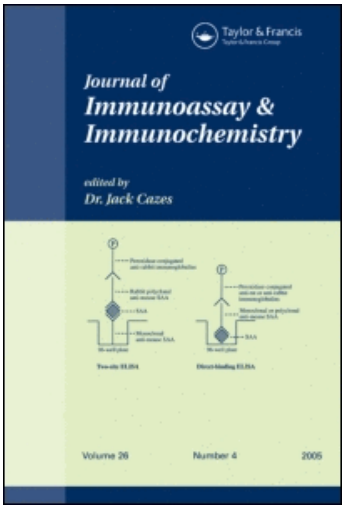

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IMPROVED PERFORMANCE OF ELISAs FOR FERTILITY ASSESSMENT USING COMMON REAGENTS AND ASSAY PROTOCOL AS EVIDENCE FROM QUALITY CONTROL STUDIES

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ABSTRACT

At our Institute, a panel of reproductive hormones, viz., estrone glucuronide (E_1G), pregnanediol glucuronide (PdG), luteinising hormone (LH), and follicle stimulating hormone (FSH) are estimated by ELISA for the assessment of fertility from a single urine sample collected from a subject. In order to make the estimates less cumbersome, the selection and mode of presentation of immunoreagents of the assay were modified in such a way that, either on reconstitution or single dilution, would result in ready-to-use reagents in the assay. Retrospective analysis on the

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performance of these ELISAs with uniform protocols ($n = 86$) was compared with assays having individual assay protocols ($n = 116$). The performance of the assay, based on the standard curve characteristics and quality control pools, was better and the rate of acceptance of these assays improved from 87.9 to 97.6%. The simplification of assay protocols, thus, had better impact on the quality and reproducibility of immunoassay of the four analytes.

INTRODUCTION

Hormone estimations play a vital role in the diagnosis and management of endocrine disorders.(1) Many clinical situations, particularly fertility assessment, require estimation of a panel of hormones in a single sample. This can easily be carried out by employing either multianalyte or automated immunoassay systems.(2,3) Smaller laboratories find it difficult to adopt multianalyte or automated systems, as it may remain underutilized or commercially not viable. In these laboratories, from the same sample, hormones are estimated individually using a set of protocols described for each hormone. These protocols usually involve a series of manual steps which are cumbersome, prone to errors, and may affect the quality of results.

For assessment of infertility, our laboratory developed indigenous ELISAs for four reproductive hormones: estrone glucuronide (E_1G), pregnanediol glucuronide (PdG), follicle stimulating hormone (FSH), and luteinising hormone (LH), each having an individual assay methodology.(4–7) A few of the clinical research projects required simultaneous estimation of three or four of the above hormones from a single urine sample. It was not possible to employ a multianalyte system or an autoanalyzer system in these projects. Earlier, due to the individualised assay protocols available for each hormone, the laboratory work used to be repetitive and tiresome. In order to reduce the number of assay steps and simplify the methodology, these assays were modified into prototype kit formats having uniform assay protocols.(8–10)

We retrospectively assessed the impact of introduction of assays in the prototype kit format having uniform protocols on the performance characteristics of assays by comparing performance data of these assays with earlier assays having individual protocols.

EXPERIMENTAL

Antisera and Enzyme Labelled Analytes

Antisera to E_1G, PdG, LH, and FSH were raised at the Institute, purified, and characterised as described earlier.(11) E_1G and PdG were coupled to enzyme β-lactamase by a mixed anhydride method, whereas LH and FSH were coupled to enzyme β-lactamase by the glutaraldehyde method. Preparation of these enzyme labelled hormones/metabolites have been previously described in detail.(6)

For comparison of the performance characteristics, 116 randomly selected assays for E_1G, PdG, FSH, and LH, having individualised assay protocols called as regular assays [E_1G assays ($n=40$), PdG assays ($n=46$), FSH assays ($n=15$)], LH assays ($n=15$), and 86 kit-based assays with uniform protocols for the same hormones ($n=30$ E_1G assay, $n=30$ PdG assay, $n=13$ FSH assay, and $n=13$ LH assays) were considered in the study.

