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Effect of sample dilution on recovery of serum free light chains with Freelite™

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In this study the Freelite™ application for quantification of free light chains in serum (FLC) was investigated. Despite the single reagent source, literature shows methodological differences. We aimed to compare behaviour of Freelite™ calibrators, controls and diluent reagents to that of pool serum and serum from patients with increased FLC, using different diluents. For this investigational purpose an analyzer with an open application and user defined methods was needed. The routine chemistry analyzer Architect® from Abbott Diagnostics, an open system with open applications, was used to study the effect of diluent specimen and dilution on Freelite™ recovery. Results revealed a significant increase of recoveries for both controls, calibrators and pooled serum when the recommended diluent from Beckman Coulter was used in serial dilution series, while using human serum as a diluent normal theoretical expected recoveries were obtained. Different behaviour of the assay with the recommended diluent from Beckman and with human pooled serum as diluent has been observed on both Architect® and Immage®. Differences can be explained by non-parallelism of the Beckman Coulter diluent in serial dilution experiments.

Keywords: free light chains; commutability; non-parallelism; recovery

It has been reported in several studies that the introduction of the Freelite™ immunoassay for measurement of kappa and lambda free light chains (KFLC and LFLC) offers an improvement in monitoring disease progression and response to treatment, particularly in those situations where the quantification of the M-protein by immunofixation electrophoresis is inadequate, e.g. in light chain myeloma, in case of very low M-protein levels, or in amyloidosis (1,2). The Freelite™ immunoassay is an improvement compared to conventional monitoring methods with lower sensitivity. However, despite the single reagent source, it has been described that the FLC assays for κ and λ in UK NEQAS show significant interlaboratory coefficient of variations of over 100% (3). Also, R.J. Pattenden et al. show significant methodological differences for free KFLC and LFLC on two different analyzers (4). These differences also amplify the deviation in kappa:lambda ratios between the methods, and bring along method depending reference intervals.

Because of the clinical consequences, traceability and transferability are important prerequisites to reach interlaboratory harmonization across different applications. Currently, no international standard is available and trueness verification is impossible. Referencing to manufacturer's in-house reference materials is the maximum level of traceability. Notwithstanding the JCTLM standardization efforts (www.bipm.org), laboratory professionals are still confronted with analyzer-specific reference intervals, which is unsatisfactory (5).

Analytical studies on FLC methodology are sparse. They are focused mainly on revealing variation coefficients, linearity, method comparison, determination of the reference intervals and description of preanalytical issues (6). The poor performance of the test when using different applications from the same manufacturer challenged us to study behaviour of controls, calibrators and diluent formulations in serial dilution experiments. For this purpose, a system with open applications was selected to be able to investigate the recommended parameter settings. In our case, the Architect® analyzer (Abbott Diagnostics) with open channels was used to investigate the recommended analytical parameter settings of the closed FLC application on Immage® (Beckman Coulter). It should be noted that Beckman Coulter Diluent 1 (BMD1) is recommended by the manufacturer for dilution of the samples. We aimed to evaluate the entire application on the Architect® and focused on behaviour of calibrators and controls as compared to native pooled serum and some sera of patients with monoclonal M-protein.

Materials and Methodology

Parameter settings of the KFLC and LFLC assays as recommended by the manufacturer were copied from the Immage® to the Architect® analyzer. All settings, like sample volumes, dilution factors, temperature, were identical, except the wavelength of detection and the order of pipetting. Detection wavelength at Immage® was 940 nm, while at Architect® the detection was performed at 804 nm. The order of pipetting on

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List of abbreviations: SHI: serum from healthy individuals, KFLC: kappa free light chains, LFLC: lambda free light chains, BCD1: Beckman Coulter Diluent 1

Immage® is: 1) 195 µL dilution buffer (BCD1), 2) 60 µL latex reagent, followed by incubation, 3) sample 5.0 (λ) or 15.0 (κ) µL, 4) 5.0 µL supplemental reagent (0.099% sodium azide as a preservative), followed by reaction. The order of pipetting on Architect® is: 1) 195 µL dilution buffer (BCD1) + 5.0 µL supplemental reagent (0.099% sodium azide), 2) sample 5.0 (λ) or 15.0 (κ) µL, followed by incubation, 3) 60 µL latex reagent, followed by reaction. On both systems a dedicated cuvet was selected and single reagent and calibrator lots were used to reduce analytical variation.

