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Biochemistry Assays in Uncoated Microwells

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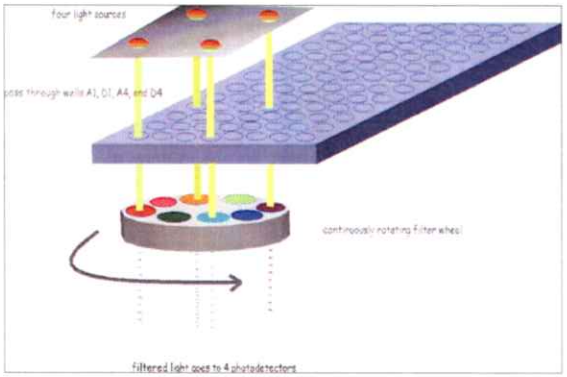
INTRODUCTION:

Microplate technology has offered great advances in clinical diagnostics, first with an explosion of enzyme immunoassay (EIA) developments; and in recent years, with a focus on enabling technologies such as high throughput analysis. This laboratory examined the use of microwells in the "lower tech" arena of routine clinical biochemistry. Because a large part of the world's population lacks access to the standard of medical care generally available in modern developed cities, this research began as a quest for more economical alternatives to current automated diagnostic instrumentation.

Microwell technology could offer cost advantages by reducing reagent volume requirements, employing low cost, reusable and readily available plastic ware, and reducing instrumentation to a single analyzer capable of performing both biochemistry assays and microplate EIA. Thus we explored the feasibility of adapting commercially available biochemistry assays to a microplate format.

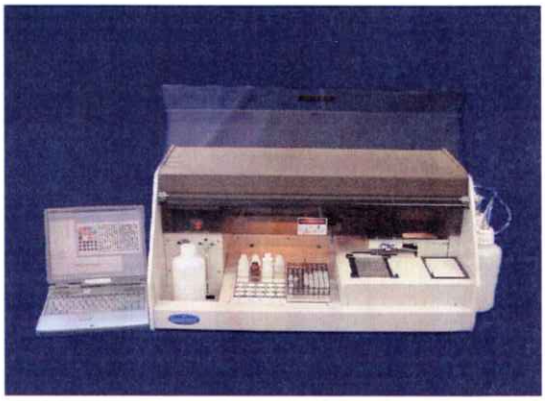
MATERIALS AND METHODS:

To accommodate these studies, we configured a four channel vertical photometric system to simultaneously illuminate four microwells positioned in the four corners of a square (A1, D1, A4, and D4, for example). A continuously rotating wheel containing 8 embedded hard-coat interference filters was fixed below the moveable microplate carrier. The filters were positioned so that 4 of them aligned with the 4 illuminated wells above, and 4 photodetectors below, generating 32 near-simultaneous voltage readings (4 channels at 8 wavelengths). Under external computer control the voltage data appropriate for the test assigned to each microwell were selected and employed in the calculations of analyte concentrations.



This photometer was fitted about a 37°C temperature-controlled microplate holder with X and Y precision movement. A 37°C temperature-controlled , self washing, single probe: and two syringe pumps (50uL and 2.5mL) comprised the pipetting system. Moveable racks for reagents and samples, and an 8-manifold microplate washer completed the instrument system we called ChemWell.

Flat-bottom Immulon brand break-apart microwell strips manufactured by Dynex Technologies (Chantilly, VA, USA) were used for all assays. The wells were washed and reused numerous times. Washing was done by the on-board automatic microplate washer using 2 cycles of 500uL of a wetted phosphate buffer followed by 1 rinse cycle with 0.015N H Cl.



Before introducing biochemistry reagents, the accuracy and precision of the ChemWell instrument were established by comparing the absorbance results of instrument-made dilutions of PNP in 0.5N NaOH, with expected results based on readings from a reference instrument (Shimadzu Model UV-1601) having NIST traceable calibration using Corion Filters MC-10, MC-50, and MC-100. See FIG 1. Equivalent data have also been generated at an independent test site.(1) An additional study measured the effect of volume changes on absorbance readings. In Experiment A, various volumes of a PNP solution were read, and absorbance readings increased with volume as expected due to increased pathlength. In Experiment B, 10uL of concentrated PNP solution was pipetted into each well. Varying volumes of colorless diluent (0.5N NaOH) were added. Absorbance readings, over the range of diluent volumes from 100 to 300uL, were measured using ChemWell's vertical photometer. See FIG 2.