Regular Assays

A competitive ELISA was developed for each analyte as described previously. All antisera were preserved at $-20^{\circ}C$ in aliquots of 0.1 mL diluted 100-fold in phosphate buffered saline (100 mmol/L sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride). All enzyme labeled analytes were preserved, diluted 10-fold in phosphate buffered saline containing 1% bovine serum albumin and 0.1% sodium azide. Table 1 gives details of optimum dilutions, working dilutions required, and procedure employed for dilution.

Prototype Kit-Based Assays

Regular laboratory based assays were modified to suit prototype, kit-based assay format having uniform assay protocols. Accordingly, plates pre-coated with appropriate dilution of antisera were supplied in the kits.

Similarly, all conjugates were preserved at a predetermined dilution so that optimum dilution (working dilution) is obtained by diluting 50 μL to 10 mL.

Table 1. Optimum Dilutions, Working Dilutions of Enzyme Conjugates, and Standards in Regular Assays

Component	Concentration/ Dilutions Preserved	Dilutions Required	Procedure
E_1G Enzyme conjugate	1 : 20	1 : 4000	0.05 mL to 10 mL
PdG Enzyme conjugate	1 : 30	1 : 6000	0.05 mL to 10 mL
LH Enzyme conjugate	1 : 4	1 : 800	0.05 mL to 10 mL
FSH Enzyme conjugate	1 : 10	1 : 2000	0.05 mL to 10 mL
Standard E_1G Standard PdG	1.6 µg/0.1 mL 16 µg/0.1 mL	12.5–800 pg/0.1 mL	1) 50 µL of E_1G and 50 µL of PdG in 19.9 mL called NSB vial. 2) 0.1 + 0.9 mL buffer NSB to get first standard (E_1G = 800 pg/0.1 mL, PdG = 8000 pg/0.1 mL). 3) Double dilution for further standards.
Standard FSH	3.2 Iu/mL	2.5–160 mIu/mL	0.1 + 1.9 mL buffer to get first standard.
Standard LH	3.2 Iu/mL	2.5–320 mIu/mL	0.1 + 0.9 mL buffer to get first standard. Double dilutions for further standards.

Assay Procedure

In the case of regular assays, plates were coated as follows: For each assay, 250 μL of diluted antisera was dispensed to each well of microtitre plate. Plates were kept at 2–8°C for 16 h and washed 3–4 times with wash solution (150 mmol/L NaCl containing 0.05% Tween 20).

To the antisera coated plate, 100 μL of buffer or respective diluted standard or sample was added followed by 100 μL of diluted enzyme labelled conjugate. After incubation at 37°C for 2 h, plates were washed with wash solution and enzyme activity was measured in the bound fraction as described earlier.(6)

Steps and assay procedures for carrying out regular assays and kit-based assays are given in Tables 2(a) and (b).

Data Analysis

The performance of the assays was judged on the basis of the following:

i) Standard curve characteristics, which included slope of the logit-log transformed curve. The intercepts at defined B/Bo bindings (88, 50, and 12%).
ii) Precision profile of the standards.
iii) Variations in estimates of 3 quality control pools.

RESULTS AND DISCUSSION

The advent of automation in immunoassay and the development of multianalyte systems have considerably decreased the workload for laboratory analysts. Being automated, these systems are more precise and less prone to human errors. However, the use of these state-of-art innovations remains restricted to sophisticated laboratories where the workload is sufficiently high and, hence, installation of expensive automated systems are either affordable or commercially more viable. The present study depicts an example of how a comparatively better precision can be achieved by adopting a judiciously/intelligently formulated uniform assay protocol without any monetary input.

