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ImmunoNephelometric Determination of Free Light Chains of Immunoglobulins in Urine for the Detection of Bence Jones Proteins, and Comparison with Immunofixation

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Non standard Abbreviations

BJP	Bence Jones Proteins
EF	Electrophoresis
FLC	Free Light Chains
FRK	Kappa Free Light Chains
FRL	Lambda Free Light Chains
IFE	Immunofixation
IN-FLC	FLC ImmunoNephelometry
LPIP	Liquid Phase ImmunoPrecipitation
LC(b&f)	Total Light Chains (bound & free)
MC	Monoclonal Component
Ig	Immunoglobulins
MGUS	Monoclonal Gammopathy of Undetermined Significance
BDL	Biological Detection Limit
SS	Spiked Sample
BS	Blank Sample

Key Words

Bence Jones Protein, Kappa Free Light Chains, Lambda Free Light Chains

Summary

We have evaluated a commercial kit for the direct immunonephelometric determination of kappa and lambda free light chains (FRK – FRL) of immunoglobulins in unconcentrated urine on automatic nephelometer with a view to verifying its utilisation amongst the methods for the detection of Bence Jones Proteins (BJP).
The kit is based on an antiserum reagent with reactivity against the epitopes “hidden” in light chains; that is, those which are not shown when the light chain is bound to the heavy chain of the immunoglobulin.

We primarily assessed the following analytical performances:

- Reproducibility of the calibration curves, linearity and parallelism, imprecision: very good
- Sensitivity: analytical limit – FRK = 0.8 mg/l – FRL = 0.5 mg/l; biological limit: FRK and FRL = 3 mg/l
- Cross-reaction with intact Ig: practically absent
- Antigen excess: not demonstrable until over 30,000 mg/l

We then verified the qualitative value of the method, that is the ability of the method to act as an “alarm” sufficiently reliable in identifying the definitely negative samples and those in which the presence of free light chains would be followed by immunofixation in order to determine mono- or polyclonality.

For this objective, we carried out, side by side, nephelometric determination and immunofixation on all the urine samples in our Laboratory requesting the detection of Bence Jones Proteins in three different periods of three months, each between 1998 and 1999, for a total of 572 samples from 526 patients.

All in all, the method - for its characteristics of simplicity, rapidity, automation and reliability - is well placed as a first level test in our routine procedure for the qualitative detection of Bence Jones Proteins.
Such characteristics, together with the availability of the method for the most widely used nephelometers, means we can hypothesize that it can have its role in the process which aims to give uniformity to the quality of response in the field of BJP detection.

The quantitative value of the method could be of use, even though with many limits and fine details, in the follow up of Monoclonal Gammopathy of Undetermined Significance (MGUS), of myeloma and gammopathy in general.

Introduction

The qualitative detection and the quantitative determination of Bence Jones Proteins (BJP) and Free Light Chains (FLC), similarly to that of the other immunoglobulins (Ig), are more complex than those of other proteins, which fact is connected to the heterogeneous characteristic of immunoglobulins and their fragments.
Frequent anomaly of the Ig and their fragments is the reduction of heterogeneity resulting in the presence of the so-called Monoclonal Component (MC) which, in the case of FLC in urine, is known as BJP; the anomaly which characterises BJP is the reduced heterogeneity of the FLC present in urine.
Perhaps it is not a mere coincidence that, as a pathological sign, a wide heterogeneity of clinical and sub-clinical situations, in which it does not seem able at the time to make out a single lead, matches the heterogeneity of the Ig and their components and the variability of such heterogeneity.
Leaving aside the polyclonal increase of Ig in lupus, rheumatoid arthritis, etc., and dwelling, instead, on the so-called Monoclonal Components (MC) it should be said that these on the one hand characterise multiple clinical situations and on the other a sub-clinical situation whose current definition, Monoclonal Gammopathy of Undetermined Significance (MGUS), demonstrates the uncertainty in the matter. BJP are no exception, as they can be an indication of MGUS and myeloma, or AL amyloidosis or Light Chain Deposition Disease (LCDD).
In this area of work the Laboratory is requested to evaluate the presence and the entity of the anomalies of the Ig and their fragments, as already mentioned above, and amongst these the detection of BJP in urine has an important place. In the evaluation of these anomalies the separative techniques and the immunological techniques used individually have their limits whereas the combined use of both types of techniques guarantees the best results. The detection of BJP is rendered even more uncertain and complex by the particular metabolism of the FLC which constitute it and which involves renal function and, consequently, by being their evaluation as well as "sample-dependent".

From the qualitative point of view, the same definition of BJP, that is monoclonal FLC in urine, means that the **detection of their presence** must take into account two characteristics:

- *antigenic individuality or composition*
  The effective presence of FLC in the sample or in the fraction must be ascertained, and to do this we need an immunochemical technique specifically for FLC.
- *electrophoretic mobility or distribution*: monoclonal, that is homogenous, or polyclonal, heterogeneous.
  This characteristic will have to be investigated with electrophoretic techniques or those that at least provide for electrophoresis.

The simultaneous verification of both characteristics in the current Laboratory practice is only possible with a method which is at the same time electrophoretic and immunological, a condition achieved by IFE which, in reality, is a "protocol" made up of two techniques: the separative technique, electrophoresis, followed by ImmunoPrecipitation on Support, as immunological technique.

The alternative is that of resolving the problem in the two components by providing protocols of two or more methods able to verify "composition" and "distribution" separately.

A first-level screening test for one of the two characteristics would be utilised, followed by, on the positive samples in the first test, a second test to verify the second characteristic, either separately from, or together with, the first.
Depending on which of the two parameters is chosen for screening, ***two types of protocol*** can be formalised.

- *parameter screening: distribution – first level test: Electrophorosesis.*
- *parameter screening: composition – first level test: direct Liquid Phase ImmunoPrecipitation (LPIP) – nephelometry/turbidimetry specifically for FLC, indirect LPIP – nephelometry/turbidimetry of LC (b&f) and Ig and relevant "indices".*

As regards the quantitative evaluation, that is the concentration of the BJP, this would seem important above all in the follow up of MGUS, myeloma and other gammopathies.

Similarly to that for the other analytes "absent" (or almost) in a normal subject and whose presence is thus a sign of "abnormality", and therefore warning of a potential disease, *the "perfectly effective" method or protocol also for BJP (and FLC) will be that characterised by:*

a. no "false negative"
It is the principal and absolutely essential characteristic, for which maximum intra and inter-laboratory reproducibility is consequently required; that is, it is of primary importance that samples result BJP-positive or BJP-negative in all the Laboratories.

b. no "false positive"
The more the number of "false positives", the lower the efficacy becomes.

c. good "precision" and "accuracy"
The value of this element depends upon the how great the importance for quantitative determination is felt; but it must be remembered that "precision" affects reproducibility, and "accuracy" affects the

opportunity to compare the performances of the different methods.

