An Immunofixation Tutorial

Presented by
John O’Keefe
Sha Robinson, PhD
Rita Ellerbrook, PhD
Jeff Spencer, PhD
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This guide is intended as a primer to the use and interpretation of immunofixation for characterizing monoclonal proteins (M-proteins) in human serum and urine.

Whenever a restricted band is identified in serum protein electrophoresis, the next step is to run immunofixation electrophoresis (IFE) for definitive identification of specific M-proteins. IFE may also be directly ordered by the physician if there are sufficient indications that a plasmacytoma or lymphocytoma is present. IFE is a procedure that separates the serum proteins by electrophoresis, followed by treatment of the proteins with specific antiserum against IgG, IgA, IgM, IgD, IgE, kappa, and lambda. If an M-protein is present, a precipitin band will form. The gel is washed with saline to extract all unprecipitated proteins, then stained, destained, and dried. A normal pattern looks like the image below.

The patient is initially screened for the most common M-proteins first: IgG, IgA, IgM and the two light chains, kappa and lambda. Normal or uninvolved immunoglobulins have a blush or color that is darker or lighter depending on the concentration of that particular immunoglobulin. Since IgG is the most prevalent immunoglobulin in the circulatory system, it is usually fairly dark. IgA is lighter than IgG but still very evident on most specimens. Unless IgM is elevated there will be no color or blush at all in that lane. Kappa is present in a 2:1 ratio with lambda, so kappa is usually darker than lambda. If there is an imbalance in this ratio then you should look more closely at the IFE pattern. This would be an indication that there is an M-protein present.
Interpreting IFE Patterns

When interpreting an IFE pattern, it is best to describe what you see. For example, statements in the interpretation could be 1) IgG kappa present or 2) Presence of a poorly-defined IgG kappa or 3) Presence of an IgG kappa in the presence of polyclonal IgG. Each conveys a different meaning to the clinician. Since this is not a quantitative test, the interpretation must contain all useful characteristics about the pattern, especially if the final report does not include an image of the gel. If the image is not reported, additional characteristics may be noted in the interpretation that might not otherwise be included such as “an abnormal band in the protein pattern that is not seen to have any immunological counterpart in this test is probably fibrinogen contamination of the sample”.

Polyclonal Increase

The image below shows a polyclonal pattern. In order to be classified as a polyclonal, both kappa and lambda must be elevated. The pattern below does have an increased intensity in all five lanes so it is easily identified as a polyclonal. Remember that a polyclonal pattern does not exclude the possibility of the presence of an M-protein. Examine the pattern closely to see if there is an M-protein hidden within the dark blush. If you suspect that you see a band, you can try different dilutions to make the band more visible. Remember that as you dilute the polyclonal blush, you are also diluting the band. The report is Polyclonal increase present; no M-proteins observed.
IgG

Let’s look at simple patterns first. Most of the M-proteins you identify will be IgG since IgG is the most prevalent immunoglobin. You look for a well-defined restricted band in the protein lane with a corresponding band in a heavy chain lane (G,A,M) and light chain lane (kappa, lambda). It is best if both the top and bottom edge are straight and easily seen as separate from the blush. Sometimes that is not possible and only one well-defined edge can be seen. Below are two different IgG lambda patterns. In the first, the bands are at the edge of the gamma region. Notice also that the band in the IgG lane is darker than the corresponding band in the protein lane. This is a common phenomena because the antibody bound to the band is contributing to the total stain seen. IgG typically migrates anywhere in the gamma region, but it can be found in the beta and alpha region also.

It is helpful to the physician if you note when the uninvolved immunoglobulins are decreased or essentially absent, which is the case in the example below. Note also that this band is more in the middle of the gamma. The report is IgG lambda with uninvolved immunoglobulins decreased.
IgA

There are several things to note about IgA. It frequently will polymerize and show two or even three bands. IgA also is often very elevated so the pattern will be distorted. IgA can also undergo posttranslational modification such as glycosylation. Due to the uneven arrangement of charges in the glucose the band will migrate in a “squiggly” fashion as it is below. Some techs may want to make the bands look perfect and be tempted to repeat this specimen, however doing so will not add anything to the clinical or diagnostic picture for the physician. Remember that this is an expensive test and need not be repeated for aesthetic reasons.