The characteristics of the regular assays and kit-based assays having uniform protocols are given in Tables 3(a) and (b). All assays fulfilled the validatory criteria described for labeled immunoassays. As expected, the

Table 2(a). Regular and Uniform Assay Protocols

Regular Assay Protocol				
Assay	Component	Preserved	Required	Procedures
E_1G	Excess standard (NSB)	0.1 mg/mL	0.1 mcg/mL	0.01 mL Stock + 9.99 mL IAB
	Working standards (WS)	NSB	12.5–800 pg/mL	A. 0.8 mL NSB + 0.2 mL IAB B. 0.1 mL 'A' + 0.9 mL IAB (800 pg) C. Serial double dilutions 'B'
	Label	1 : 10	1 : 4000	0.03 mL Stock + 11.97 mL IAB
	Antisera	1 : 100	1 : 8000	A. 0.3 mL Stock AS + 23.7 mL CB B. 0.25 mL of above to each well of ELISA plate C. Incubate 2–8°C overnight, wash, and use for assay
	Sample/QC pools	1 mL (EMU)	1 : 50	0.050 mL Sample/pool + 2.45 mL IAB; vortex thoroughly
PdG	NSB	0.1 mg/mL	1 mcg/mL	0.01 mL Stock + 9.99 mL IAB
	WS	NSB	0.125–8 ng/mL	A. 0.8 mL NSB + 9.2 mL IAB B. Serial double dilution 'A'
	Label	1 : 10	1 : 6000	0.020 mL Stock + 11.98 mL IAB
	Antisera	1 : 100	1 : 15 000	A. 0.150 mL Stock AS + 22.35 mL CB B. 0.250 mL of above to each well of ELISA plate C. Incubate overnight 2–8°C, wash, and use for assay
	Sample/QC pools	1 mL (EMU)	1 : 200	0.1 mL Sample/QC pool + 1.99 mL IAB; vortex thoroughly

Uniform Assay Protocol				
Kit	Component	Provided	Required	Procedures
E_1G/PdG	NSB	Lyophilised Standard	E_1G: 0.08 mcg/mL PdG: 0.08 mcg/mL	To vial, add 1.2 mL distilled water (DW)
	WS	NSB	E_1G: 12.5–800 pg PdG: 125–8000 pg	Serial double dilutions
	Label	Concentrated solution	E_1G: 1 : 4000 PdG: 1 : 6000	0.1 mL Conc. solution + 3.9 mL IAB
	Antisera	Coated plate	Coated plate	Discard buffer and use directly for assay
	Quality control pools	Lyophilised	E_1G: 1 : 4000 PdG: 1 : 6000	To vial, add 1 mL DW E_1G: 0.1 mL A + 0.4 mL IAB PdG: 0.1 mL A + 1.9 mL IAB
	Sample	EMU	1 : 100	3.2 0.02 mL Sample + 1.980 mL IAB

IAB: Immunoassay buffer.
CB: Coating buffer.
EMU: Early morning urine samples.
QC: Quality control.

Table 2(b). Regular and Uniform Assay Protocols

Regular Assay Protocol				
Assay	Component	Preserved	Required	Procedures
LH	NSB	15 IU/mL	3.2 IU/mL	0.320 mL Stock + 1.18 mL IAB
	WS	NSB	2.5–320 mIU/mL	A. 0.1 mL NSB + 1.9 mL IAB B. Serial double dilutions 'B'
	Label	1 : 10	1 : 800	0.125 mL Stock + 9.875 mL IAB
	Antisera	1 : 100	1 : 8000	A. 0.300 mL Stock + 23.7 mL CB B. 0.250 mL of above to each well of ELISA plate C. Incubate overnight 2–8°C, wash, and use for assay
	Sample/QC pools	1 mL (EMU)	1 : 5	0.1 mL Sample/QC pool + 0.400 mL IAB; vortex thoroughly
FSH	NSB	15 IU/mL	3.2 IU/mL	0.320 mL Stock + 1.18 mL IAB
	WS	NSB	2.5–160 mIU/mL	A. 0.1 mL NSB + 1.9 mL IAB B. Serial double dilution 'A'
	Label	1 : 10	1 : 2000	0.05 mL Stock + 0.950 mL IAB
	Antisera	1 : 100	1 : 15 000	A. 0.150 mL Stock antisera + 22.35 mL CB B. 0.250 mL of above to each well of ELISA plate C. Incubate overnight 2–8°C, wash, and use for assay