A series of six calibrators with assigned antigen concentrations for both KFLC and LFLC included in the Freelite™ test kit were assayed to set up calibration curves on both the Immage® and Architect®. On both analyzers concentrations of the calibrators were measured as such, i.e. no predilution was performed, which is in line with the manufacturers recommendations. Calibrators span a range of about 0.7-20.0 mg/L and 0.9-29.0 mg/L for KFLC and LFLC, respectively, which concurs with physiological KFLC (3.3-19.4 mg/L) and LFLC (5.7-26.3 mg/L) reference intervals provided by the manufacturer.

QC-materials were used to check the calibration curve at both systems. In contrast to calibrators, QC materials in Immage® are prediluted 1:10 with BCD1 diluent before measuring. According to the package insert intended for use on Immage®, set point values of the QC-samples are 17.9 mg/L and 30.4 mg/L for KFLC-low and KFLC-high. For LFLC-low and LFLC-high set point values were 28.8 mg/L and 59.2 mg/L, respectively. This means that by 1:10 predilution of the QC-samples, the absolute values of the prediluted QC-samples coincides with the low ends of the calibration curves, i.e. 1.8-3.0 mg/L for KFLC and 2.9-5.9 mg/L for LFLC, meaning that the part of the calibration curve 3.0-20.0 mg/L for KFLC and 5.9-29.0 mg/L for LFLC are not controlled.

The values of the QC-low of KFLC and LFLC samples (17.9 mg/L and 28.8 mg/L, respectively) are just within the calibration range and could in principle be measured without predilution for fitting on the calibration curve. Because of the closed application and automatic predilution on Immage®, it is impossible to measure undiluted specimens. On Architect® these QC-samples were measured both undiluted an with a 1:10 predilution to check whether results are exchangeable.

Independent serial dilutions of serum from a patient with increased monoclonal KFLC or LFLC (tested by gel electrophoresis), controls and calibrator 6 (the highest concentration of the calibration curve) with either BCD1 or with a serum pool from healthy individuals (SHI) were prepared to test recovery and parallelism. SHI was obtained from nine healthy individuals with KFLC and LFLC within the reference intervals.

Recoveries of the serial dilution series are presented in Figure 1A-C. KFLC and LFLC concentrations in SHI contain a physiological amount of KFLC (0.5 mg/L) and LFLC (1.7 mg/L) when measured in undiluted form with Architect®. We adjusted for the amount of FLCs in SHI before calculating the recovery.

Results

Table 1 shows results of the QC recovery measurements on Immage® and Architect®. Prediluted QCs (1:10 diluted with BCD1) correspond with the set point values provided by the manufacturer on both systems. However, direct (undiluted) measurements of QC-low for KFLC and LFLC on Architect® show decreased values as compared to the 1:10 prediluted QC samples. It has been clearly demonstrated that diluted samples provide an increased recovery compared to the undiluted samples on the Architect®.

Figures 1A-C show absolute and relative recoveries of KFLC and LFLC in independent serial dilutions of pathological patient sample (figure 1A), calibrators (figure 1B) and QC materials (figure 1C). All series were measured using the open application on Architect®. Dilution series were prepared with BCD1 (solid lines) or SHI (dashed lines). The result of the 100:00 ratio (sample: diluent = 100:00) is by definition 100% recovery. It is striking that the figures show the same trend in results for both KFLC and LFLC dilution series. Dilution with BCD1 (solid lines) yields increased recoveries compared to dilutions with SHI (dashed lines). Serum of the patient with known monoclonal KFLC and LFLC disease (figure 1A) was prediluted with SHI to enable measurements without further dilutions due to substrate excess. Further dilution of these patient samples with BCD1 (solid lines) yields extreme increases of recoveries up to 664% and 449% for KFLC and LFLC, respectively, while in corresponding dilution series with SHI (dashed lines) a much smaller increase up to 109% and 121% was obtained. In the series also polyclonal SHI was diluted with BCD1. It has been observed that this series also yield extremely increased recoveries of 861% for KFLC and 449% for LFLC (figure 1A). This indicates that the increase of the recoveries can not be attributed to monoclonality of the patient's KFLC of LFLC.