Biochemistry reagents were donated by Sigma Diagnostics, (St. Louis, MO, USA) for albumin (both bromcresol green and purple), alkaline phosphatase, ALT, AST, cholesterol, creatinine, GGT, glucose, total protein, triglycerides, BUN and uric acid. Each assay was programmed to be automatically processed by ChemWell. In most cases, this involved reducing reagent and specimen volumes proportionally, and programming all other parameters exactly as provided in the manual method. For each method, within-run precision and total precision were determined using normal and abnormal levels of ACCUTROL Control Serum (Sigma Diagnostics). The two runs were made on different days; and the ChemWell was re-blanked and re-calibrated with fresh standard material before each run.

Sensitivity was measured by replacing serum with a saline solution, and run 10 times for each method. Correlations were made to the manual method. Either 25 or 45 different serum samples were assayed both on ChemWell and also using a manual method read on Stat Fax 1904 (Awareness Technology). Data were plotted with regression analysis comparing the microwell method (y) with the manual (reference) method (x). A series of dilutions were made using an appropriate high sample, either Multi-Analyte LIN-TROL (Sigma Diagnostics), Multi-Enzyme LIN-TROL (Sigma Diagnostics), or ACCUTROL control serum (Sigma Diagnostics). The assigned values were compared with the measured values to determine assay linearity and reportable range. Accuracy was measured using bi-level controls (normal and abnormal ACCUTROL control serum (Sigma Diagnostics). Each was run in duplicate on two different days as an unknown patient sample. For assays that require a calibration standard, the standard was also run in duplicate as an unknown. Data were compared with the means and recovery ranges stated by the control manufacturer. (3).

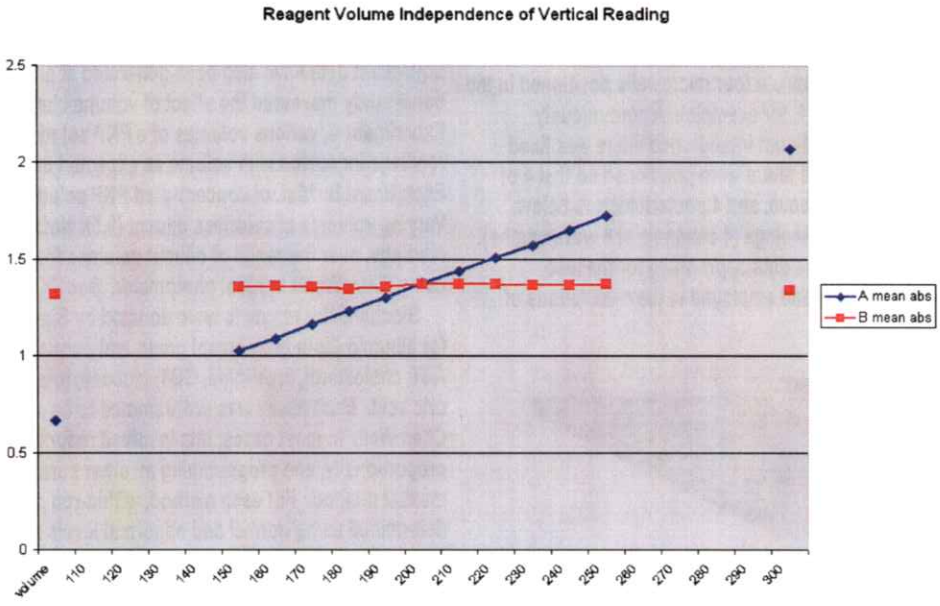
DATA:

FIG. 1 Typical Instrument Performance Check Data

Performed by Ethel Robbins on May 7, 2001 using serial number 2900-1003 (SR4/680, 71/78) Blank absorbance= -0.0005, filters: 405-630nm

dye volume	target absorbance	mean, n=8	SD	%CV	%Difference
18uL	2.4190	2.4155	0.0184	0.7601	-0.1439
10uL	1.3440	1.3463	0.0110	0.8136	0.1735
2uL	0.2690	0.2762	0.0049	1.7741	2.6743

