Multicentre Evaluation of Routine Commercial Methods for the Detection of Bence Jones Proteins in Urine

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Inter-Regional Study Commission ***"Forlì"*** *on "Bence Jones Proteins and Free Light Chains"*

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Introduction

At present, research into Free Light Chains (FLC) is for the detection of Bence Jones Proteins (BJP – Monoclonal Free Light Chains in urine), while Polyclonal Free Light Chains (PFLC) are considered to be of secondary importance unless one specifically intends carrying out determination, as a tubular proteinuria index. In such case, the choice method would appear to be the direct quantitative estimation with ImmunoPrecipitation in liquid phase (IPL), Turbidimetry and Nephelometry achieved with specific reagents for FLC.
An adequate standardization of methods, protocol, references, controls and reporting – all justly felt absolutely essential by the operators – does not currently appear to match the importance of the BJP (and FLC) research.

Presentation of the Study Groups

The need for overall standardization for the detection of BJP is strongly felt by the operators in the sector as is the opportunity to compare concrete experiences.
The New Scientific Company (NSC) has collated these needs, taking care of the organisational part of the study group which was formed at the time of the meeting in Forlì on 28th May, 1993.
Since then the "Forli" Commission has held nine meetings, the last being on 18th June, 1999.
In 1997 the Liguria Sections of SIBioC - AIPAC – SIMEL decided to jointly undertake an analogy initiative co-ordinated by the NSC.
Since then the "Liguria" Commission has had three meetings, the last held on 1st July,1999.

Method of Work

The study groups hold periodic "co-ordination meetings", round table and open debate. At the meetings the results of work previously carried out are examined and discussed and the work to be completed before the following meeting is defined.

Objectives and General Strategy

The objective of the study groups is to verify the possibility of making uniform the evaluation, the interpretation and the reporting of Bence Jones Proteins (BJP) and, more generally, for Free Light Chains (FLC) in urine.

It is hoped that a specimen at least results

"BJP positive" or "BJP negative"

in all the laboratories

regardless of the method or the protocol, that is the screening and second stage methods, utilised.

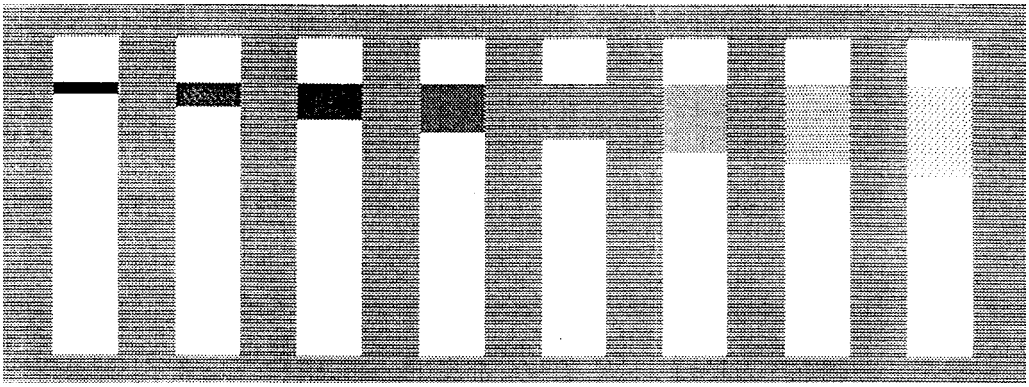
The strategy adopted is that of comparing operating experiences of the daily routine in the many laboratories, dimensionally, structurally and organisationally diverse, starting with the verification of the *"real characteristics"* of the methods practised.
To that end it is necessary to evaluate the "sensitivity" and the "precision" of the individual methods in order then to place them in the strategy of BJP detection.
Evaluation of the "precision" is not only preliminary to the possibility of an eventual quantitative determination but is also indispensable for the correct evaluation of the "sensitivity".
As an initial verification we have taken the basic but clear model made up of dilutions of samples with high BJP and only traces of other proteins. This allows us to evaluate and compare "sensitivity and precision" of methods in revealing the **"abnormality of the sample"** setting aside any consideration of the absolute concentration of the BJPs.
This approach seemed indispensable, as a starting point, despite not being complete due to the heterogeneity of the BJPs and the FLCs:

- The BJPs themselves are antigenically different, even amongst the same type, and this fact can give varying results in the ImmunoPrecipitation technique in Liquid phase (IPL) (Nephelometry, Turbidimetry) and in ImmunoFixation (IFE) while it does not affect Electrophoresis (EF).
- The Polyclonal Free Light Chain (PFLC) will be highlighted with less sensitivity by EF and IFE compared to an equal quantity of BJP since, due to the distribution on a wider surface compared to the narrow band typical of BJP, PFLC will have a lower concentration per surface unit. Figure 1(1)

Figure 1 From Monoclonal to Polyclonal – Simulation by Computer

Effect of the same quantity of colour (stain) distributed on a narrow surface comparable to the homogeneous BJP band and on a surface eight times larger like polyclonal FLC.

This demonstrates that, for the same concentration of protein, **the sensitivity of EF and of IFE decreases** proportionately to the increase in the surface on which the proteins are distributed; in the case of FLC, from the monoclonal band of BJP, to the diffused aspect of the polyclonal FLC.



Details of the Items on the Agenda

During a wider discussion, the commissions retained the evaluation of the methods to be preliminary, which details are as follows:

Analytical Standardisation

The objective of the standardisation should be the comparability of results and report:
- in the patient and among patients
- for the first recurrence and for subsequent checks
- at the same laboratory and among laboratories

To achieve this objective it is necessary to make the following points uniform:

Quality of the Methods

This is the work principally carried out by the Commissions until now and it is the first fundamental step for the standardisation of the testing. However, it still remains necessary to define other points mentioned below.

The uniform evaluation of the methods utilised in the research of Bence Jones Proteins (and of Light Free Chains in general) should be reached and maintained, and to this end:
- insist on the method of periodical multicentre checks
- define a control set to use constantly
- insist on the method of periodical meetings for common verification and discussion of results

Limits of Sensitivity in the Methods or Limits Utilised in the Operating Strategy

- Screening limit
- Confirmation limit
- To be made explicit in the report?

Type of Sample

- **Urine** to be used: 24 hours – first of the morning – second of the morning – random
- **Preservative** to be used to take and/or store the sample
- To be made explicit in the report?

Diuresis - Creatinine Urine

- *Diuresis – Creatinine Urine - nothing* to be evaluated
- To be made explicit in the report?

Reporting Strategy – provisional and final, and relative communication

Need for -
- provisional report
- final report

and for every one –
- information on the sample
- unit of measurement, also in relation to the sample and the eventual evaluation of diuresis or creatinine urine
- information on the absolute limits and relative to the preceding points
- positive and negative type of communication
- descriptive only, quantitative only, both
- images
- notes, conclusions, suggestions for checks and other

Standardisation of Rating

At the last meeting it emerged that there is a notable variation.

Other Operating Points

- Specific and separate report for positive samples
- Invitation to produce previous reports
- Conservation of the information

Proposing of Documents

- Informative document for requesting Doctor
- Informative document for the Institute
- Proposing document for the category

Multicentre Epidemiological Survey

The survey would have various complementary values:
- Overall analytical and pre-analytical characteristics of BJP detection
- Characteristics of the people of the region
- Characteristics of the people afferent to the laboratory

Desired Characteristics for Methods and Protocol

For the BJPs and FLC the **"perfectly effective"** method or protocol should be characterised by:

a) no "false negative "– Sensitivity and Precision
This is the principle and irremissible characteristic and consequently maximum intra- and inter-laboratory reproducibility is required. The "sensitivity" and "precision" of each method should be defined.

b) no "false positive" – Specificity of the Positivity Marker
The effectiveness gradually diminishes as the number of "false positives" increases.

c) good quantitative information
One should evaluate beyond the "precision" to the "accuracy".
The value of this depends upon how much the need for absolute quantitative determination is felt.