It follows that in the evaluation of the methods for the detection of BJP the parameters, as for any other analyte, which must be borne in mind are:

i) analytical sensitivity limit: that is, the smallest quantity of analyte detectable in any one real sample with sufficient reliability and reproducibility (intra and inter-laboratory)
ii) analytical specificness of the positivity signal
iii) reproducibility of the positivity signal
iv) reliability in its execution: manual, semi-automatic, automatic; identification of the sample etc.
v) reliability in the interpretation of the result: subjective, semi-subjective, objective

Evaluation of the aforementioned points, with the exception of iv), must take into account that the detection of BJP in urine is **<u>"sample-dependent":</u>**

- on the one hand, if the production of BJP remains unchanged, its concentration is influenced by diuresis;
- on the other hand, the performance of the methods could be influenced by variability in the proteinuria, from physiological to mixed (such as diluted serum with the addition of micro-molecular tubular components).

It is without doubt that in the detection of BJP, despite being so delicate, a multitude of approaches has generated starting with the type of sample to utilise and the method and/or analytical protocol, arriving at the interpretation of the result, the reporting method and language. In fact, probably on account of the very nature of BJP, not only is there no standard or control- not even of an imperfect or provisional kind - but neither are there any clear indications as to the criteria for evaluation and choice of methods or for reporting and interpretation of the result.

Objectives – Methodology - Samples

Kits for the direct immunonephelometric determination (on unconcentrated urine) of kappa and lambda free light chains by automatic nephelometer have been available commercially for several years. The kit is based on an antiserum reagent with reactivity against epitopes "hidden" in the FLC; that is, those which are not indicated when the light chain is bound to the heavy chain of immunoglobulin.

The general objective of the work is to evaluate the potentiality of including nephelometric determination of the FLC (IN-FLC) with the kit in question in the routine protocol for the detection of BJP as a first level test able to identify, with sufficient reliability, the definitely negative samples and those in which the presence of FLC should be followed by IFE in order to determine mono or polyclonality.

To be more precise, the objective was to verify whether the use of this protocol could offer concrete advantages in terms of objectivity and uniformity in the final result compared to the use of EF and IFE notoriously affected by :

a. a certain lack of reproducibility both intra-laboratory and inter-laboratory, even for the same commercial product utilised (1);
b. the semi-automatic operation, at least in our set-up, and therefore the possibility of error in identification of the sample;
c. and lastly the subjectivity of result interpretation.

In other words, if the IN-FLC method had proved itself analytically reliable in detecting the presence of FLC in the sample, this information would have served as an objective and reproducible basis - full stop - for identifying those samples to be considered BJP-negative compared to those potentially BJP-positive and subject to quality IFE so as to highlight the FLC polyclonal or monoclonal (BJP) detected by IN-FLC. In this way, the IN-FLC would have served as quality control of IFE; avoiding sample exchanges and inversion of antisera, providing a guide on sample concentration, etc.

From this viewpoint, in the first place we examined the analytical performances of the IN-FLC method.

We also verified the qualitative value of the method as first level test in the sense indicated above and therefore made a parallel comparison between IN-FLC and IFE on all the urine samples coming to our Laboratory with the request for the detection of Bence Jones Proteins in three different periods of three months, each between 1998 and 1999, for a total of 572 samples from 526 patients.

It must be stated that the vast majority of requests for the detection of BJP which come to the ImmunoHematology Laboratory are "aimed at" and supported by precise clinical motives, diagnostic suspect or follow-up.

The results were compared and analysed only subsequently; for this reason the verification of dubious situations was carried out only on a few samples.

Materials and Methods

A. <u>ImmunoNephelometric Determination of Free Light Chains (IN-FLC)</u>

We used the "New Scientific Company" kit, code K.BNA.FRK.FRL, (New Scientific Company, Cormano, Milan, Italy) on the "Dade-Behring" BNII Nephelometer, (Dade–Behring, Milan, Italy).

The calibration and analysis procedures were those proposed by the manufacturer.

The kit contains reagents and calibrators for both light chains.

The manufacturer states that the calibrators are made up of an opportune dilution of urine from a Micromolecular Myeloma patient, containing only Bence Jones Proteins and no others.

The concentration of the calibrator is indicated at ≈ 20 mg/dl for both kappa and lambda and is obtained for internal standardisation measuring the Total proteins with the Bradford method.
As prescribed by the manufacturer, the urine was examined unconcentrated.
The analytical protocol for programming of the BNII to carry out the test has developed over time and since 1999 provides:

a. execution of "pre-reaction" to control the phenomenon of antigen excess;
b. a calibration curve of five points from 5 to 80 mg/l;
c. the automatic repetition on a more diluted sample for signals which exceed the highest point of the curve (80 mg/l) and
d. "zero interpolation" of the curve which is able to give us results in concentration even for values below the lowest point of the curve (5 mg/l).

B. Serum Electrophoresis (s-EF) and Serum Immunofixation (s-IFE)

We used the "Sebia" Hydrasys equipment (Sebia, Florence, Italy) and their kits.
The method is that suggested by the manufacturer.

C. Standard Urine Immunofixation (u-IFE)

The "Sebia" Hydrasys equipment and kits were used. The anti Free Light Chain antisera are those of Sebia and of the New Scientific Company.
u-IFE was carried out on all the samples concentrated 25 times, using the manufacturer's method for serum IFE.
IFE was also carried out on 236 unconcentrated samples, as per the relative method of the manufacturer.

D. Urine Electrophoresis and Urine Immunofixation – Protur (u-EF-Protur, u-IFE-Protur)

In few cases (15 in all) Beckman-Coulter's Protur-HISI and Protur Plus kit was utilised.

Analytical Performances Evaluation

The evaluation results of the performance of the methods are reported.

BJP Positive Urine Samples

For some of these tests we selected the samples from the 9 BJP positive samples - 5 BJ-kappa and 4 BJ-lambda - already subject of the Forlì and Liguria Groups multi-centre evaluations. The average of the concentrations obtained in the multi-centres (Table 1) was assigned as theoretical concentration of BJP.

A) Calibration Curves and Reproducibility

As already stated, parameterisation of the standard method requires a 5-point calibration curve, ranging from 5 – 80 mg/l, and zero interpolation, that is the rectilinear extension from the lowest point (5 mg/l) to the origin of the axes (Figure 1).
This means we can have concentration values even for signals below the lowest point of the curve and therefore allows us to discriminate between a sample just below 5 mg/l, for example 4.99 mg/l, and a sample decidedly lower, for example 1 mg/l.

