparation
Sample Application
Electrophoresis
Fixative Application
Pre-dry
Wash
Stain
Destain
Dry

Sample Preparation	Volume (µL)	19
	Primary Wash Time (mm:ss)	00:06
	Primary Wash Cycles	4
	Secondary Wash Time (mm:ss)	00:03
	Secondary Wash Cycles	0
	Samples per gel	4
	Application per sample	6
	Dilutions	Neat

Sample	SP	G	A	M	K	L
1-4	Neat	Neat	Neat	Neat	Neat	Neat

Sample Application	Applicator Load Time (mm:ss)	00:25
	Applicator Load Speed	115
	Application Rows	2
	Row 1 Location (mm from gel edge):	47.0
	Apply Time (mm:ss)	00:25
	Apply Cycles	3
	Absorption Time (mm:ss)	02:00
	Inter-Gel Start Delay (mm:ss)	08:00
Electrophoresis	Voltage	400
	Minimum Current (mA)	10
	Maximum Current (mA)	100
	Temperature (°C)	21
	Time (hh:mm:ss)	00:05:00
Fixative Application	Absorption (hh:mm:ss)	00:02:00
	Blot Time-Comb Blotter(hh:mm:ss)	00:02:00
	Blot Time-Flat Blotter(hh:mm:ss)	00:00:10
Pre-Dry	Temperature (°C)	53
	Time (hh:mm:ss)	00:13:00
Wash	Cycles	1
	Time (hh:mm:ss)	00:10:00
Stain	Stain Type	Acid Violet
	Absorption Time	00:02:00
Destain	Cycles	4
	Time (hh:mm:ss)	00:02:00
	Agitate Destain	No
Dry	Temperature (°C)	60
	Time (hh:mm:ss)	00:12:00
Scan	Sequence	1-4
	Aperture	5
	Gain Mode	Auto
	Smooth	No
	Auto Interpretation	No
	Image Contrast	0.0
	Fraction Detection	
	Sensitivity	5.0
	Force Fractions	No
	Combine Split Beta	No

c. Proceed to Section IV. Electrophoresis

2. Urine Volumes less than 500 µL

a. Pipette 20 µL of each urine specimen into the Sample Tray.

b. Place the Sample Tray with specimens into the Sample Tray Holder.

c. The SPIFE 4000 User Setup menu should be used to create “Add a Test” parameters for small volumes of urine which need to be manually pipetted. The test parameters will be the same as those given in Section III B.1 except that “Sample Preparation” will not be checked.

d. Proceed with the testing according to steps in Section IV.

IV. Electrophoresis

1. Click START on the screen and respond to the analyzer prompts. The analyzer will load samples when appropriate, apply samples, electrophorese, immunofix, wash, stain, destain, dry and scan the gels.
2. After scanning, the cassette with the gel will be dropped into the cassette receptacle.
3. Remove the cassette(s) from the receptacle. If gel storage is required, remove and discard the two gel blocks. If not, discard the used gels, blotters, applicator blades, sample trays and blotter combs as biohazardous waste.
4. Cassettes must be washed after each run with deionized water. The Antisera Template should be cleaned with a mild biocidal detergent. The template may also be scrubbed with a soft brush to remove any antisera residue. Rinse with deionized water. Dry the Antisera Template thoroughly with a lint free tissue.

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

INTERPRETATION 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. Because of the increased sensitivity of the procedure, it is not uncommon to see a fixed band that is not visible in the serum protein procedure.

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.

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 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 of the band, while 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 dilution. 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.
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.

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 antisera, may require further testing as follows:

1. Serum samples which have a precipitin band with Kappa or Lambda Light Chain Antisera but none corresponding 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 ImmunoFix IgD and IgE antisera.
2. A CRP band may be detected in patients with acute inflammatory response.[7, 8] CRP appears as a narrow band on the most cathodic end of the high resolution agarose protein electrophoresis pattern. Evaluated alpha$_1$ 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

Fifty abnormal and twenty normal serum specimens were tested using the SPIFE 4000 ImmunoFix Procedure and the SPIFE ImmunoFix Procedure. Of the test results, all showed good clinical interpretative agreement between methods. Twenty abnormal and ten normal urine specimens were tested using the SPIFE 4000 ImmunoFix Procedure and the SPIFE ImmunoFix Procedure. Of the test results, all showed good agreement between methods. Any sensitivity differences observed are within acceptable test variation.

BIBLIOGRAPHY

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