Matters Discussed at the Last Meetings

The matters discussed at the last Meetings at Forlì and Genoa and mentioned in this presentation are:
- assessment of the results obtained on the multicentre, multimethodological evaluation of dilutions of four samples of urine; two with lambda BJP and two with kappa BJP
- definition of a control set
- choice of sample type on which to carry out BJP testing
- choice of evaluation parameter for diuresis

Objectives of Multicentre Experimentation

This experimentation intends to verify the effectiveness of methods in use in the participating laboratories, in terms of "sensitivity" and "precision".
The evaluation of the "specificity" and the "accuracy" will be the object of future experimentation.

Samples

The samples were obtained by diluting the original pre-selected urine in order to have the approximate concentrations of BJPs agreed upon by the participants.
The New Scientific Company attended to the preparation of samples and distribution to the participants.
The dilutions were carried out in PBS and distributed in 5 ml phials clearly labelled with the conventional name of the samples, the type of BJP present and the dilution.
Forms for noting the results were distributed together with the identified samples.
The table shown under (Table 1) bears the samples and the dilutions examined by both work groups.

Table 1 – Samples

Sample	**λ A Bellaria**		**λ B Lavagna**		**κ A Forlì**		**κ B Lavagna**	
	λ A 1	λ A 2	λ B 1	λ B 2	κ A 1	κ A 2	κ B 1	κ B 2
Dilution	1:100	1:200	1:100	1:200	1:30	1:60	1:200	1:400
Indicative concentration mg/dl	1.1	0.6	2.5	1.25	3	1.5	2.5	1.25

Nota bene:
The indicative concentration is shown for clarity. It was obtained afterwards and is the average of the assays carried out with the quantitative methods.

The Query

If the eight samples had arrived at the participating laboratories with a request for "Detection of Bence Jones Proteins", what would have been the reply?

Operating Procedure

All the dilutions were analysed by participating laboratories with both screening and second stage methods in routine use for the BJP assay.
Some laboratories carried out two determinations in two separate analytical sessions.

Stability of the Dilutions Distributed

The time that elapsed between the preparation of the dilutions distributed and the execution of the laboratory tests varied greatly.
The stability of the dilutions distributed was thus checked, determining the FLC by the immunoturbidimetric manual method with a specific anti-FLC reagent.
It was revealed that the 1:60 dilution of the k-Forlì sample showed rapid deterioration, then stabilising itself, whilst all the other prepacked dilutions showed no appreciable variation during the period of observation - June, 1998 to June, 1999.

Positive Result

The "sign of abnormality", typical of every method, was considered to be a "positive result".

Expected Result

All the samples should have resulted positive for the BJP type indicated on the identification label on the phial.