We compared the six calibration curves obtained in the last eight months with four different lots and that shown on the sheet accompanying the product. The results are given in Table 2 and Table 3 and relative Figures.
The overall coefficient of variation resulted more than acceptable, being between 7.6% and 24.2% (5 mg/l) for FRK and between 8.6% and 21.8% (5 mg/l) for FRL.

B) Linearity Limit and Parallelism

Although evaluation of the performances of IN-FLC for the quantitative dosage of BJP and comparison with those of the other methods were not objects of this work, we retained it opportune to look at the subject for both completeness of the evaluation and for the influence these parameters can have on sensitivity.
One of the most frequent criticisms of immunochemical determination of BJP (and more generally of the monoclonal Ig) is the lack of parallelism between the calibrator reaction trend and the sample reaction trend (2, 3). Since the FLC or the BJP of the Calibrator can be antigenically different from the FLC or BJP of the sample, they can react in different ways, not proportionally, with the anti-FLC antibodies of the antiserum. The lack of parallelism is expressed with an unpredictable lack of accuracy, which would be specifically characteristic of the individual BJP, and in theory could lead to false-negative results where a particular BJP does not in fact react with the antiserum in use.
It must be stated that, should the reaction signal of the sample go beyond the highest calibration point, which is 80 mg/l, the BNII carries out subsequent opportune dilutions to fall within the range of the curve. Therefore, linearity and parallelism can be found only within the extension of the calibration curve.
We diluted in PBS 6 of the BJP samples of the Study Groups, as stated above - 3 BJP-K and 3 BJP-L - so as to obtain a mother solution with a theoretical concentration of between 50 and 60 mg/l; thus from the mother solution we obtained scaled dilutions in PBS. IN-FLC was carried out on the dilutions twice over.
The curves of all the determinations are shown in Table 4.
Conclusions: the coefficients of correlation obtained between the theoretical value assigned to the dilutions on the basis of the value obtained on the lowest dilution (100%) and the average value of the measurements of each dilution demonstrate the linearity and the parallelism between reaction trend of the calibrator and reaction trend of the samples. This does not exclude the fact that a particular sample may have a non-parallel trend and therefore produce an imprecise concentration, but ***wherever this is suspected it can be ascertained by carrying out a series of dilutions.***

C) Cross-Reaction with Bound Light Chains – Intact Ig

Cross-reaction between anti FLC antiserum and Light Chains Bound to Immunoglobulins (Ig), that is intact Ig, is one of the reasons for advising against the use of such antisera for the detection of BJP in urine on both IFE

and the immunochemical techniques (2, 3). Such cross-reaction would actually be minimal and unimportant provided that it did not involve urine with only slightly selective glomerular proteinuria and therefore with a significant concentration of Ig.
For this verification, we carried out three tests:

- "Strategic Biosolution" Standard Reference Serum, code "CA4"
  The concentration of Ig-kappa and Ig-lambda was measured with anti Total Light Chain (LC(b&f)) reagent on the BNII nephelometer.
  The concentration of FLC was measured twice over on 4 dilutions of "CA4": the results are reported in Table 5.
- "Dako" Standard Reference Serum – High values
  The standard serum was diluted 1:20 and then ultra-filtered by 30,000 MWCO membrane in order to remove any FLC present.
  The concentration of Ig was measured twice on the BNII with relative reagents, and the concentrations of FLC were also measured twice over thus. The results can be seen in Table 6
- 10 sera with monoclonal component (MC): 5 kappa types and 5 lambda types
  We measured the Ig and the FLC on these 10 sera neat and on dilutions 1:2, 1:5, 1:10. The results are reported in Table 7

Conclusions: All the results agree in the ruling out of cross-reaction between the FLC reagents used and the intact Ig.

D) Evaluation of the "sensitivity"

From the theoretical point of view, in the case of BJP the **precise** (reproducible intra and inter laboratory) and **general definition** (valid for any sample, for any BJP) of the "sensitivity limit" is very difficult, if not impossible, whatever the method: Nephelometry, Electrophoresis, Immunofixation.
The problem is the same as that of the quantitative determination of MC in serum: what is the IFE "sensitivity limit" for the MC IgA analyte in serum, and what is that of EF and nephelometry?
Such difficulty is due to the intrinsic characteristics of the analyte and, for BJP in urine, it is aggravated by the variability of the sample in which that BJP is present both in terms of diuresis, which under the same production influences the concentration, and for the presence of proteinuria. Depending on the method, this can interfere with or disguise the BJP, as the proteinuria can vary from being physiological to mixed, like diluted serum, with important tubular components, and with the frequent presence of beta-2 micro.
In order to have an indication – even though not perfect – of the sensitivity, we evaluated the "Biological Detection Limit" (BDL) in "Spiked Samples" (SS).
To obtain the "SS" we utilised as "Blank Sample" (BS) the Dako standard reference serum – high value – diluted 1:40, with the aim of reproducing the case of a non-selective glomerular proteinuria. The BS was ultra-filtered by 30,000 MWCO membrane in order to eliminate any FLC present.

The BS was utilised for scaled dilutions on the kit Calibrators in evaluation and 2 BJP-positive samples - 1 BJP-kappa and 1 BJP-lambda - selected from those of the multicentre 2001 Forlì and Liguria Groups; this was to provide a minimum "agreed value" for their composition and concentration.
The results confirmed that which was expected.

- IN-FLC is not influenced by the protein composition of the sample (see cross-reaction paragraph), and likewise IFE with anti FLC antisera.
- On the other hand, unpredictably influenced by the protein composition of the sample are both EF (co-migration of BJP with Transferrin, other proteins of homogeneous band, haemoglobulin, polyclonal Ig) (Figure 2) and IFE with anti LC(b&f) antisera (co-migration of the BJP with monoclonal intact Ig, disguise for polyclonal Igs). (Figure 3)
  For these methods, the increase in sensitivity is clearly only counterproductive since it ends up having an effect also on the "interfering proteins".

Conclusions: for IN-FLC we found:

- Lower Detection Limit (LDL): FRK = 0.8 mg/l – FRL = 0.5 mg/l.
- Biological Detection Limit (BDL): FRK and FRL = 3 mg/l

As mentioned in relation to the calibration curves, it must be underlined that, where retained necessary, a notable increase in the "sensitivity" of IN-FLC is obtained utilising the so-called "High-Sensitivity" procedure. In addition, use of the concentrated sample could always be reverted to.

E) Imprecision - Repeatability

We used the positive Bence Jones samples which the Forlì and Liguria Commissions had selected as "provisional controls" and named: BJ-kappa Lavagna, BJ-lambda Bellaria.
9 repeats were carried out for 2 days on 6 dilutions of the afore-mentioned samples and the results are in Table 8.
Conclusion: The imprecision was very good and similar to that of the other nephelometric determinations of specific proteins.