Notice that the kappa band looks much nicer than the IgA band. The kappa is usually diluted more than the IgA lane and gives a neater appearance in this case. It is still an IgA kappa no matter how pretty or ugly it looks. IgA typically migrates in the beta region but like IgG it can be found cathodal or anodal to this location. The report is IgA kappa present.

IgA may not be denatured by the fixative in such a way that prevents it from washing out of the protein channel. If this occurs, there will be no IgA in the fixed protein lane, but it appears under standard protein electrophoresis.
**IgM**

IgM is the most troublesome M-protein to interpret. IgM is the largest of the immunoglobulins and often exists as a pentamer. IgM immunoglobulins can polymerize and remain at the application point making it impossible to identify an M-protein.

The specimen below shows normal to elevated levels of uninvolved immunoglobulins. This is information that the physician may find helpful. The report on this would simply be *IgM kappa present*. Some laboratories add the area of migration and/or the level of uninvolved immunoglobulins. It does not hurt to add more information. It may be the last piece of the puzzle.

![IgM Kappa](image)

The image below shows a more typical IgM. Notice the bands in all six lanes. They are all the same intensity and width and migrate at the same point. In this instance, the IgM immunoglobulins have polymerized and remain at the application point. If the specimen is mixed with beta mercaptoethanol (BME) or dithiothreitol, disulfide bonds will be broken causing the molecule to unravel and the pattern will be resolved as in the second image. The report would be *IgM kappa present*.

![IgM Immune Complex and Specimen treated with BME: IgM Kappa](image)
**IgD and IgE**

The general IFE screen looks for IgG, IgA, and IgM because these comprise 99% of all M-proteins seen in the laboratory. Only 1% of the M-proteins seen will be IgD, and IgE is very rarely seen. If the finished gel shows a band only in the light chain lanes, the tendency is to assume that it is a free light chain. This would be correct 99% of the time. It is incumbent on the laboratory to strive for 100% accuracy, particularly since IgD myelomas are extremely aggressive. It is, therefore, very important to identify IgD and report it to the physician. Any time a band is seen only in the light chain lane the specimen should be checked for IgD and IgE. A lambda light chain will be present in 90% of IgDs.

The first image that follows shows the initial gel. As you examine the lanes, note there is a band in the lambda with no corresponding bands in the heavy chain lanes. You need to repeat the specimen with a protein lane and IgD and IgE. This is shown below.

The unlabelled lanes have SP, IgD, and IgE. The band in the middle lane identifies the heavy chain as IgD. The report on this is *IgD lambda present*. 
MGUS, Poorly-Defined and Suspicious Patterns

Progressing to patterns that are a little more difficult, the cardinal rule is describe what you see. If the immune system is dysfunctional, many types of immunofixation patterns can be seen. It is difficult, if not impossible, to tell what the disease state is from looking at the individual pattern. Depending on your patient population, many of the M-proteins that you identify will be MGUS or Monoclonal Gammopathy of Unknown or Undetermined Significance. Some studies have shown that up to 80% of the M-proteins will be MGUS. Even an M-protein of 2.5 g/dL can be MGUS. About 1% of MGUS each year will progress to myeloma. This percentage increases with age.

The following pattern always causes consternation while trying to interpret. There are arguments pro and con about whether to report this or leave it as normal. It is better to give the physician the information and allow him or her to decide whether to ignore it or follow up.