Table 1. Recovery (mg/L) of FLC in diluted and undiluted commercial QC materials on Architect® and Immage®. CVs (%) are presented between brackets. Undiluted samples (Architect® undiluted) yield decreased recoveries of controls compared to diluted samples (Architect® 1:10 diluted). QC-materials were analyzed during a period of 31 days.

Set point value according to manufacturer, 1:10 diluted	Kappa low QC 17.9 (10%)	Kappa high QC 30.4 (10%)	Lambda low QC 28.8 (10%)	Lambda high QC 59.2 (10%)
Immage® 1:10 diluted*	17.2 (1.8)	29.7 (2.3)	28.6 (3.5)	59.7 (1.1)
Architect® 1:10 diluted**	19.5 (3.9)	31.6 (2.4)	30.4 (2.1)	59.7 (0.9)
Architect® (undiluted)*	12.8 (0.8)	Outside Calibr. Interval	17.9 (1.0)	Outside Calibr. interval

* Within-run precision, N =3. ** Between-run precision, N = 11.

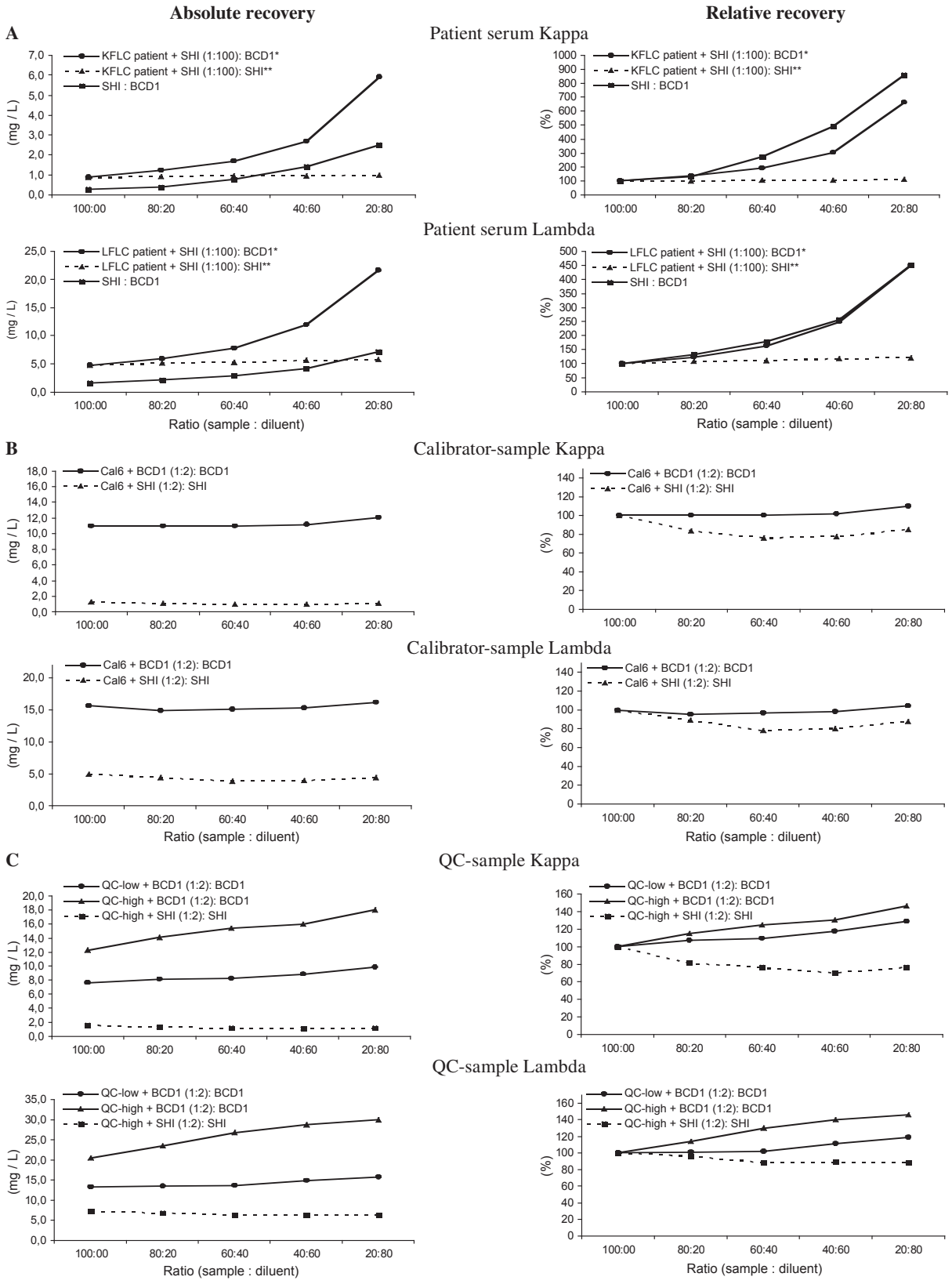

Figure 1. Recovery experiments in serial independent dilutions using (A) samples of patients known with monoclonal FLC-kappa and FLC-lambda,(B) calibrators for FLC-kappa and FLC-lambda and (C) QC samples for FLC-kappa and FLC-lambda on Architect®. Left: absolute recoveries, i.e. deviations of recoveries in mg/L from undiluted samples (100:00). Right: relative recoveries, i.e. deviations of recoveries in percentages from undiluted samples (100:00).
On the X-axis the ratio's of (sample : diluent) are represented. The legend in the figures shows the ratios of the sample and the diluent used. Dilutions with Beckman Coulter Diluent (BCD1) are represented by solid lines, dilutions with serum of healthy individuals (SHI) are represented by dashed lines. The ratio 100:00, which is the undiluted sample, is by definition 100% recovery.