F) Cross-Reaction with Free Light Chains of the opposite type

We ran the kappa Calibrator with the lambda Reagent and vice versa, and the result was below the Sensitivity Limit of the method.
Lack of this type of cross-reaction is confirmed by the results obtained on the samples with the exclusive presence of BJ-kappa with the lambda reagent and vice versa.

G) Antigen Excess

Application of the IN-FLC method on the BNII provides for the control of excess antigen by means of the so-called "pre-reaction"; in practice, the entity of the

reaction is first tested with a small quantity of the sample. If the result does not exceed a determined limit, the analyser proceeds with the determination, adding the missing part of the sample. If, on the other hand, the result exceeds the limit, the analyser indicates so by an alarm and tests again with a stronger dilution.
To evaluate in practice the efficacy of such control we concentrated the K-B and L-G Multicentre Samples in order to have a theoretically very high value of BJP in the concentration. We thus ran the dosage obtaining a correct measurement for values above 38,000 mg/l for both samples – Table 9

H) Matrix Effect – Dynamic Blank Sample – IN-FLC Determination in the serum

Apart from the kit Calibrators, we utilised the SS above and 10 neat sera with MC: 5 kappa and 5 lambda.
The IN-FLC test was effected, substituting the Antiserum Reagent with the PBS.
The results obtained were all far below the BDL reported above.
The result on the neat sera would indicate assessing a possible application of the method to detect FLC in serum.

Evaluation of the samples and IN-FLC/IFE comparison

We carried out parallel IN-FLC and IFE with anti FLC antisera on all our laboratory urine samples requesting the detection of Bence Jones Protein in three different periods of three months, each between 1998 and 1999 for a total of 572 samples from 526 patients. Patient recurrence was: 31 patients present twice, 6 patient three times and 1 patient four times.
It must be emphasised that the vast majority of the requests for the detection of BJP in our ImmunoHematology Laboratory are "aimed at" and supported by precise clinical motives, diagnostic suspect or follow-up. In fact, in our Hospital a Centralised Laboratory works with a protidology section.

Positivity Marker

Standard IFE was interpreted by the usual reader and, in case of doubt, by a second person. The cases remaining dubious by both were classified thus, that is "dubious".
IN-FLC was considered positive if one of the two FLC had concentration > 5 mg/l.

Result Analyses and Verifications

The results of standard IFE and of IN-FLC were compared and analysed as a whole only subsequently, and consequently verification of dubious situations was carried out on few samples - 15 in all - of the most recent. EF on Protur HISI and IFE on Protur Plus with anti LC(b&f) and anti FLC antisera were used for this, and the samples were opportunely concentrated or diluted in such a way as to obtain as accurate a result as possible.

Monoclonal Component Serum and Standard Urine IFE

The overall results in terms of MC obtained with the serum IFE and standard urine IFE can be seen in Table 10.
The important elements are:

- Total frequency of BJP: 91 cases out of 572 urine IFE (16%)
- Frequency of BJ-K and BJ-L: 8% for both
- Frequency of BJP-positive urine without MC in the serum: 10 cases (0.9% of the total urine IFE)
- One case of BJ-K in urine with MC serum IgG-L
- Two cases of BJP in urine: double BJP-K + BJP-L and in both, in the serum, double IgM-K + IgM-L MC.

Comparison between Standard Urine IFE results and FLC ImmunoNephelometric results

In order to simplify the comparison layout between standard IFE and IN-FLC, the results have been grouped based on the result of the standard IFE and are shown under the following points. Table 11.

A) Standard IFE – BJP-kappa positive
42 samples resulted positive on IFE (7.3% of the total) and 2 of these resulted negative on the IN-FLC test.
On the unconcentrated sample on IFE, one of these two resulted negative and the other dubious.
Verifying of such discrepancy between standard IFE and IN-FLC, which is in fact the only check, was not possible.

B) Standard IFE – BJP-kappa suspect
There were 3 samples all positive on IN-FLC. It was possible to carry out IFE on Protur Plus as verification on 2 of these samples and both resulted BJP positive.
One of these samples was of a current patient with a case history of two recurrences:
   - September 1998 – IN-FLC FRK = 62 mg/l – standard IFE = BJP-kappa positive
   - May 1999 – IN-FLC FRK = 27 mg/l (suspect) – standard IFE = BJP-kappa dubious

C) Standard IFE – double BJP: kappa + lambda
Two samples, both positive on IN-FLC.
Both samples, from two different patients, had double IgM-K + IgM-L MC in the serum, which are also the only MC of this type on record.

D) Standard IFE – BJP-lambda positive
40 samples (7% of the total) all positive on IN-FLC.

E) Standard IFE – BJP-lambda suspect
4 samples, all positive on IN-FLC.
It was not possible to do further checks on any of these.

F) Standard IFE – polyclonal and ladder kappa and lambda.
51 samples (9% of the total) all positive on IN-FLC.

G) Standard IFE – negative
430 samples (75% of the total) resulted negative on standard IFE; out of these, 284 (66% of IFE negative) were also negative on IN-FLC, whilst 146 (34% of IFE negative) resulted positive.

Verification by IFE on Protur Plus of 12 of these samples was carried out, chosen amongst those with IN-FLC values more suspect and the result was that 3 were BJP-kappa positive and 4 positive for polyclonal-FLC.

In conclusion, including the suspects, standard IFE detected 91 (16%) BJP positive samples and of these 2 resulted negative on IN-FLC (2.2% of the IFE positive).
Furthermore, IN-FLC showed all the samples as BJP suspect by IFE, of which the two checkable cases were confirmed BJP positive.
Also, IN-FLC indicated 146 samples suspect of those negative on standard IFE and, of the 12 checkable ones by IFE on Protur, 3 resulted BJP positive, that is "false negatives" on standard IFE.

Analysis of the Recurrences

As stated, patient recurrences were: 31 patients present twice, 6 patients three times, 1 patient four times.
Table 12 shows the results of a selection of noteworthy cases.
Analysis of the recurrences is not within the objectives of this work – also because of data scarcity - but it would seem possible to assume that IN-FLC could also have a role here.
Here are some examples

a) Patient B.G.
- 2 samples 7 days apart
- Urine IFE: BJ-L in both
- IN-FLC: significant reduction (approx. 3 times less) of the FRL
- Conclusion: possible effect of the therapy.

b) Patient P.A.
- 2 samples 7 months apart
- Urine IFE: first sample negative, second BJ-K
- IN-FLC: FRK first sample: 13 mg/l (suspect); second: 38 mg/l
- Conclusion: IN-FLC already showed a positivity signal on the first sample whilst IFE was still negative.