Clinical significance is 50 mg/dL for an M-protein. The band in the pattern above is around that level of concentration and has a size and intensity similar to an alpha1 in a serum protein pattern. The most visible band is in the lambda lane and there are two fairly well-defined edges. It is barely visible in the protein lane but you can see a darkening of the gamma at the same migration area as the lambda. As you examine the IgG, IgA, and IgM lanes you can see something in the IgG lane that again corresponds to the lambda and the protein band. In order to give accurate information to the physician, you should more fully describe this band. Some laboratorians say suspicious or poorly-defined. You could add that the level is very low and the significance is unknown. The report is suspicious or poorly-defined IgG lambda. In cases like this you should always suggest a follow up in 3 to 6 months if clinically indicated. Many times, when repeated, the band is gone. Because of the extreme sensitivity of IFE systems, transient M-proteins are sometimes detected.
One of the reasons to be cautious about these suspicious bands is illustrated in the next example. The initial sample showed a poorly-defined IgG kappa. Two years later the sample showed a large IgG kappa. If it had been reported initially, the physician would have been following the patient. If followed up at 3 to 6 months, the IgG kappa would have shown an increase which would have alerted the physician that it was not an MGUS but probably a smoldering myeloma. Further tests could have been run, identifying it and allowing for treatment. If it had been ignored initially, it might not have been seen until the patient was at Stage III or even Stage IV. This would complicate treatment and lessen the likelihood of a better outcome. It is better to report these small M-proteins and let the physician decide how to react to it.

The image shows the initial sample with suspicious IgG kappa bands and the sample two years later with a large IgG kappa. The bands in the initial sample are light with a well-defined edge at top and bottom. The report should be:

**Suspicious or poorly-defined IgG kappa; Suggest repeat in 3 to 6 months if clinically indicated.**

Next is the patient two years later. This is a sample you might want to repeat with a higher dilution for the kappa lane. It appears to be prozoning or antigen excess but it could be an IgG kappa with a free kappa.

Whenever a sample is repeated in an attempt to clarify it, you only need to repeat the lanes that you are unsure about. In the case above, you would repeat the kappa with a couple of dilutions, such as 1:20, 1:30, and 1:40. This should make the banding easier to interpret. If you are trying to tease out a small M-protein hidden in normal or elevated blush, you use dilutions on either side of your normal dilution. Sometimes a lesser dilution will make it more clear. If the normal dilution is 1:10 for an IgG, you would repeat with dilutions of 1:5 and 1:20. Remember that as you dilute out the uninvolved immunoglobulins you also dilute the M-protein. So, sometimes the process is not successful and you have to describe what you see.
This next sample points out of couple of difficulties. The application point is quite distinct. It is the space devoid of stain between the beta2 and gamma. Any stained portion that is right next to that void appears to have a band. This is due to the sharp contrast of stain and no stain. In patterns like this if you start at the outer edge and work your way in toward the application point, the more gradual increase in color intensity is easier to differentiate. For instance, look at the kappa and lambda lanes toward the bottom of the staining. The lambda has a more distinct edge at the bottom and migrates with the heavy chain band seen in the IgA lane. The kappa has a less distinct edge at the bottom as you follow the color in from the very bottom up to the point of application. It is an idiosyncrasy that takes some experience to interpret correctly. You could repeat this with lambda run neat and it should be more distinct. After a few times of seeing this, you will be able to distinguish it with less effort. The report for this is IgA lambda present.
Multiple M-Proteins

The next pattern has several M-proteins. You can identify them in decreasing order of intensity. There is an IgG lambda that is very distinct in the middle of the gamma region. If you look at the very edge of the gamma region you see that it ends abruptly. Usually the gamma region starts as a light blush and gradually increases to a darker color in the middle, then gradually decreases in intensity as it reaches the end limit. The abrupt end should signal you to look more closely at the pattern because many times IgG migrates here. It is helpful with these complicated patterns to lay the gel on a lined surface to assist in lining up the edges of bands. The more visible band that lines up with the cathodal edge of the IgG is the kappa band. If you follow the migration point through all three heavy chain lanes you see a darker area in IgG at the very end. There is a nice straight line at the bottom edge which shows this as an IgG kappa. There is also a distinct band in the IgA lane. At first you may have difficulty seeing a band in one of the light chain lanes lined up with the IgA band but this is an IgA kappa. The report would be IgG lambda, IgA kappa, and IgG kappa present.