	Sample/QC pools	1 mL (EMU)	1:5	0.1 mL Sample/QC pool + 0.400 mL IAB; vortex thoroughly

Uniform Assay Protocol

Kit	Component	Provided	Required	Procedures
LH/FSH	NSB	Lyophilised Standard	3.2 lu/mL PdG: 0.08 mcg/mL	To vial, add 1.2 mL distilled water LH: 0.1 mL A + 0.9 mL IAB FSH: 0.1 mL A + 1.9 mL IAB
	WS	NSB	2.5–320 mLu/mL FSH 1 : 2000	0.3 mL A + 0.9 mL IAB 0.4 Serial double dilutions
	Label	Concentrated solution	LH 1 : 800 FSH 1 : 2000	0.1 mL Conc. solution + 3.9 mL IAB
	Antisera	Coated plate	Coated plate	Discard buffer and use directly for assay
	Quality control pools	Lyophilised	LH—1 : 5 FSH—1 : 5	To vial of pool, add 0.5 mL distilled water
	Sample	EMU	1 : 5	0.1 Sample + 0.4 mL IAB

IAB: Immunoassay buffer.
CB: Coating buffer.
EMU: Early morning urine samples.
QC: Quality control.

Table 3(a). Characteristics of the Regular Assays

Analyte	Specificity Slope of Dose Response Curve		Sensitivity Well (0.1 mL)	Precision Coefficient of Variation of 3 Pools (%)	
	Standard	Samples		Intra	Inter
E_1G	−2.12	−2.05	25 pg	7–9	9–10
PdG	−2.01	−2.07	125 pg	4–6	10–14
FSH	−2.15	−2.07	0.25 mIU	6–9	9–12
LH	−2.94	−3.04	0.25 mIU	5–6	9–10

Table 3(b). Characteristics of the Prototype Kit-Based Assays

Analyte	Specificity Slope of Dose Response Curve		Sensitivity Well (0.1 mL)	Precision Coefficient of Variation of 3 Pools (%)	
	Standard	Samples		Intra-	Inter-
E_1G	−2.00	−2.02	12.5 pg	5–7	8–10
PdG	−2.09	−2.14	100 pg	4–5	9–10
FSH	−2.14	−2.20	0.20 mIU	5–7	8–10
LH	−2.02	−3.16	0.20 mIU	4–5	6–8

Table 4. Comparison of STD Curve Characteristics of Assays with Individual Protocols and Assays with Uniform Protocols

Hormone	Standard Curve Characteristics	Assays with Individual Protocol		Assays with Uniform Protocols	
		Observed Range	Assays Rejected	Observed Range	Assays Rejected
E_1G	Slope	−1.75–−2.37	7/40	−2.08–−2.32	1/30
	Intercept	3.33–4.94		4.16–4.92	
	88%	12.18–20.56		8.68–16.68	
	50%	79.9–140		100–141.2	
	12%	41–1143		714–1326	
PdG	Slope	−1.60–−2.16	6/46	−1.722–−2.29	0/30
	Intercept	5.11–6.31		4.82–7.14	
	88%	61.2–128.4		50.3–124.3	
	50%	609–1609		365–1413	
	12%	4580–21548		2035–16 363	
FSH	Slope	−1.77–−2.35	0/15	−1.811–−2.37	0/30
	Intercept	3.73–4.318		3.81–4.656	
	88%	8.34–10.86		9.52–14.08	
	50%	61.86–119.8		78.4–135.9	
	12%	347–1419		462.76–1558.7	
LH	Slope	−1.76–−2.22	1/15	−1.82–−2.77	1/13
	Intercept	3.67–4.38		3.76–4.42	
	88%	8.78–12.76		8.17–11.97	
	50%	76.75–147.5		56–119.14	
	12%	479–1831		295–1295	

kit-based assays, having uniform protocols, were observed to have a narrow range of inter- and intra-assay coefficient of variation (CV) of quality control pools (<10%) and were more sensitive due to better inherent precision.