In general, evaluation of the results of the few recurrences available highlights also the quantitative "sense" of IN-FLC and, at least in these cases, its greater sensitivity as an alarm bell compared to IFE.

Conclusions

IN-FLC has demonstrated that it can be well placed as a first level test in the routine protocol for the detection of Bence Jones Proteins and could take on a role in uniformity of the quality of response in that area.

IFE will always have to be used as a test to indicate the mono- or polyclonality of the FLC shown up in IN-FLC, but the latter should, as it were, "pilot" the quality and sensitivity of IFE.
In other words, if a suspect sample on IN-FLC results "negative" on standard IFE, IFE with greater sensitivity should be reverted to, for example concentrating the sample further.

Bibliography

1. Pallotti G. et altri
Commissione InterRegionale "Forlì" su Proteine di Bence Jones e Catene Leggere Libere
Valutazione Multicentrica di Metodi e protocolli per le Catene Leggere Libere e Proteine di Bence Jones in urine - Primi Risultati.
Biochim Clin 1995;19: 410-425
2. Graziani MS, Merlini GP, Petrini C.
Linee guida per la ricerca della proteina di Bence Jones
Gruppo di Studio Proteine – Società Italiana di Biochimica Clinica
Biochim Clin 2001; 25:23-32
3. Graziani MS, Merlini GP
Measurement of Free Light Chains in Urine – To the Editor
Clin Chem 2001; 47:2069

Tables

Table 1	Multicentre Samples				
Sample Identification	BJP-K				
	K-Lavagna	K-A	K-B	K-C	K-E
Assigned Concentration (*) mg/l	***2000***	***825***	***2420***	***2200***	***880***
Sample Identification	BJP-L				
	L-Bellaria	L-F	L-G	L-I	
Assigned Concentration (*) mg/l	***900***	***770***	***5280***	***1760***	

(*) Average of Concentrations obtained by the Multicentres

Table 2 – Calibration Curves and Reproducibility – FR-kappa BNII

Comparison of Calibrations FR-kappa BNII		
Method	Standard	
Cal. mg/l	Average raw data	CV%
5	57	24.2%
10	235	13.3%
20	764	7.6%
40	1945	12.2%
80	4056	10.9%

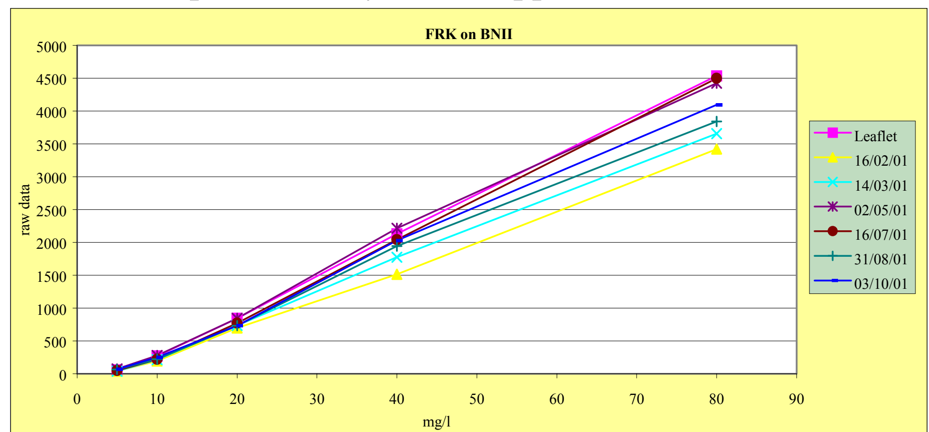


Table 3 – Calibration Curves and Reproducibility – FR-lambda BNII

Comparison of Calibrations FR-lambda BNII		
Method	Standard	
Cal. mg/l	Average raw data	CV%
5	166	21.8%
10	607	14.5%
20	1753	13.1%
40	3984	9.3%
80	6807	8.6%

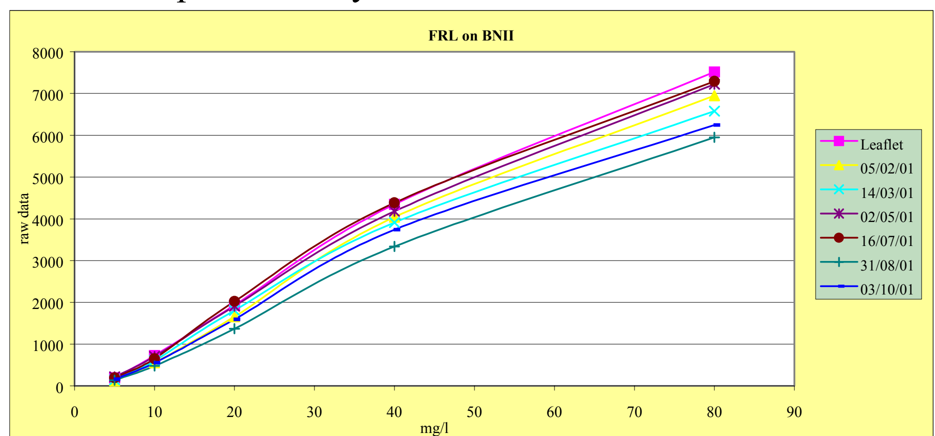
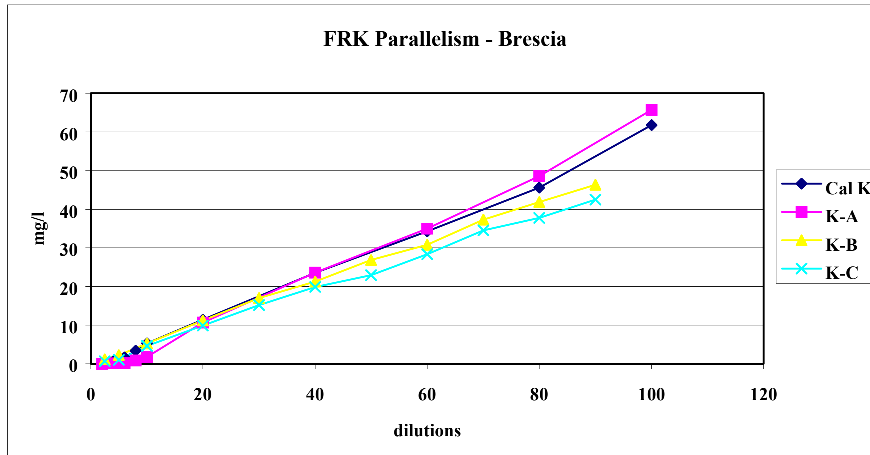


Table 4 Linearity and Parallelism

Correlation

Cal K	0,999
K-A	0,997
K-B	0,999
K-C	0,999
Cal L	0,999
l-G	0,995
l-F	0,998
l-I	0,996