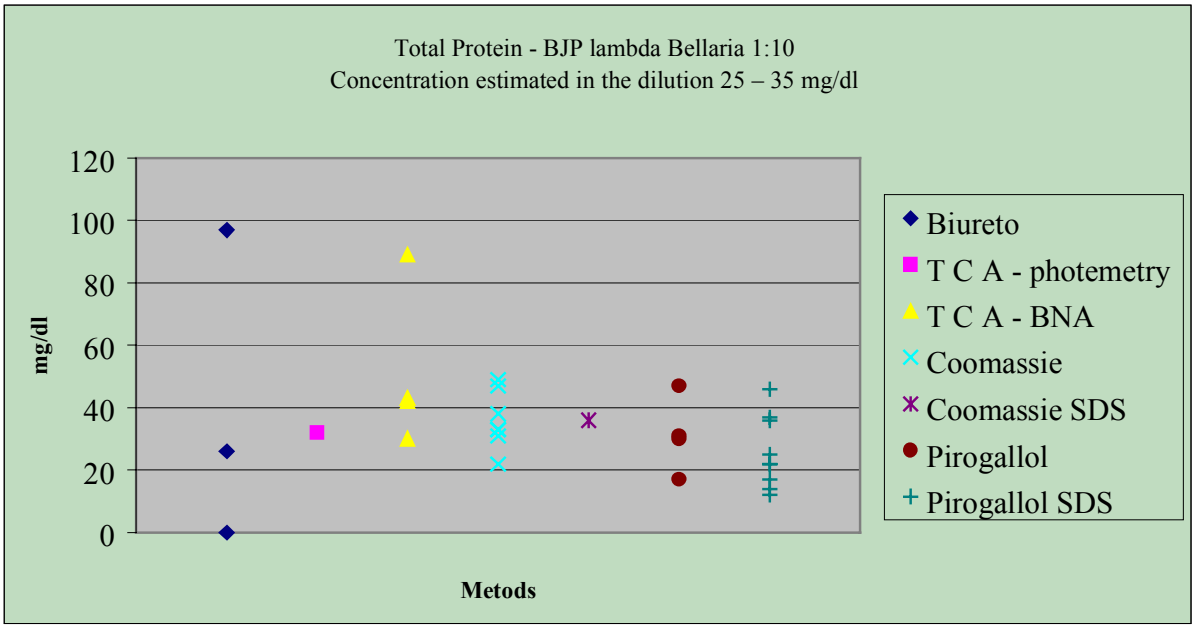
Results of Previous Work

In the previous work undertaken by Forlì Group (1), results of the multimethodological multicentre evaluation of dilutions of a urine sample with lambda BJP were presented.
Since that work and since subsequent multicentre evaluations conducted in recent years by both the "Forlì" and also the "Liguria" group, it has emerged that the following methods are not very reliable for BJP testing and therefore should not be utilised **for the time being:**

- the dosage of Total Proteins (TP) in urine
- the stick for the dosage of Total Proteins in urine

Leaving aside the stick, whose use for the screening of BJP is almost out-of-date, it is worth mentioning that the dosage of Total Proteins in urine is, on the other hand, more widely used.
By way of an example, the graph in Figure 2 indicates the results obtained from the determination of TP on a 1:10 dilution of the lambda BJP Bellaria sample. The concentration of lambda BJP in this dilution was estimated between 25 and 35 mg/dl. It can be seen how none of the methods used should be considered reliable. Still with regard to the determination of the Total Proteins in urine, a multicentre experimentation took place on dilutions of urine with glomerular proteinuria, and it showed that the methods utilised should be considered unreliable for concentrations inferior to 20 mg/dl.

Figure 2



Present Results and Discussion

The eight samples were examined in **23 hospital laboratories** plus in the Beckman Laboratory, and in total **769 determinations** were carried out.
The comparison of the results reached and grouped together under five types of techniques utilised is illustrated in the graph in Figure 3.

Electrophoresis (EF)

The results are shown on the graph in Figure 4 and more analytically in Table 2. **8 Laboratories** carried out EF in a total of **96 tests** with **17 negative results (17.7%).**
The sensitivity of Electrophoresis with the kits and methods for "unconcentrated urine" on unconcentrated sample resulted potentially discreet but affected by excessive variability intra- and inter-laboratory even with the same method.

ImmunoFixation (IFE) with Antisera anti-Total Light Chains (bound & free) (TLC)

The results are shown on the graph in Figure 5 and more analytically in Table 3.
The methods utilised are fairly reliable. **12 Laboratories** carried out **127 tests** with **3 negative results** equal to **2.4%.**

ImmunoFixation (IFE) with Antisera anti-Free Light Chains (FLC)

The results are shown on the graph in Figure 6 and more analytically in Table 4.
The technique was followed by **12 Laboratories,** one having followed two methods, for a total of **146 tests** with **59 negative results, equal to 40.4%.**
The sensitivity of IFE with specific anti-FLC antisera was potentially good but that also showed, as for EF, excessive inter-laboratory variability, due, in part, to the variability previously found for electrophoresis, partially attributed to the diverse quality of the antisera utilised. The NSC antisera showed the correct result on three different gels.

Nephelometry/Turbidimetry with anti-Total Light Chain (bound & free) (TLC) Reagents

The results are shown on the graph in Figure 7 and more analytically in Table 5.
The technique was followed by **6 Laboratories** for a total of **80 Tests** with **14 negative results,** equal to 17.5%.
Turbidimetry had 2 negative results out of 8 tests carried out.
The other 12 negative results were obtained on the APS nephelometer and are all relative to the tests carried out on lambda BJP samples. This was due to the limit of sensitivity established by the producer for lambda TLC.
Both the sensitivity and the precision were good all round but the limit of sensitivity of the methodology must be better than 0.5 mg/dl.
Table 7 indicates the averages of the concentration obtained with the various methods and the relative CV.