The standard curve (logit-log transform) characteristics are the front line of an internal quality control (IQC) program. They provide a simple and immediate check on the performance of immunoassays.(12) In this study, standard curve characteristics used are slope and intercepts at three different binding levels (88, 50, and 12% B/Bo of the curve). Table 4 gives standard curve characteristics of kit-based assays and regular assays. It was observed that, in kit-based assays, the intercept range at all the 3 binding levels was narrower than those observed for assays with individual protocols. In some of the assays, the slopes and intercepts fell outside the expected limits and, hence, they were interpreted with caution. These assays were subsequently rejected on the basis of QC pool values.

In addition to the above parameters, the random error or imprecision profiles of standard curves of assays were also checked (Figure 1). It was observed that the kit-based assays were more precise than the coefficient of

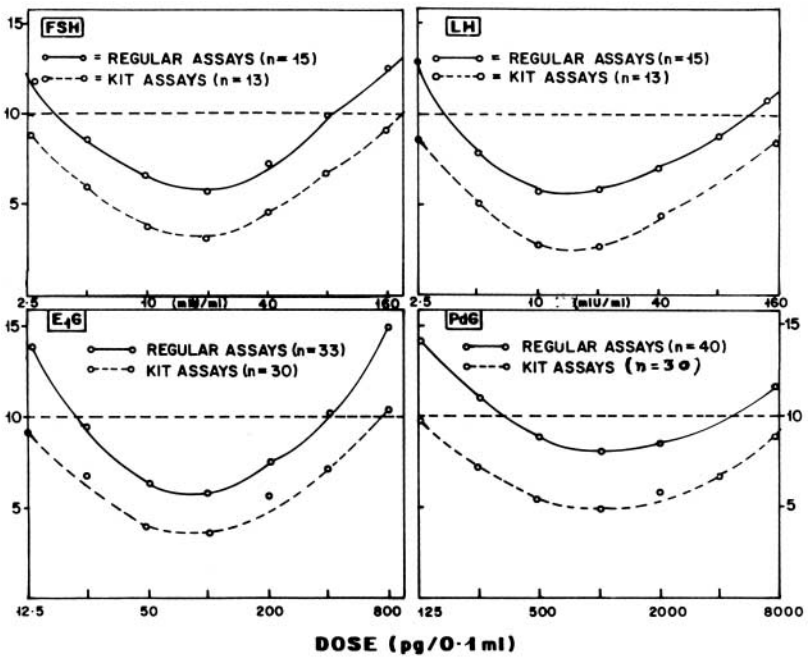


Figure 1. Precision profiles of regular and kit assays for four analytes.

variation (CV) at each standard or dose concentration, which was <10%, as compared to the regular assays wherein the CV ranged from 10–15%, indicating better performances shown by the kit-based assays.

Standard curve characteristics and precision profiles are not routinely used parameters for assessing performance of an assay, as they are cumbersome and require special calculator or computer programmes.