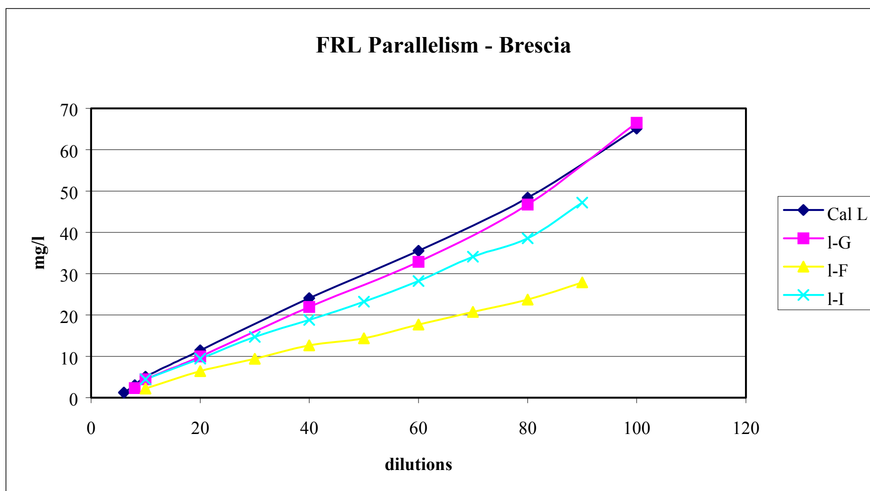


Table 5 Evaluation of Cross-Reaction between FLC reagent and intact Ig in standard sera

		Light B&F		Free Light Chains								Ratios		
				Free Kappa				Free Lambda						
Standard reference Serum Strategic Biosolution (ex ATAB) code CA4 Lot: A003-3	Dil. 1:	Ig-K	Ig-L	Test 1	Test 2	Average	CV	Test 1	Test 2	Media	CV	Average Free-K / Ig-K	Average Free-L / Ig-L	B&F sum /Free sum
		mg/l	mg/l	mg/l	mg/l	mg/l	%	mg/l	mg/l	mg/l	%			
	5	553	249	0,59	0,38	**0,49**	30,62	1,62	1,41	**1,52**	9,80	*0,09%*	*0,61%*	0,25%
	10	280	135	0,19	0,14	**0,17**	21,43	1,50	1,79	**1,65**	12,47	*0,06%*	*1,22%*	0,44%
	20	138	69	0,33	0,16	**0,25**	49,06	0,61	0,59	**0,60**	2,36	*0,18%*	*0,88%*	0,41%
	40	72	37	0,57	0,47	**0,52**	13,60	0,16	0,24	**0,20**	28,28	*0,72%*	*0,55%*	0,66%

The ratio between the FLC concentration and that of the respective Light Chain bound to the Ig is < 1% except for the Lambda-FLC of the 1:10 dilution, which results do not agree with the sequence of dilutions.

All the values are below 2 mg/l (Sensitivity Limit = 3 mg/l)

Table 6	Cross-Reaction Evaluation between FLC reagent and intact Ig in standard serum												
Standard reference Serum Dako High Values code X0940 Lot: 117(901)	Dil. 1:	IgG (BNII)		Free Light Chains									Ratio
				Free Kappa				Free Lambda					IgG Average /Free sum
		Test 1	Test 2	Test 1	Test 2	Average	CV	Test 1	Test 2	Average	CV		
		mg/l	mg/l	mg/l	mg/l	mg/l	%	mg/l	mg/l	mg/l	%		
	20	**1040**	**1010**	0,45	<0,0	**0,45**	n.c.	3,01	2,45	**2,73**	14,50		**0.2%**

The ratio between the FLC concentration and that of the IgG is < 1%.

Table 7 — Cross-Reaction Evaluation between FLC reagent and intact Ig in sera with MC

Samples	IFE MC serum	IFE Urine	BNII-Ig Serum Classe	BNII-Ig Serum mg/dl	Dil. 1:	IN-FLC Urine F-K mg/l	IN-FLC Urine F-L mg/l	IN-FLC Siero F-K mg/l	IN-FLC Siero F-L mg/l	F-K+F-L /Ig-tot
1	**G-K**	**BJ-K**	Ig-G	3230	1	10	0	65	51	0,4%
			Ig-A	<22,5	2	3	0	24	22	0,3%
			Ig-M	24	5	0	0	8	8	0,2%
			Totale	**3254**	10	0	0	3	1	0,1%
2	**G-K+ A-K?**	**no BJ**	Ig-G	899	1	62	20	55	40	0,7%
			Ig-A	503	2	29	9	16	13	0,4%
			Ig-M	36	5	12	2	5	6	0,4%
			Totale	**1438**	10	4	0	1	0	0,1%
3	**A-K**	n.e.	Ig-G	1180	1	0	0	24	21	0,3%
			Ig-A	190	2	0	0	10	12	0,3%
			Ig-M	<18	5	0	0	3	6	0,3%
			Total	**1370**	10	0	0	2	1	0,2%
4	**A-K**	**BJ-K**	Ig-G	298	1	66	0	199	26	1,3%
			Ig-A	1430	2	32	0	99	12	1,3%
			Ig-M	<18	5	13	0	44	2	1,3%
			Total	**1728**	10	6	0	15	0	0,9%
5	**M-K**	n.r.	Ig-G	1290	1	0	0	49	72	0,4%
			Ig-A	830	2	0	0	24	21	0,3%
			Ig-M	918	5	0	0	8	9	0,3%
			Total	**3038**	10	0	0	2	5	0,2%

Samples	IFE MC Serum	IFE Urine	BNII-Ig Serum Class	BNII-Ig Serum mg/dl	Dil. 1:	IN-CLL Urine F-K mg/l	IN-CLL Urine F-L mg/l	IN-CLL Siero F-K mg/l	IN-CLL Siero F-L mg/l	F-K+F-L /Ig-tot
6	**Free L**	**BJ-L**	Ig-G	641	1	5	104	71	233	4,4%
			Ig-A	27	2	1	55	43	114	4,5%
			Ig-M	22	5	0	21	15	45	4,3%
			Total	**690**	10	1	11	8	18	3,7%
7	**A-L**	**BJ-L**	Ig-G	1170	1	27	12	60	68	0,6%
			Ig-A	852	2	11	5	42	44	0,8%
			Ig-M	38	5	3	1	11	13	0,6%
			Total	**2060**	10	1	0	5	5	0,5%
8	**G-L**	n.e.	Ig-G	899	1	0	0	15	13	0,3%
			Ig-A	110	2	0	0	7	8	0,3%
			Ig-M	41	5	0	0	2	5	0,3%
			Total	**1050**	10	0	0	0	1	0,1%
9	**G-L**	n.e.	Ig-G	1990	1	1	0	37	38	0,2%
			Ig-A	1610	2	0	0	12	14	0,1%
			Ig-M	32	5	0	0	3	5	0,1%
			Total	**3632**	10	1	0	0	1	0,0%
10	**G-L**	**no BJ**	Ig-G	1710	1	3	0	37	35	0,4%
			Ig-A	67	2	2	0	13	16	0,3%
			Ig-M	58	5	0	0	3	5	0,2%
			Total	**1835**	10	0	0	0	2	0,1%

= not run

In the serum, the ratio between the sum of the FLC concentrations measured in the various dilutions and that of the Ig measured on the whole sample and related to the dilutions is < 1% for all the samples except no. 4 (IgA-K MC serum, BJ-K urine) and no. 6 (Free L MC serum, BJ-L urine).