Serum of the patient with known monoclonal KFLC and LFLC disease was prediluted with SHI (1:100) to enable measurements without further dilutions due to substrate excess. * Average of duplicate series. ** Average of triplicate series. Cal6 (highest concentration of the calibration line) and QC samples for KFLC and LFLC were prediluted with BCD1 or SHI (1:2). The differences in absolute recoveries are explained by this predilution. Predilution with BCD1 yields increased recoveries.

Serial dilutions of QC materials and calibrators do not show such extremely increased recoveries as for patient samples. Dilution of calibrator 6 (Cal6) with BCD1 shows recoveries close to 100% for both KFLC and LFLC over the whole interval of the calibration curve (figure 1B).
Dilutions of QC-low and QC-high with BCD1 show an increase of the recovery of the same order for both series (figure 1C). Like for Cal 6, sample dilutions of QC with SHI show a slight decrease of the recoveries for both KFLC and LFLC.
Considering the concentrations at 100:00 in Figure 1B and Figure 1C, there should be in principle no difference in the recoveries between the series. The absolute differences between the curves on the left site of figure 1B and 1C are due tot the effect that the sample is 1:2 prediluted with either SHI or BCD1. The predilution had to be performed to prevent that Cal 6 or QC-low in undiluted form can not be read from the calibration curve, since their values are in the upper limit range of the calibration curve. The absolute difference is in line with the observation that dilutions with BCD1 result in absolute higher recoveries, like it is demonstrated in table 1.