FIG. 2 Vertical Photometer Data



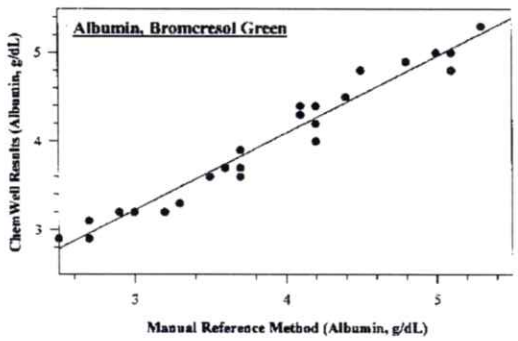
A, in blue, shows the increase in absorbance with increasing volumes of a constant concentration of PNP solution. These results support Beers/Lambert law because the pathlength increases with volume.

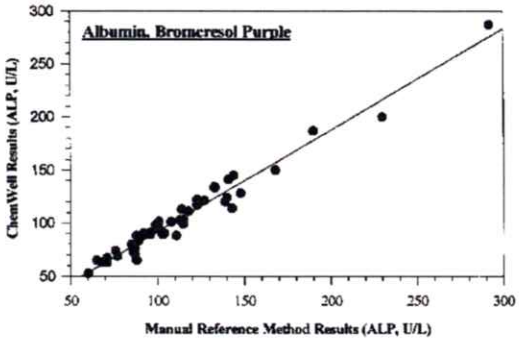
Abs = ecl, where
e is the extinction coefficient,
c is the concentration, and
l is the pathlength

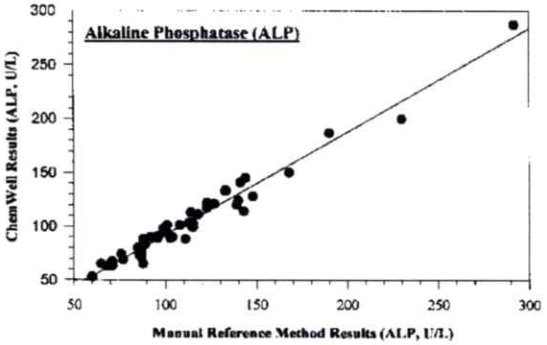
B, in red, shows that the pathlength increase is proportional to the concentration decrease as a fixed amount of PNP is diluted with increasing amounts of colorless buffer.

