145

Location Dependent Biases in Automatic 96-Well Microplate Readers

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Procedures performed in 96-well microplates and quantitated by automatic readers assume instruments to be precise, accurate, and free of well location dependent bias. Instrument specifications generally focus on precision and accuracy without specifically addressing biases which are dependent on well location. These biases appear to be meniscus dependent and can be demonstrated in varying degrees in automatic readers of many designs by using a reverse plate wet test, which compares repeated readings of a dye loaded plate in normal and reversed positions. This test analyzes differences between readings and is, therefore, independent of pipetting error or other experimental variables such as protein binding or immunoassay variability. Different plates increased or decreased the magnitude of observed errors but did not themselves cause the errors measured by the reverse plate wet test. Error patterns were consistent for each reader and varied widely among the 16 instruments tested. Only 4 of 16 instruments passed an existing manufacturer's specification for precision, and only one of the 16 readers tested passed a similar specification for accuracy. The severest location dependent bias was found in an instrument which exhibited excellent repeatability and consistently passed its built-in diagnostic tests. One reader with significant bias was returned to the manufacturer for routine service and calibration, but it was not demonstrably improved. The reverse plate wet test is an extremely useful diagnostic tool for quality control at all stages of instrument manufacture and use.

The use of automatic 96-well microplate readers in quantitative analytical methods is becoming increasingly widespread. Microplate-based immunoassays have been described for a broad range of natural and synthetic toxins, including aflatoxin B_1 (1), benomyl and thiabendazole (2), paraquat (3), and many others (4). Microplate-based immunoassays are also important in clinical applications (5) and plant diagnostics (6). Microplate-based immunoassays are gaining favor as official methods of analysis for hazardous environmental contaminants such as pentachlorophenol (7) and for foodborne pathogens such as *Salmonella* (8). Other non-immunoassay quantitative microplate applications include enzyme assays (9, 10), direct biochemical assays (11), and protein assays (12). The use of 96-well microplates and automatic readers is likely to keep increasing rapidly because of the advantages of microplate-based immunoassays over other analytical methods' cost, speed, and convenience.

Differing from the familiar horizontal beam spectrophotometers, microplate readers are vertical beam spectrophotometers having one boundary of the reading cell defined by the meniscus of the contained liquid. This difference poses a couple of potential sources of error: First, the volume of liquid can vary in the well and is, therefore, outside the control of plate and reader manufacturers. Second, the meniscus and the light beam can misalign during reading. This alignment problem can lead to variations in surface scatter due to variable incident angle and variations in absorbance due to variable average path length, but neither of these is a problem in horizontal beam spectrophotometers. If these inaccuracies vary across the plate, there will be well location dependent biases inherent in the reader. In addition, multichannel instruments (i.e., nearly all those presently available) must have their channels matched optically and electronically to avoid other channel dependent errors. Misalignment and inaccurate interchannel calibration should be manifested as channelwise errors in a comparison of reversed readings for one plate.

The potential for location dependent biases in microplate reading is great and has not been addressed systematically by instrument manufacturers. These biases have been observed in single channel Dynatech instruments, using a comparison of reversed plate readings as a convenient detection method (R. O. Harrison and J. O. Nelson, unpublished data). In the present study, a wider range of instrument designs was included to illustrate the potential severity of location dependent biases and the crucial importance of bias testing in quality control procedures. A case study is presented demonstrating the need for bias testing by instrument manufacturers at all stages of design and manufacture of vertical beam microplate readers. Another case study is presented illustrating the difficulty in servicing and calibrating readers with location dependent biases.

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METHODS

Materials

Instruments tested were either demonstration units provided by company representatives or laboratory units in routine use at the University of California, Davis. The selection was arbitrary, and results are not claimed to be representative of all units of that model or manufactuer.

Reverse Plate Wet Test

Wash a new plate with neutral pH phosphate buffered saline plus 0.05% Tween 20, tap plate on paper towel to remove excess liquid, and load plate with 100 μL/well of 0.02 mg/mL *p*-nitrophenol in buffer plus 0.05% Tween 20. The buffer composition is probably unimportant, and water plus Tween 20 may work equally well if a realistic meniscus is produced. This approximates a typical enzyme immunoassay endpoint, giving 0.700–1.000 absorbance at 405 nm in a flat-bottom polystyrene plate. Subsequent data handling can be facilitated by placing an additional 10 μL dye solution in a chosen well to mark plate orientation (not done for example calculation).

Set reader for air blank after warm-up, and verify optical and electronic stability (<0.002/min).

Read plate 3 times, twice in normal orientation (N1, N2) and once in reversed orientation (R1), and calculate mean of all individual wells for each reading ($\bar{x}_{N1}$, $\bar{x}_{N2}$, or $\bar{x}_{R1}$). Rearrange R1 values to correct for reversal (well A1 becomes H12, etc.). Tables 1 and 2 show the N2 and R1 readings, with the R1 data set already corrected for plate reversal.