Discussion and Conclusion

By predilution of the QC-samples, like it is performed on Immage®, only the low ends of the calibration curves, 1.8-3.0 mg/L for KFLC and 2.9-5.9 mg/L for LFLC, are controlled. Calibrators span a broader range of about 0.7-20.0 mg/L and 0.9-29.0 mg/L for KFLC and LFLC, respectively. The manufacturer apparently supposes that results which coincide with the intervals 3.0-20.0 mg/L for KFLC and 5.9-29.0 mg/L for LFLC on the calibration curves do not have to be controlled. Our preliminary results from the QC experiments show that there is a significant difference when samples are offered in pure or in prediluted form (table 1). Measurements of the same undiluted QC samples result in significantly lower recoveries than prediluted QC samples, or one can say that diluted samples result into too high concentrations if the measurements of undiluted samples are correct. The effect of the predilution with diluent is also observed in the series of figure 1B and figure 1C, where significantly increased recoveries are observed for the 100:00 ratios of samples that are prediluted with BCD1.
For patients with multiple myelomas the monoclonal FLC can be raised far above the calibration interval and should be prediluted. Patient samples measured on Immage® are, irrespective of the concentration, diluted 1:10 with diluent. In case the concentration of the monoclonal still exceeds the calibration interval, the sample is diluted 1:100. Based on our results, it is deduced that monitoring of high FLC concentrations, as in multiple myelomas, goes hand in hand with large dilutions and possibly strong deviations from the expected value. If in such patients the FLC concentrations decrease, the dilution factor can be changed while monitoring. This brings along results which deviate strongly from the expected value and changes the KFLC/LFLC ratio.
Recovery experiments in Figure 1 clearly demonstrate strong non-parallelism between serial dilutions of patient serum with BCD1 and SHI (figure 1A). Ideally, a polyclonal sample with high FLC concentrations should be compared with the kit calibrator and QC-materials. We are aware that monoclonal FLC may show non-parallelism as compared to the polyclonal calibrator, and that this effect may reflect heterogeneity in the test. However in our case we demonstrate that the non-parallelism effect changes significantly when changing the diluent while the monoclonal SFLC and all other parameters are unchanged. This clearly indicates an effect of the diluent that is independent from the clonality.
Dilutions of Cal 6 with BCD1 seem to have no effect on relative recoveries (figure 1B), however the absolute concentrations differ significantly. These differences are due to the effect that the sample is 1:2 prediluted with either SHI or BCD1. This is in line with the results of table 1, where it is demonstrated that predilution with BCD1 yields higher recoveries. The recoveries of the serial dilutions clearly show that the processed materials, i.e. QCs and calibrators behave different in series with BCD1 or SHI as diluent. This indicates that BCD1 diluent can be a cause of the large differences between the methods, as shown by R.J. Pattenden *et al.* and UK NEQAS (3, 4).
We realize that the application for FLC on the Immage® has been transferred to the Aeroset® analyzer with some modifications. The order of pipetting is different in the two methods. Because corresponding calibration curves and reproducible QC results are obtained on both Immage® and Architect® when sample preparations are identical, the order op pipetting is not believed to have an effect on the results. The reaction volumes are identical and a different detection wavelength is also not of influence on the results.
In conclusion, the Freelite™ application for testing KFLC and LFLC shows, independent from clonality, different behaviour when using serial independent dilutions with the recommended BCD1 diluent as compared to pooled serum (SHI) as diluent. Therefore, the commercial diluent may be an obstacle to method harmonization.
Following the JCTLM standardization efforts (www.bipm.org), manufacturers should investigate these features before making their kit/diluent formulations available for diagnostic purposes. Secondly, calibrators, QCs and patient samples should behave identically for reliable transferability of the manufacturer's measurement system. Also QCs should span the complete measuring interval, which is because of automatic predilution not the case for the application of the Freelite™ kit on Immage®. We recommend that manufacturers should realize that entire applications should be figured out first to avoid confusion in the professional field. In the current status, quantification of FLC in patients using Freelite™ can be useful but should be performed with care, preferably only when other methods fail to provide clinical solutions.

References

1. Durie BGM, Harousseau J-L, Miguel JS, Bladé J, Barlogie B, Anderson K, Gertz M, et al. on behalf of the International Myeloma Working Group. International uniform response criteria for multiple myeloma. Leukemia 2006; 20: 1467-1473.

2. Rajkumar SV, Kyle RA, Therneau TM, Melton LJ, Bradwell AR, Clark RJ, Larson DR, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. Blood 2005; 106: 812-817.
3. Sheldon J, Free light chains: author's response. Ann Clin Biochem 2007; 45: 444- 448.
4. Pattenden R, Rogers SY, Wenham PR. Serum free light chains; the need to establish local reference intervals. Ann Clin Biochem 2007; 44: 512-515.
5. Katzmann JA, Clark RJ, Abraham RS, Bryant S, Lymp JF, Bradwell AR, Kyle RA. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. Clin Chem 2002; 48: 1437-1444.
6. Tate JR, Gill D, Cobcroft R, Hickman PE. Practical considerations for the measurement of free light chains in serum. Clin Chem 2003; 49: 1252-1257.

Samenvatting

Gammeren AJ van, Unen V van, Smeekens C, Cobbaert CM. Het effect van monsterverdunning op de recovery van vrije lichte ketens in serum met de Freelite™. Ned Tijdschr Klin Chem Labgeneesk 2010; 35: 41-45.