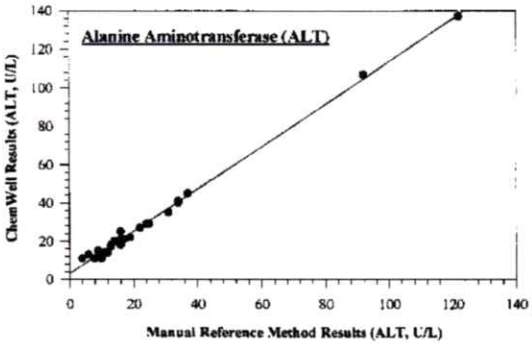
Assay	Units Level 1	PRECISION/Accuracy mean SD %CV Level 2 mean SD %CV within-run precision (n=10) between-run precision (n=20) published mean (range for control)						SD of zero (n=10)	Lin-earity
Albumin Bromcresol Green	g/dL	4.2 4.1 4.0	0.10 0.10 (3.3 - 4.7)	2.3 2.5	5.4 5.4 5.4	0.09 0.09 (4.2 - 6.6)	1.7 1.7	0	9.0
Albumin Bromcresol Purple	g/dL	4.1 4.2 3.9	0.22 0.24 (3.0 - 4.8)	5.4 5.6	5.6 5.7 5.2	0.30 0.31 (3.9 - 6.5)	5.4 5.5	0.08	7.8
Alkaline Phosphatase	U/L	95 95 87	3.1 3.8 (57 - 117)	3.2 4.0	201 204 207	6.4 6.5 (145 - 269)	3.2 3.2	2.7	2955
Alanine Amino Transferase(ALT)	U/L	52 54 41	2.8 3.8 (26 - 56)	5.4 7.0	117 114 104	1.8 2.7 (82 - 126)	1.6 2.4	2.1	500
Aspartate Amino-Transferase (AST)	U/L	70 71 57	2.4 2.3 (40 - 74)	3.4 3.3	195 195 163	5.7 5.4 (130 - 196)	2.9 2.8	2.7	500
Cholesterol	mg/dL	148 148 150	5.1 5.1 (125 - 175)	3.4 3.4	214 216 222	6.7 7.5 (189 - 255)	3.1 3.5	0.7	780
Creatinine	mg/dL	2.0 2.2 1.7	0.09 0.10 (1.0 - 2.4)	4.4 5.2	7.3 7.5 7.7	0.21 0.25 (5.5 - 9.9)	2.9 3.3	0.03	26
Gamma-Glutamyl-Transferase (GGT)	U/L	35 35 29	1.8 2.1 (20 - 38)	5.2 6.0	78 78 73	2.0 2.1 (55 - 91)	2.6 2.7	3.6	1765
Glucose	mg/dL	98 99 96	2.8 3.1 (77 - 115)	2.9 3.1	355 356 340	9.5 10.8 (300 - 380)	2.7 3.0	0	700
Protein, total	g/dL	6.2 6.2 6.6	0.12 0.12 (5.9 - 7.3)	1.9 1.9	8.1 8.1 8.1	0.13 0.15 (7.0 - 9.2)	1.6 1.8	0.05	14
Triglycerides	mg/dL	65 65 64	3.0 3.3 (50 - 78)	4.7 5.1	154 153 148	5.1 5.0 (118 - 178)	3.3 3.3	0.3	1190
Urea Nitrogen (BUN)	mg/dL	13 13 13	0.7 1.1 (9 - 17)	5.7 8.0	45 46 49	1.9 2.3 (40 - 58)	4.3 5.0	0.9	100
Uric Acid	mg/dL	5.8 6.0 6.0	0.16 0.28 (5.0 - 7.0)	2.7 4.7	9.8 10.0 9.6	0.15 0.30 (8.1 - 11.1)	1.5 3.0	0	33