Calculate N2 − N1 and N2 − R1 for each well, giving 2 data sets of individual well differences for repeated normal and repeated reversed readings. The N2 − R1 data set is shown in Table 3.

For each data set N2 − N1 and N2 − R1, determine absorbance grand mean ($\bar{x} = [(\bar{x}_{N2} + \bar{x}_{N1 \text{ or } R1})/2]$). Example: absorbance grand mean for N2 − R1 = 0.827, according to the equation $\bar{x} = [(\bar{x}_{N2} + \bar{x}_{R1})/2]$ [(0.825 + 0.830)/2]. This is the mean of all 192 N2 and R1 absorbance values. All results shown are rounded; only final results were rounded in the actual calculations, and, therefore, intermediate results may show rounding errors.

Table 1. N2 individual well absorbance values

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.838	0.828	0.827	0.830	0.828	0.814	0.828	0.826	0.823	0.832	0.831	0.833
B	0.831	0.831	0.826	0.832	0.835	0.820	0.831	0.827	0.830	0.827	0.833	0.833
C	0.837	0.830	0.828	0.845	0.842	0.823	0.836	0.827	0.831	0.833	0.836	0.834
D	0.831	0.827	0.822	0.827	0.830	0.810	0.827	0.822	0.828	0.833	0.835	0.832
E	0.834	0.824	0.823	0.830	0.836	0.830	0.832	0.828	0.828	0.830	0.827	0.830
F	0.832	0.822	0.834	0.830	0.828	0.821	0.830	0.814	0.834	0.830	0.835	0.834
G	0.820	0.820	0.816	0.836	0.831	0.812	0.820	0.826	0.834	0.821	0.826	0.836
H	0.765	0.821	0.767	0.820	0.780	0.821	0.751	0.830	0.769	0.844	0.795	0.824

$\bar{x}_{N2}$ = 0.825, mean of all 96 N2 absorbance values.

Table 2. R1 individual well absorbance values (corrected for reversal)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.821	0.798	0.821	0.761	0.826	0.774	0.834	0.790	0.835	0.796	0.842	0.843
B	0.818	0.819	0.816	0.827	0.830	0.823	0.834	0.835	0.841	0.835	0.847	0.854
C	0.820	0.823	0.824	0.842	0.837	0.830	0.844	0.838	0.843	0.842	0.851	0.856
D	0.815	0.817	0.813	0.826	0.827	0.815	0.836	0.835	0.842	0.844	0.847	0.852
E	0.823	0.819	0.819	0.824	0.834	0.837	0.838	0.839	0.841	0.839	0.839	0.853
F	0.823	0.818	0.826	0.826	0.827	0.827	0.836	0.827	0.847	0.841	0.849	0.856
G	0.813	0.815	0.812	0.837	0.833	0.823	0.830	0.842	0.842	0.832	0.837	0.857
H	0.817	0.815	0.814	0.824	0.808	0.830	0.830	0.846	0.826	0.855	0.845	0.845

$\bar{x}_{R1}$ = 0.830; mean of all 96 R1 absorbance values.

Table 3. N2 − R1 individual well differences ($A1_{N2} - A1_{R1}$, etc.), with columnwise and rowwise means expressed as absorbance and percent of $\bar{x}$

	1	2	3	4	5	6	7	8	9	10	11	12	RM[a]	%RM[b]
A	0.017	0.030	0.006	0.069	0.002	0.040	−0.006	0.036	−0.012	0.036	−0.011	−0.010	0.016	2.0
B	0.013	0.012	0.010	0.005	0.005	−0.003	−0.003	−0.008	−0.011	−0.008	−0.014	−0.021	−0.002	−0.2
C	0.017	0.007	0.004	0.003	0.005	−0.007	−0.008	−0.011	−0.012	−0.009	−0.015	−0.022	−0.004	−0.5
D	0.016	0.010	0.009	0.001	0.003	−0.005	−0.009	−0.013	−0.014	−0.011	−0.012	−0.020	−0.004	−0.5
E	0.011	0.005	0.004	0.006	0.002	−0.007	−0.006	−0.011	−0.013	−0.009	−0.012	−0.023	−0.004	−0.5
F	0.009	0.004	0.008	0.004	0.001	−0.006	−0.006	−0.013	−0.013	−0.011	−0.014	−0.022	−0.005	−0.6
G	0.007	0.005	0.004	−0.001	−0.002	−0.011	−0.010	−0.016	−0.008	−0.011	−0.011	−0.021	−0.006	−0.8
H	−0.052	0.006	−0.047	−0.004	−0.028	−0.009	−0.079	−0.016	−0.057	−0.011	−0.050	−0.021	−0.031	−3.7
CM[c]	0.005	0.010	0.000	0.010	−0.002	−0.001	−0.016	−0.007	−0.018	−0.004	−0.017	−0.020	−0.005	
%CM[d]	0.6	1.2	0.0	1.3	−0.2	−0.1	−1.9	−0.8	−2.1	−0.5	−2.1	−2.4		

[a] Rowwise means of individual well absorbance differences.