![Image of gel with bands labeled SP4, G, A, M, K, and lambda]

There are a couple of explanations for the difficulty in seeing the kappa band attached to IgA. The IgA lane is normally diluted 1:3 or 1:5 while kappa is diluted to 1:10. If you were to repeat this specimen and dilute kappa to 1:5, the intensity of IgA and kappa would be closer but still may not be the same. Remember you are seeing the intensity of the antigen-antibody complex. They may not be the same. A second reason could be due to the quaternary structure of the IgA molecule. The molecule may fold in on itself and sequester the epitopes of the light chain. This happens about 10% to 20% of the time with IgA. In order to elicit a reaction of the antigen-antibody precipitate you could repeat with a lesser dilution or even neat. You could also add BME to the specimen and see if you can unravel the molecule and expose the epitope so the antiserum can react with it.
In the next sample shown below, there are two IgG kappa bands present.

![Two IgG Kappa with Free Lambda](image)

Although not common, IgG can polymerize. You cannot determine this by looking at the pattern. If you were really interested in documenting polymerization, you could react the sample with BME. If it is polymerized then treating with BME would break the bonds holding the molecules together and only one IgG kappa band would be present. Since it is not relevant to the clinical, diagnostic, or therapeutic picture it is not necessary to go through all this effort. The IgA lane has a normal IgA blush and there is nothing in the IgM lane. The lambda lane has a darkening in the gamma region. There is a sharp, well-defined edge at the top and the bottom of this band. The band does not line up with the IgG band so it is presumed to be a free light chain. You should run an IgD and IgE to rule them out. There have been reports of IgG and IgD myeloma. The interest in the free light chain is for the condition of the kidneys.

A urine needs to be run on this patient to see if there are free light chains in the urine or even worse if there are heavy chains. This indicates what sort of damage has been done to the kidneys. Light chains are an indication that the tubules are malfunctioning. Normal tubules will catabolize and reabsorb low molecular weight proteins so none are present in the urine. The presence of heavy chains in the urine is an indication that the glomerulus is not functioning properly. The presence of both heavy and light chains indicates serious kidney malfunction. Whenever an M-protein is seen on serum, you should run the patient’s urine. The report on this sample is two IgG kappa with free lambda.
The IgA lambda in the sample below is easy to see. There is a second band in the lambda lane right above the lambda attached to the IgA. At the same level you can easily see the band in the protein lane. What is not easy to see is the thin band in the IgM that lines up with the lambda. Sometimes the IgM shows as a pencil thin band, possibly due to an incomplete or less than optimal antigen-antibody complex. The report is IgA lambda, IgM lambda present.

[Image of a gel with bands labeled SP, G, A, M, K, and λ]

IgA Lambda, IgM Lambda

Here is another IgA. This band looks more like a prozoned IgA. Frequently, when IgA is present, it is at a very high concentration. The antigen excess will cause the middle of the band to look empty or give the appearance of having two bands there. The report is IgA kappa with free kappa. You can repeat this sample to make it look better by using higher dilutions. It will be a nicer pattern but it will not change the interpretation. IFE is an expensive test that takes an hour to run so try to be judicious when ordering repeats.

[Image of a gel with bands labeled SP, G, A, M, K, and λ]

IgA Kappa with Free Kappa
The next pattern has an extremely cathodal IgG. It looks like either the electrophoresis time was too long or the voltage was too high. The albumin also migrated to the edge of the lane. The report is IgG lambda and IgM kappa present.

The following sample is a good example of just having to describe what you see. The Helena Technical Service Department tried several things with this sample. First, higher dilutions were used to try to better resolve the bands. It did not make it any better. Using BME did not help. Using another manufacturer’s antiserum did not help. Running free light chain antisera against kappa and lambda showed no reaction. The one thing that could have helped would have been to run a urine. A urine sample was not available and the patient had already been discharged from the hospital. In the end, the pattern was described as: Two IgM bands migrating with two kappa bands and one lambda band; suggest follow up with urine protein and urine IFE.
Free Light Chains

Using free light chain antiserum in a complex pattern like the preceding example is a good idea. However, you should be aware that the process required to manufacture free light chain antiserum uses additional steps to strip out some of the epitopes. This makes free light chain antiserum less sensitive than the light chain antisera used in the normal screen. The light chain antisera normally used contains epitopes to both bound and free light chains. This allows for more epitopes that can react. So, you need to be aware that if you use free light chain antiserum you can have a false negative but not a false positive result.