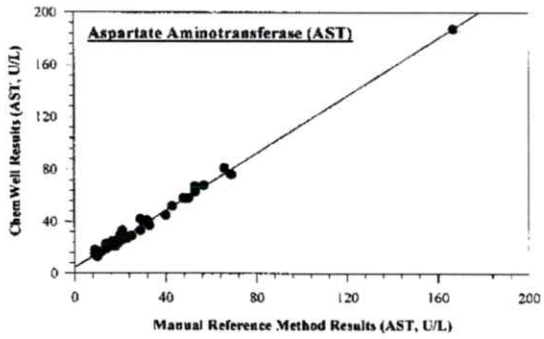
ChemWell - Manual Assay Correlation

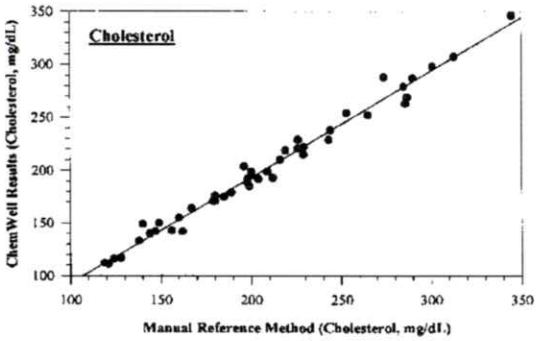


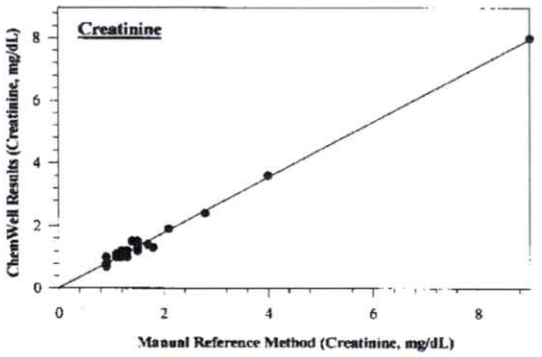


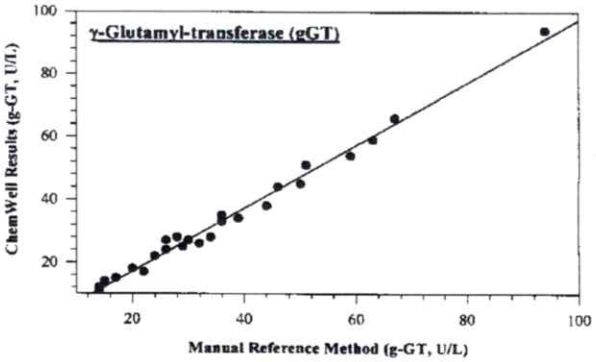


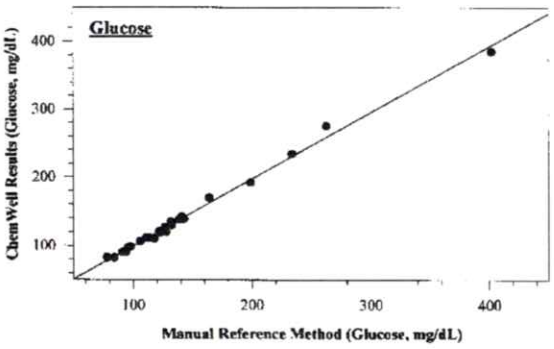


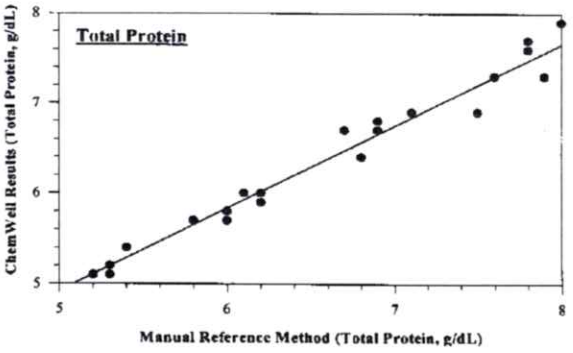


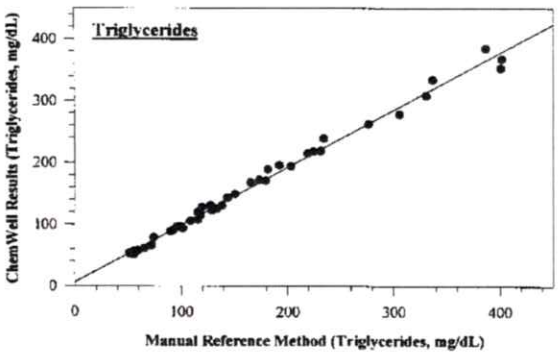


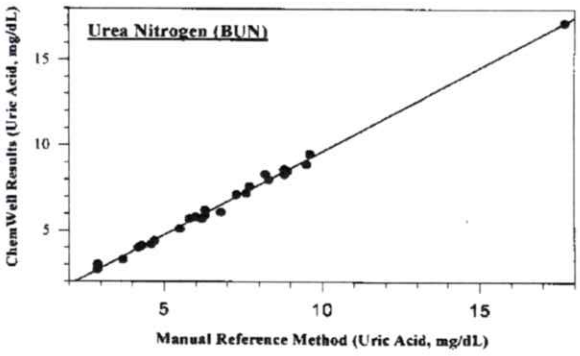

Creatinine
ChemWell Results (Creatinine, mg/dL)
Manual Reference Method (Creatinine, mg/dL)

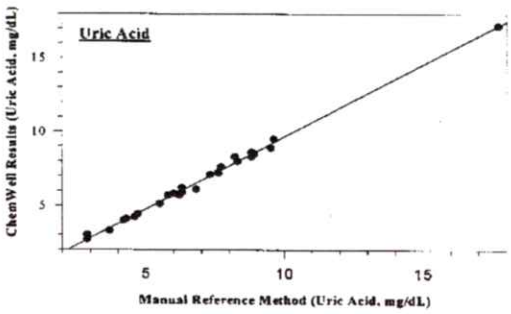

γ-Glutamyl-transferase (gGT)
ChemWell Results (g-GT, U/L)
Manual Reference Method (g-GT, U/L)