For convenience, the criteria for acceptance of an assay depend upon quality control pool charts based on the estimates of two out of the three QC pools within mean ± 2 S.D. of the target value as described by Shewhart and Levey and Jennings control charts.(13,14)

Hence, the day-to-day performance of the regular and kit-based assays were monitored by internal quality control pools which were prepared in bulk

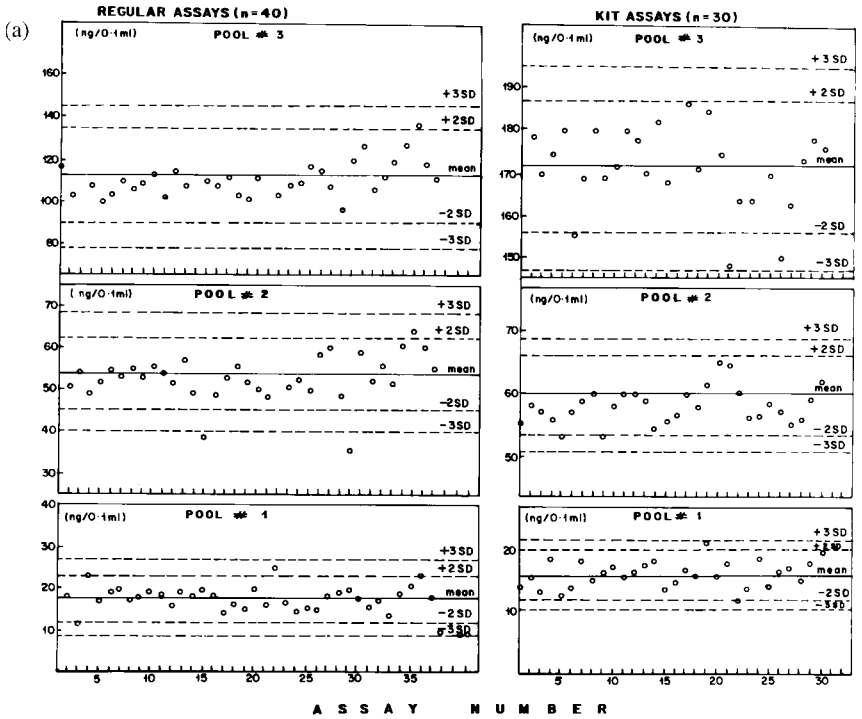


Figure 2. a) Performance of three quality control pools in regular and kit assays for estrone glucuronide (E_1G); b) Performance of three quality control pools in regular and kit assays for pregnanediol glucuronide (PdG); c) Performance of three quality control pools in regular and kit assays for FSH; d) Performance of three quality control pools in regular and kit assays for LH.

(b)

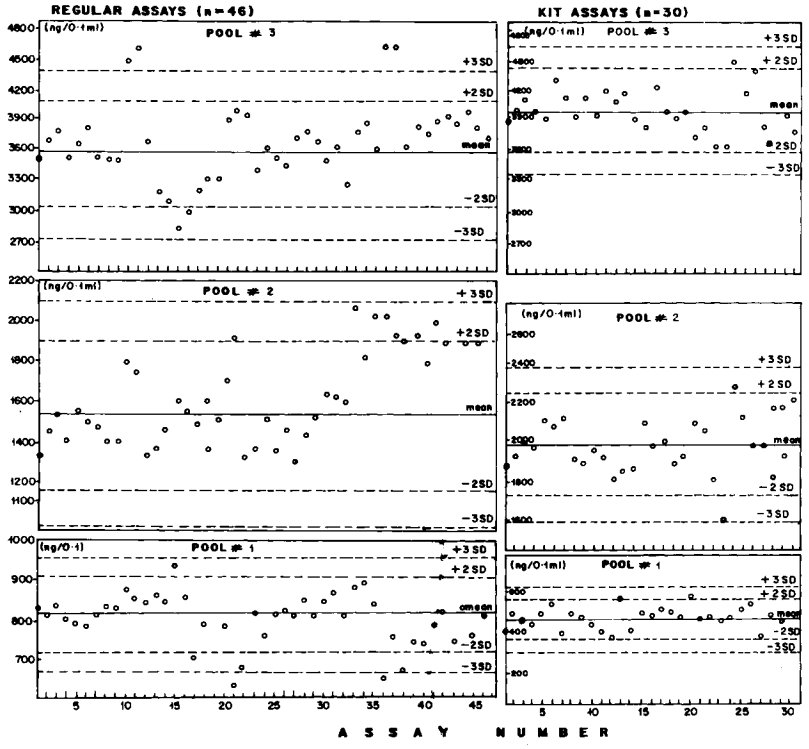
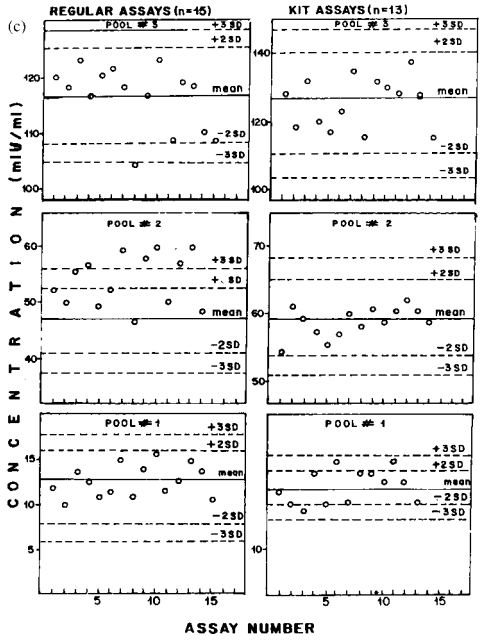