Nephelometry/Turbidimetry with anti-Free Light Chain (FLC) Reagents

The results are shown on the graph in Figure 8 and more analytically in Table 6.
The technique was followed by **23 Laboratories plus** the **Beckman Laboratory** for a total of **320 Tests** with **1 negative result,** equal to 0.3%.
Since the only negative results was for a sample which, during the test repetition carried out in the same Laboratory gave a clear positive result, one can assume that it was an operating error.
Both the sensitivity and the precision were very good all round.
Table 8 shows the averages of the concentrations obtained with various methods and the relative CV.
With the Immage nephelometer the CVs appear to be higher compared to those of BNA/BNII. We can presume that the cause is in the inevitable inter-laboratory differences in the manual preparation of the dilutions for the calibration curve. In fact, Immage, contrary to BNA/BNII, does not automatically prepare the dilutions for carrying the calibration of the non-Beckman methodology.
This idea comes from, and is confirmed by, the fact that the intra-laboratory CVs were more than satisfactory on 24 pairs. The average of the CVs were 1.8% with only two results over 5%.
The inter-laboratory CVs for the APS nephelometer were not calculated since only two Laboratories with three determinations on each sample showed the results in concentration, whilst the others showed only the rate. The APS does not give a calibration curve and therefore does not calculate the concentration value but the interpolation of the rate on the curve is calculated manually. In general, the Laboratory is not interested in the concentration value but only to distinguish between “positive” and “negative” based on the rate given by the reaction compared to a cut-off

Conclusions regarding the Multicentre Evaluation

Action to be Taken

With the co-operation of the producers, we should aim to achieve a better standardisation of the Electrophoresis and ImmunoFixation methodology.
The limit of sensitivity of the methodology, both qualitative and quantitative, should be better than 0.5 mg/dl.

Reference Samples

The multicentre evaluation has defined as "reference samples", at least in terms of "positive/negative", two dilutions of the kappa BJP Lavagna sample and two of the lambda BJP Bellaria sample. For both samples the concentration of BJP in the two dilutions was estimated in about 1 mg/dl and 0.5 mg/dl.
The "reference samples" mentioned above are available from the New Scientific Company.

Other Conclusions

Sample Type

The study groups agreed to utilise a random sample of urine for BJP detection.
The testing will be carried out preferably on a fresh sample.

Creatinine Urine

The participants agreed that it would be useful to carry out the determination of the creatinine urine concentration, together with the BJP testing. This information will initially be exclusively for internal use and the results will be discussed at the subsequent meetings.

Future Objectives

The future objectives of the study group will be:

- verification of the aforementioned "reference samples"
- to make more uniform the various aspects of the reporting

Bibliography

1) Pallotti G. et al.
Valutazione multicentrica di metodi e protocolli per le Catene Leggere Libere e Proteine di Bence Jones in urine – Primi risultati
Biochimica Clinica, 19, 1995, pp 410-425

Table 2

Electrophoresis							Samples							
Method	Sample Conc.	Dye type		Number Labor.	tests	Results Total	λ A		λ B		κ A		κ B	
							λ A 1	λ A 2	λ B 1	λ B 2	κ A 1	κ A 2	κ B 1	κ B 2
Protur HISI	N.C.	Violet		1	8	pos 5	1	0	1	1	1	0	1	0
						neg -3	0	1	0	0	0	1	0	1
Protur Plus	N.C.	Blue		1	8	pos 8	1	1	1	1	1	1	1	1
						neg 0	0	0	0	0	0	0	0	0
Paragon SPE	N.C.	Blue		1	8	pos 8	1	1	1	1	1	1	1	1
						neg 0	0	0	0	0	0	0	0	0
Hydragel 30	N.C.	Violet		3	48	pos 36	4	2	6	6	6	4	6	2
						neg -12	2	4	0	0	0	2	0	4
Hydragel 15	N.C.	Violet		1	8	pos 7	1	1	1	1	1	1	1	0
						neg -1	0	0	0	0	0	0	0	1
Acetato Cell.	N.C.	Gold Helena		1	16	pos 15	2	2	2	2	2	2	2	1
						neg -1	0	0	0	0	0	0	0	1
Total				8	96	pos 79	10	7	12	12	12	9	12	5
						neg -17	2	5	0	0	0	3	0	7