Glucose
ChemWell Results (Glucose, mg/dL)
Manual Reference Method (Glucose, mg/dL)


Total Protein
ChemWell Results (Total Protein, g/dL)
Manual Reference Method (Total Protein, g/dL)


Triglycerides
ChemWell Results (Triglycerides, mg/dL)
Manual Reference Method (Triglycerides, mg/dL)

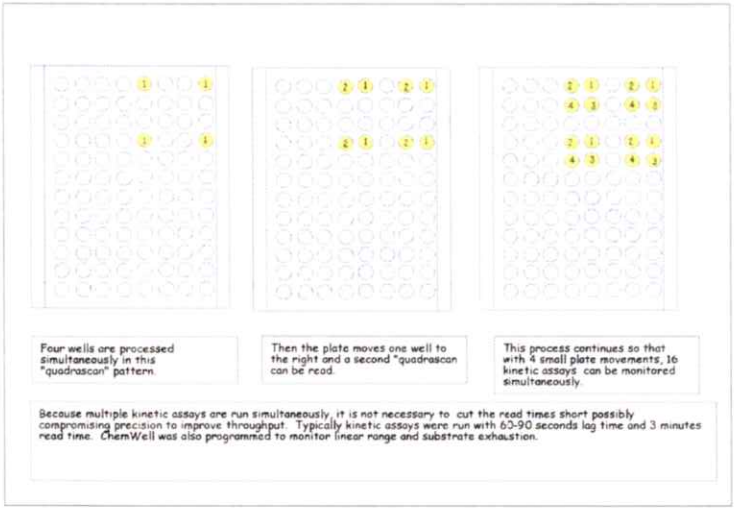

Urea Nitrogen (BUN)
ChemWell Results (Uric Acid, mg/dL)
Manual Reference Method (Uric Acid, mg/dL)


Uric Acid
ChemWell Results (Uric Acid, mg/dL)
Manual Reference Method (Uric Acid, mg/dL)

RESULTS AND DISCUSSION:

The performance of microwell assays, with throughput of greater than 150 results per hour, and 80% lower reagent volume consumption, was equivalent to manual methods for these 13 analytes. Similar studies were performed by an independent manufacturer of biochemistry reagents, Pointe Scientific (Lincoln Park, MI, USA) for 25 analytes: albumin, ALT, amylase, AST, direct and total bilirubin, calcium, cholesterol, CK and CKMB, creatinine, GGT, glucose (hexokinase and oxidase), Iron, LDH, LDL cholesterol, magnesium, phosphorous, total protein, triglycerides, BUN and uric acid with similar results. (2) Our laboratory then re-confirmed their observations, again demonstrating equivalent performance between manual methods and microwell biochemistry methods. In fact, these Sigma Diagnostics and Pointe Scientific assays were all subsequently assigned FDA 510(k) numbers and moderate CLIA complexity ratings for use in the automated microwell format described here.

Results of performance testing remain dependent upon high precision pipetting of micro-volumes, and a uniform, stable temperature control system. In addition, there are several unique aspects of the ChemWell instrument design that contributed to the reliable performance of these biochemistry assays in microplates. Firstly, the open system was completely user-programmable. Assay steps could be fine tuned by adjusting parameters such as read time, sample volume, and the speeds of aspiration and dispensing. Secondly, the 4-channel optical system allowed multiples of 4 kinetic assays to be run simultaneously (massively parallel kinetics) instead of the traditional method of one-at-a time in a flow cell. Consequently, total reaction times could readily be extended without sacrificing throughput. Often assay precision improved simply by lengthening total reaction time. Reactions were also monitored for linearity, and rates of change indicative of substrate depletion. Thirdly, the use of a wide light beam and bichromatic reading eliminated path length variations and meniscus effects such as light scattering or focusing.



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2. Pointe Scientific Laboratories, Kay Newel, www.pointescientific.com

3. Application Verification Protocol, William Gilbert, Sigma Diagnostics, St. Louis MO USA

CONCLUSION:

These data clearly demonstrate the technical feasibility and reliability of performing low cost automated endpoint and kinetic biochemistry reactions, using re-usable uncoated microwells for sample cups, and an instrument, such as ChemWell, whose design is specifically optimized for this purpose.