Using another manufacturer’s antisera may be helpful because there may be differences in reactivity, mainly due to the different animal pools used to manufacture each antiserum. Patient pools used for immunization of the animals also play a role. There is a large overlap of epitopes in both antisera but each animal will have a small percentage of antibodies that react better with some patient epitopes. If you are not getting the answer you expect or are having difficulty in interpreting a specific patient, it is a good idea to use this second set of antisera. That does not mean you should use it to make a pattern look prettier. Only use it to help define a specific result.

The next sample shows another troublesome IgM along with a technical problem. Let’s look at the technical problem first. Notice the blivet or streak down the middle of the IgM lane. It can be caused by several different circumstances. There might not be enough sample in the sample well for that lane. The tooth on the applicator blade at that lane may have been bent or twisted. A lot of techs would prefer to repeat this, especially since it appears in the lane where the M-protein is located and makes it more difficult to interpret. The report on this would be: Suspicious or poorly-defined IgM lambda with free lambda; Check for IgD and suggest repeat in 3 to 6 months, if clinically indicated along with a urine protein and IFE. All three bands identified do have well-defined edges but they are quite small. The uninvolved immunoglobulins are also at normal to elevated levels.
The following sample is an IgG kappa with free kappa present. The extra bands in the IgG lane are interesting. Sometimes IgG forms a complex in vitro with alpha1 and alpha2. Once the complex is formed the IgG migrates a little further due to the charges of the alpha1 and alpha2. When the antiserum is applied it forms a precipitate band. There are no light chains associated with that IgG that will stain. It can be reported as an IgG immune complex that has formed in vitro. There is no clinical significance attached to this complex.

There are several causes of this, none of which have real clinical significance:

1) Most likely with this patient, there is incomplete proteolysis of the immunoglobulin molecule. This is due to the presence of polyclonal IgG.

2) Heavy Chain Disease (HCD) is less likely in this case because there is a polyclonal IgG increase. Normally HCD would have a normal or decreased gamma region. HCD cannot be ascertained by IFE. There are circumstances that can otherwise prevent the reaction of light chain antisera and yield a pattern with just a heavy chain component. This is best reported as IgG with no apparent or discernible light chain attached, suggest further investigation to rule out HCD. This again, is not the case here.

3) There could be a post translational modification of the IgG molecule which yields only the Fc fragment and thus will only react to the anti-IgG antiserum. This is a remote possibility.

4) Sometimes there is an immune complex formed with IgG either in vivo or in vitro which binds with alpha-2 macroglobulin. This complex then migrates and forms a precipitate with the IgG antiserum.

It is difficult to see a band and not definitively explain what it is. However, by giving the possibilities to the physician, he or she can look more closely at the clinical situation and suggest further testing.
This sample has multiple heavy chain and light chain bands. This could be caused by the rehabilitation of the immune system following chemotherapy or radiation therapy. There are a multitude of other causes of this restricted heterogeneity such as chronic antigenic stimuli, autoimmune disease, infectious disease, age-related weakening of the immune system, immuno-deficiency, immunosuppression, or posttranslational modification of the immunoglobulin molecule.

![Image](IgA with no apparent light chain attached)

The other point to notice is the IgA band which in this case has no light chain attached. This time you need to repeat and run the kappa and lambda neat. You might also try BME to depolymerize or unravel the molecule. Due to the quaternary structure of the IgA molecule, the epitopes for the light chains can be sequestered by the folding of the molecule. This should be reported as an *IgA with no apparent light chain attached*. Though rare, IgA heavy chain disease should be considered. Heavy chain disease cannot be determined from IFE. Further testing, such as immunoselection, must be performed.

**In Conclusion**

Most IFE patterns are easy to interpret. The more difficult ones should be examined closely and reported simply. Report what you see and you will not be wrong. The actual wording of the interpretation depends on your comfort level. You can give a short description or add other comments about the implications of the bands that are present. It is important to test for IgD and IgE when you see “free” light chains. Helena’s Technical Service Department is always ready to help with suggestions for further testing or help with interpretation. There are also several clinical chemists and pathologists, experts in the field, who can help us with difficult patterns.
Bibliography


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