In deze studie werd de Freelite™ applicatie voor het kwantificeren van vrije lichte ketens in serum onderzocht. Ondanks de unieke herkomst van de reagentia laat de literatuur methodologische verschillen zien. Ons doel was het testen van kalibratoren, controles en verdunningsvloeistof van Freelite™ ten opzichte van poolserum van gezonde individuen en van serum van patienten met een verhoogde vrije lichte ketens in het serum. Voor dit onderzoek werd een analyzer met open applicatie gebruikt en vergeleken met de gesloten applicatie op het Immage systeem. De routinechemieanalyzer Architect® van Abbott Diagnostics, een open systeem met open applicatie, werd gebruikt voor dit onderzoek. Resultaten laten in onafhankelijke verdunningsreeksen een significante toename van de recovery zien voor zowel controles, kalibratoren en poolserum wanneer de door de fabrikant aanbevolen verdunningsvloeistof van Beckman Coulter wordt gebruikt, terwijl gebruik van humaan serum als verdunningsvloeistof resulteert in verwachte recoveries van nabij 100%. De verschillen in recovery bij gebruik van de aanbevolen Beckman Coulter verdunningsvloeistof of humaan serum als verdunningsvloeistof worden op zowel de Architect® als Immage® waargenomen en kunnen worden verklaard aan de hand van het non-parallellisme van de Beckman Coulter verdunningsvloeistof.

Keywords: vrije lichte ketens; commuteerbaarheid; non-parallellisme; recovery

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Een CLSI-EP5- en -EP9-validatie van een kleinformaat-chemieanalyser: de Cobas C111

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De COBAS C111 (Roche Diagnostics Ltd., Rotkreutz, Zwitserland) is een kleinformaat-chemieanalyser met een fotometrische en ISE-module. Bij een gemengd aanbod (fotometrie en ISE) ligt de monstercapaciteit tussen de 60 en 100 monsters/uur. Met de transitie van ons regulier routinelaboratorium op de locatie Waalwijk van het TweeSteden ziekenhuis naar een citolaboratorium dient de C111 als opvolger voor de Integra 800. De C111 is uitgebreid gevalideerd volgens de CLSI-normering. Precisie werd gevalideerd middels een CLSI-EP5-protocol, terwijl de correlatie met de COBAS Integra 800 werd gevalideerd middels een CLSI-EP9-protocol. Onafhankelijk van de validatieresultaten kan als minpunt worden aangedragen dat de C111 niet in staat is om serumindices te berekenen. Hiermee moet voor onze locatie in Waalwijk een afwijkende procedure voor het beoordelen van de bruikbaarheid van sera worden gehanteerd, waarbij de analisten de bruikbaarheid visueel moeten beoordelen.

Concluderend, de C111 voldoet aan alle door ons voorafgestelde validatiecriteria voor zowel het CLSI-EP5- als -EP9-protocol. Daarmee vult de C111 als kleinformaat-chemieanalyser een belangrijke niche op voor (cito)laboratoria waar het aanbod van patiëntenmonsters beperkt is.

Trefwoorden: COBAS C111; validatie; CLSI; EP5; EP9

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Het klinisch-chemisch & hematologisch laboratorium (KCHL) van het St. Elisabeth ziekenhuis in Tilburg verzorgt naast de diagnostiek van het St. Elisabeth ziekenhuis ook de laboratoriumdiagnostiek van het TweeSteden ziekenhuis, zowel voor de locatie Tilburg als Waalwijk. Met de in gang gezette centralisering van de algemene diagnostiek naar de KCHL-locatie St. Elisabeth ziekenhuis wordt op de locatie Waalwijk van het TweeSteden ziekenhuis enkel nog citodiagnostiek verricht. Met deze aanpassing van het monsteraanbod is voor het laboratorium op de locatie Waalwijk ter vervanging van de COBAS Integra 800 gekozen voor een kleinformaat-chemieanalyser: de COBAS C111 (Roche Diagnostics Ltd., Rotkreutz, Zwitserland). De COBAS C111 combineert een spectrofotometrische module met een (optionele) ionselectieve elektrode (ISE-module). Het bepalingenpakket van de COBAS C111 is geschikt voor de doeleinden waarvoor wij dit apparaat willen gaan gebruiken.