In the serum diluted 1:10, the FLC concentration measured is < 2 mg/l (Minimum Sensitivity Limit = 3 mgl) for all samples except nos. 4 and 6.

Table 8	Imprecision - Repeatability 9 repeats for 2 days					
Sample	BJ-kappa - Lavagna					
Dilution %	100%	80%	60%	50%	30%	10%
Average mg/l	5,56	4,46	3,43	2,84	1,78	0,5
CV	3,04%	1,92%	1,35%	1,87%	0,93%	4,29%
Sample	BJ-lambda - Bellaria					
Dilution %	100%	80%	60%	50%	30%	10%
Average mg/l	7,98	5,03	4,03	3,23	2,21	0,77
CV	3,19%	1,68%	1,74%	1,44%	1,79%	1,65%

Table 9	Antigen Excess	
Multicentre Samples		
BJP-K - B Sample		
Assigned Concentration (*) mg/l	Concentrated approx.	Concentration obtained mg/l
2420	18 times	**39400**
BJP-L - G Sample		
Assigned Concentration (*) mg/l	Concentrated approx.	Concentration obtained mg/l
5280	10 times	**50400**
(*) Average of the concentrations obtained by the Multicentres.		

Table 10		Serum IFE - Monoclonal Components (MC)																																	
Urine IFE concentrated 25 times		Total run	A-K	A-K+M-K	A-L	A-L+G pesanti	A-L+G-K	A-L+M-L sosp	D-K+G-K	D-L + Free L	G-K	G-K+A-K	G-K+G-L	G-K+G-L+M-K	G-K + Free K	G-K+K poli	G-K+M-K	G-L	G-L+A-K	G-L+G-K sosp	G-L+M-K	G-L+M-L	Free K	Free L	M-K	M-K+G-K min	M-K+M-L	M-L	no cm	Ig-Kappa	Ig-Lambda	Double: Ig-K+Ig-L	Free Kappa	Free Lambda	Ig-K&L + Free K&L
Total Urine IFE	572	446	17	1	16	1	1	1	1	1	88	4	4	1	1	1	3	80	1	1	2	1	4	6	40	1	2	13	154	157	113	12	4	6	2
%	100	78	4	0	4	0	0	0	0	0	20	1	1	0	0	0	1	18	0	0	0	0	1	1	9	0	0	3	35	35	25	3	1	1	0
Negative	430	338	11	0	9	0	0	0	0	0	71	3	3	1	0	1	1	64	0	1	2	1	0	0	29	1	0	8	132	117	82	7	0	0	0
%	75	76	65	0	56	0	0	0	0	0	81	75	75	100	0	100	33	80	0	100	100	100	0	0	73	100	0	62	86	75	73	58	0	0	0
BJ-K	45	39	3	0	0	0	0	0	1	0	13	0	1	0	1	0	2	1	1	0	0	0	4	0	10	0	0	0	2	30	1	2	4	0	1
%	8	9	7	0	0	0	0	0	2	0	29	0	2	0	2	0	4	2	2	0	0	0	9	0	22	0	0	0	4	19	1	17	100	0	50
BJ-L	44	36	0	0	7	1	1	1	0	1	0	0	0	0	0	0	0	11	0	0	0	0	0	6	0	0	0	5	3	0	26	1	0	6	1
%	8	8	0	0	16	2	2	2	0	2	0	0	0	0	0	0	0	25	0	0	0	0	0	14	0	0	0	11	7	0	23	8	0	100	50
BJ-K+BJ-L	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	0	0	0
%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	17	0	0	0
Total BJP	91	77	3	0	7	1	1	1	1	1	13	0	1	0	1	0	2	12	1	0	0	0	4	6	10	0	2	5	5	30	27	5	4	6	2
%	16	17	3	0	8	1	1	1	1	1	14	0	1	0	1	0	2	13	1	0	0	0	4	7	11	0	2	5	5	19	24	42	100	100	100
Ladder & poly	51	31	3	1	0	0	0	0	0	0	4	1	0	0	0	0	0	4	0	0	0	0	0	0	1	0	0	0	17	10	4	0	0	0	0
%	9	7	6	2	0	0	0	0	0	0	8	2	0	0	0	0	0	8	0	0	0	0	0	0	2	0	0	0	33	6	4	0	0	0	0

Table 11

Samples 572 — Comparison results - IFE and IN-FLC

Standard Urine IFE concentrated 25 times			**Nephelemotry Free Light Chains**			**Hydragel Urine IFE** unconcentrated				**Urine IFE** Protur Verification			
Type	No.	% of totale	type resulted	no. of samples	% of group	run	BJ-	BJ+	BJ-?	run	BJ-	BJ+	BJ-?
BJ-K	42	7,34%	K<,L<	**2**	4,8%	19	1			0			
			suspect	40	95%		1	15	2				
BJ-K suspect	3	0,5%	K<,L<	0	0%	3				2			
			suspect	3	100%		2		1			**2**	
BJ-K+BJ-L	2	0,3%	K<,L<	0	0%	1				0			
			suspect	2	100%			1					
BJ-L	40	7,0%	K<,L<	0	0%	24				0			
			suspect	40	100%			23	1				
BJ-L suspect	4	0,7%	K<,L<	0	0%	2				0			
			suspect	4	100%		1		1				
poly and ladder κ & λ	51	8,9%	K<,L<	0	0%	23				1			
			suspect	51	100%		5	0	18				1
negative	430	75%	L<,K<	284	66%	236							
			suspect	146	34%		235		1	12	5	**3**	4