Figure 2. Continued.

(continued)

and stored at −20°C. In our earlier study, it was observed that the concentration of the analyte does not change at this temperature for a year. For each pool, the mean value (target value) was assigned by estimating each pool at least 5–6 times in a single assay. The control limits of pools were established on the basis of standard deviation (S.D.) of multiple estimates. A value greater than ±2 S.D. from the mean was considered suspicious and ±3 S.D. as abnormal. The data obtained from each pool value for the different analytes from consecutive assay batches are depicted in Figures 2(a–d). The criteria for acceptance of an assay was that at least two of the three quality control pool values should lie within normal limits for acceptance of an assay. It was observed, from these charts, that quality control pool estimates (P1, P2, P3) supplied in kits fell within the control limits while, in regular assay, some of the pool values fell outside the 3 S.D. limit, suggesting



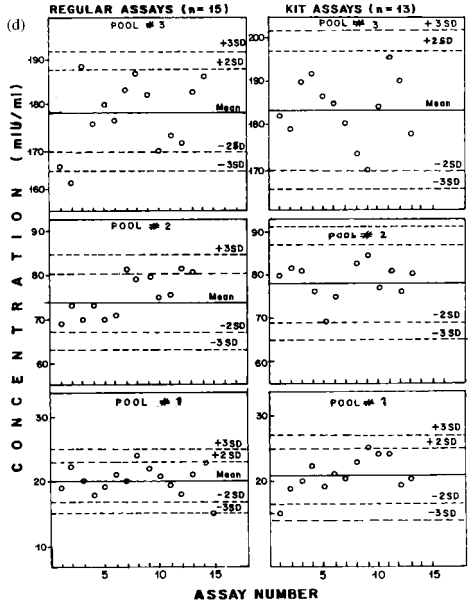


Figure 2. Continued.

abnormal values, thereby casting doubt on the performance of the assays. Out of 40 assays carried out for E_1G with individual protocols, 7 assays were rejected on the basis of estimates of quality control pools. Similarly, in case of PdG, 6 of the 40 assays and 1 assay for LH were rejected, giving an overall acceptance rate of 87.93%. However, when uniform assay protocols were followed, only 2 assays were rejected, giving an acceptance rate of 97.6%. The reproducibility of assays with uniform protocols was better and the acceptance rate improved from 87.94 to 97.6%. This could be attributed to the simplification of the methodology and further reduction in the individual steps of the assay.

Workload and stress have detrimental effect on the performance of individual assays in the laboratory. In the absence of provision for automation, the steps are repetitive and more prone to human errors. The study has judiciously documented how to overcome this aspect.

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