Table 3

ImmunoFixation - TLC Antisera							Samples							
Method	Conc. sample	Dye Type	Antisera Supplier	Number Labor.	tests	Result Total	λ A		λ B		κ A		κ B	
							λ A 1	λ A 2	λ B 1	λ B 2	κ A 1	κ A 2	κ B 1	κ B 2
Protur Plus	N.C.	kit	Beckman	7	80	pos 78	10	8	10	10	10	10	10	10
						neg -2	0	2	0	0	0	0	0	0
Hydragel 4IF	N.C.	kit	Sebia	5	47	pos 46	6	5	7	6	7	5	6	4
						neg -1	0	0	0	0	0	0	0	1
Totale				12	127	pos 124	16	13	17	16	17	15	16	14
						neg -3	0	2	0	0	0	0	0	1

Table 4

ImmunoFixation - FLC Antisera							Samples							
Method	Conc. sample	Dye Type	Antisera Supplier	Number Labor.	tests	Result Total	λ A		λ B		κ A		κ B	
							λ A 1	λ A 2	λ B 1	λ B 2	κ A 1	κ A 2	κ B 1	κ B 2
AutoIFE	N.C.	kit	Helena	1	8	pos 8	1	1	1	1	1	1	1	1
						neg 0	0	0	0	0	0	0	0	0
ImmunFix	N.C.	kit	Helena	1	16	pos 5	0	0	2	0	1	0	2	0
						neg -11	2	2	0	2	1	2	0	2
AutoIFE	N.C.	kit	NSC	1	8	pos 8	1	1	1	1	1	1	1	1
						neg 0	0	0	0	0	0	0	0	0
Protur Plus	N.C.	kit	NSC	1	16	pos 16	2	2	2	2	2	2	2	2
						neg 0	0	0	0	0	0	0	0	0
Hydragel 4IF	N.C.	kit	NSC	1	16	pos 16	2	2	2	2	2	2	2	2
						neg 0	0	0	0	0	0	0	0	0
Hydragel 2IF	C x 10	kit	Sebia	1	8	pos 8	1	1	1	1	1	1	1	1
						neg 0	0	0	0	0	0	0	0	0
Hydragel 4IF	C x 25	kit	Sebia	1	16	pos 4	1	0	2	1	0	0	0	0
						neg -12	1	2	0	1	2	2	2	2
Hydragel 4IF	N.C.	kit	Sebia	6	58	pos 22	5	0	7	3	4	0	2	1
						neg -36	2	7	1	4	4	7	5	6
Total				12	146	pos 87	13	7	18	11	12	7	11	8
						neg -59	5	11	1	7	7	11	7	10

Nota Bene: A laboratory used two methods, so the total of laboratory doesn't correspond to the relative column sum.

Table 5

Nephelometry/Turbidimetry Total LC							Samples							
Analizer			Reagents supplier	Number Labor.	Number tests	Results Total	λ A		λ B		κ A		κ B	
							λ A 1	λ A 2	λ B 1	λ B 2	κ A 1	κ A 2	κ B 1	κ B 2
BNA/BNII			Behring	3	48	pos 48	6	6	6	6	6	6	6	6
						neg 0	0	0	0	0	0	0	0	0
APS			Bechman	2	24	pos 12	0	0	0	0	3	3	3	3
						neg -12	3	3	3	3	0	0	0	0
Cobas			Dako	1	8	pos 6	1	1	1	1	1	0	1	0
						neg -2	0	0	0	0	0	1	0	1
Total				6	80	pos 66	7	7	7	7	10	9	10	9
						neg -14	3	3	3	3	0	1	0	1