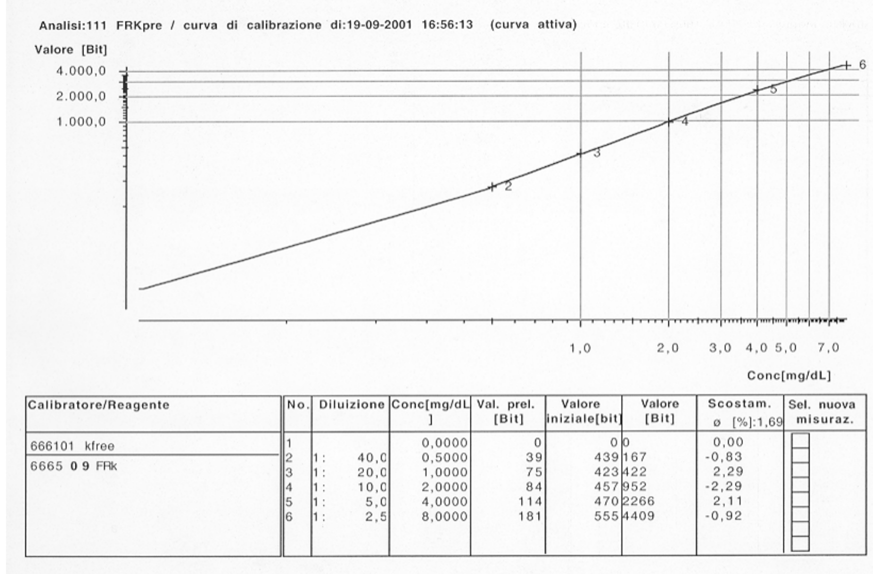
Type	No.	% of totale	type resulted	no. of samples	% of group	run	BJ-	BJ+	BJ-?
Total BJP & BJP suspect	91	16%	K<,L<	**2**	2,2%	49	1		
			suspect	89	97,8%		4	39	5

Table 12 — Evaluation of Recurrences

Ident.	Date	FRK	FRL	F-K/F-L	u-IFE	s-IFE	Notes
B.G.	11/05/99	18	4960	0,0037	BJ-L	micro L	Interval: 7 days s-IFE and u-IFE: equal
	18/05/99	6	1650	0,0035	BJ-L	micro L	IN-FLC: significant dosage variation Ratio F-K / F-L: unchanged
C.R.	02/04/98	<5	11	n.c.	BJ-L	**no cm**	Interval: 1 year s-IFE: varied - u-IFE: unchanged
	13/05/99	7	365	0,018	BJ-L	**micro L**	IN-FLC: significant dosage variation Ratio F-K / F-L: unchanged
C.G.	24/09/98	<5	7	n.c.	BJ-L	A-L	Interval: 5 days and 7 days
	29/09/98	17	43	0,404	BJ-L	A-L	s-IFE and u-IFE: unchanged
	06/10/98	19	58	0,324	BJ-L	A-L	IN-FLC: dosage varies
D.A.	04/09/98	52	17	3,041	BJ-K	G-K+G-L	Interval: 14 days
	18/09/98	**10**	<2.5	n.c.	**neg.**	G-K+G-L	u-IFE and IN-FLC: varies
M.B.	21/03/98	78	48	1,603	BJ-K	M-K	Interval: 3 days
	24/03/98	**17**	8	2,024	**neg.**	M-K	u-IFE and IN-FLC: varies
M.GF.	28/09/98	62	<2.5	n.c.	BJ-K	M-K	Interval: 8 days
	27/05/99	**27**	<5	n.c.	**BJ-K suspect**	M-K	u-IFE and IN-FLC: varies
P.A.	12/05/98	**13**	<2.5	n.c.	**neg.**	M-K	Interval: 7 months
	14/12/98	38	<2.5	n.c.	BJ-K	M-K	u-IFE and IN-FLC: varies
T.D.	12/10/98	<5	5	n.c.	BJ-L	no mc	Interval: 3 days
	15/10/98	<5	**<2.5**	n.c.	**neg.**	no mc	u-IFE and IN-FLC: varies
Z.T.	20/10/98	9	4	2,261	BJ-L	A-L	Interval: 3 days
	23/10/98	12	**5**	2,192	**neg.**	A-L	u-IFE and IN-FLC: varies

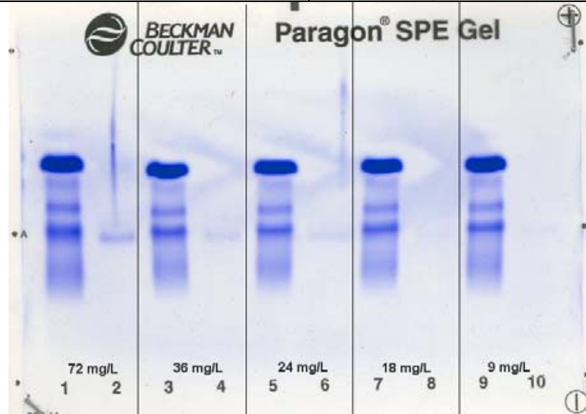
Figures

Figure 1 | **IN-FLC** – Calibration example



Calibratore/Reagente	No.	Diluizione	Conc[mg/dL]	Val. prel. [Bit]	Valore iniziale[bit]	Valore [Bit]	Scostam. ø [%]:1,69	Sel. nuova misuraz.
666101 kfree	1		0,0000	0	0	0	0,00	
6665 0 9 FRk	2	1: 40,0	0,5000	39	439	167	-0,83	
	3	1: 20,0	1,0000	75	423	422	2,29	
	4	1: 10,0	2,0000	84	457	952	-2,29	
	5	1: 5,0	4,0000	114	470	2266	2,11	
	6	1: 2,5	8,0000	181	555	4409	-0,92	

Figure 2 | **EF** – Analytical Sensitivity and Sample Dependence

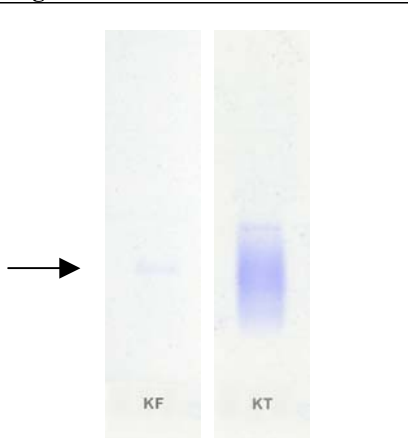


Right:
- sample BJP positive
- dilutions in PBS
- suspicious band up to 9 mg/l

Left:
- spiked sample
- same dilutions in a urine sample with Mixed Proteinuria
- suspicious only at 72 mg/l

Note: For these methods, the increase in sensitivity is only counterproductive since it ends up having an effect also on the "interfering proteins".

Figure 3 | **IFE** – Analytical Sensitivity and Sample Dependence



Comparison between:
- As. LC(B&F) – right
- As. FLC – left

BJP about 10 mg/l – co-migrant with Polyclonal Ig

Note
For these methods, the increase in sensitivity is clearly only counterproductive since it ends up having an effect also on the "interfering proteins".