Table 6

Nephelometry/Turbidimetry Free LC							Samples							
Analizer			Reagents supplier	Number Labor.	Number test	Results Total	λ A		λ B		κ A		κ B	
							λ A 1	λ A 2	λ B 1	λ B 2	κ A 1	κ A 2	κ B 1	κ B 2
BNA/BNII			NSC	15	200	pos 199	25	24	25	25	25	25	25	25
						neg -1	0	1	0	0	0	0	0	0
Immage			NSC	4	56	pos 56	7	7	7	7	7	7	7	7
						neg 0	0	0	0	0	0	0	0	0
APS			NSC	5	56	pos 56	7	7	7	7	7	7	7	7
						neg 0	0	0	0	0	0	0	0	0
Cobas			NSC	1	8	pos 8	1	1	1	1	1	1	1	1
						neg 0	0	0	0	0	0	0	0	0
Total				24	320	pos 319	40	39	40	40	40	40	40	40
						neg -1	0	1	0	0	0	0	0	0

Nota Bene: A laboratory used two methods, so the total of laboratory doesn't correspond to the relative column sum.

Table 7

Nephelometry/Turbidimetry Total Light Chains - Interlaboratory Averages and CV

Analizer	Reagent Supplier	Number Lab.	Number Tests	λ A 1 Aver. mg/dl	λ A 1 CV	λ A 2 Aver. mg/dl	λ A 2 CV	λ B 1 Aver. mg/dl	λ B 1 CV	λ B 2 Aver. mg/dl	λ B 2 CV	κ A 1 Aver. mg/dl	κ A 1 CV	κ A 2 Aver. mg/dl	κ A 2 CV	κ B 1 Aver. mg/dl	κ B 1 CV	κ B 2 Aver. mg/dl	κ B 2 CV
BNA/BNII	Behring	3	+ 48 / - 0	1.68	12%	0.97	18%	2.83	10%	1.64	13%	4.29	6%	1.40	12%	3.00	10%	1.30	13%
APS	Beckman	2	+ 12 / - 12	neg	n.c.	neg	n.c.	neg	n.c.	neg	n.c.	4.36	18%	1.39	16%	3.42	2%	1.50	9.0%
Cobas	Dako	1	+ 6 / - 2	5.82	n.c.	1.54	n.c.	6.31	n.c.	3.73	n.c.	4.94	n.c.	neg	n.c.	4.94	n.c.	neg	n.c.

n.c. = indefined, non-calculable; + and - = positive and negative

Table 8

Nephelometry/Turbidimetry Free Light Chains - Interlaboratory Averages and CV

Analizer	Reagent Supplier	Number Lab.	Number Tests	λ A 1 Aver. mg/dl	λ A 1 CV	λ A 2 Aver. mg/dl	λ A 2 CV	λ B 1 Aver. mg/dl	λ B 1 CV	λ B 2 Aver. mg/dl	λ B 2 CV	κ A 1 Aver. mg/dl	κ A 1 CV	κ A 2 Aver. mg/dl	κ A 2 CV	κ B 1 Aver. mg/dl	κ B 1 CV	κ B 2 Aver. mg/dl	κ B 2 CV
BNA/BNII	NSC	15	+ 199 / - 1	1.04	10%	0.54	10%	2.26	8%	1.12	10%	2.94	18%	0.79	27%	2.29	13%	1.15	12%
Immage	NSC	4	+ 56 / - 0	1.19	20%	0.66	30%	2.14	15%	1.11	22%	2.64	23%	0.91	71%	2.81	23%	1.26	23%
APS	NSC	5	+ 56 / - 0	0.74	n.c.	0.34	n.c.	1.65	21%	0.75	9%	2.75	28%	0.45	n.c.	1.88	17%	0.85	9%
Cobas	NSC	1	+ 8 / - 0	0.97	n.c.	0.50	n.c.	2.20	n.c.	1.06	n.c.	3.21	n.c.	1.52	n.c.	2.87	n.c.	2.12	n.c.

n.c. = indefined, non-calculable; + and - = positive and negative