[b] Rowwise means of individual well absorbance differences as a percent of the absorbance grand mean; row A example: [(0.016/0.827) × 100] = 2.0%.

[c] Columnwise means of individual well absorbance differences.

[d] Columnwise means of individual well absorbance differences as a percent of the absorbance grand mean; column 1 example: [(0.005/0.827) × 100] = 0.6%.

For each data set N2 − N1 and N2 − R1, calculate columnwise and rowwise mean as % of $\bar{x}$; plot these against row or column number to show error patterns clearly.

Calculate the range of columnwise and rowwise difference means, the coefficient of variation of individual well differences, and the maximum absolute value individual well difference (all expressed as percent of $\bar{x}$) as measures of error distribution. The following examples refer to the N2 − R1 data set, which is shown in Table 3.

Example: Maximum absolute value individual well difference (as percent of $\bar{x}$): N2 − R1 well H7 value is −0.079; absorbance grand mean is 0.827; [(| − 0.079|/0.827) × 100] = 9.5%.

Example: Rowwise range = 2.0% − (−3.7%) = 5.7%; (row A %RM − row H %RM).

Example: well CV = [(standard deviation for N2 − R1 data set/$\bar{x}$) × 100]; [(0.019/0.827) × 100] = 2.3%.

The N2 − R1 columnwise and rowwise ranges are estimates of bias across the width and height of the plate. The well coefficients of variation for repeated normal and repeated reversed readings are respectively single number estimates of the distribution of individual well imprecision and imprecision plus inaccuracy of the plate reader. The maximum individual well differences correspond to Bio-Tek's existing specifications (13) for precision (N2 − N1) and accuracy (N2 − R1). These calculations and graphs were done easily by computer and Lotus 1-2-3 (Lotus Development Corp.) using the /File Combine and Import commands to merge multiple data sets into one spreadsheet for further manipulation.

Results and Discussion

The reverse plate wet test described above was used for testing microplate readers. All data presented are from 405 nm single wavelength readings of Flow Linbro EIA plates using a 100 μL/well unless indicated otherwise. All absorbance grand means were between 0.7 and 1.0 unless indicated otherwise. A summary of plates used is given in Table 4.

Meniscus Dependence

To demonstrate the meniscus dependence of location dependent biases, a plate was read by a Flow Titertek Multiskan with no pre-wetting of the wells and using a *p*-nitrophenol solution in water. The hydrophobicity of the polystyrene plate greatly reduced meniscus formation, and the individual well liquid levels appeared flat when viewed from the side. When the same plate was read after pre-wetting and with the dye in buffer containing 0.05% Tween 20, the repeatability deteriorated dramatically for both repeated normal and re-

Table 4. Plates used for reverse plate wet test

No.	Company and name	Catalog No.	Material[a]	Well shape
1.	Flow Linbro EIA	76-381-04	PS	flat
2.	Dynatech Immulon 4	unknown	PS	flat
3.	Beckman Enhanced Protein Binding		PS	flat
4.	Nunc Immunoplate II	442404	PS	flat
5.	Dynatech Immulon II	3450	PS	flat
6.	Dynatech	2801	PVC	flat
7.	Nunc Immunoplate II	449824	PS	round
8.	Dynatech Immulon II	3650	PS	round
9.	Dynatech	2401	PVC	round
10.	Dynatech	2602	PS	vee
11.	Dynatech	2601	PVC	vee

[a] PS, polystyrene; PVC, polyvinyl chloride.

Table 5. Results of reverse plate wet test; effect of meniscus reduction on repeatability of Flow Titertek using Flow plate

	Normal repeat		Reverse repeat			
Meniscus	Well[a] CV	Well[b] max.	Well[a] CV	Well[b] max.	Col.[c] range	Row[d] range
Reduced	0.3	1.0	0.5	1.6	0.7	1.0
Normal	1.4	3.7	3.8	11.0	9.5	6.5

[a] CV of individual well differences (% of absorbance grand mean).
[b] Maximum individual well differences (% of absorbance grand mean).
[c] Range of columnwise means of individual well differences (% of absorbance grand mean).
[d] Range of rowwise means of individual well differences (% of absorbance grand mean).

peated reverse readings (Table 5). These data suggest that reduction of meniscus formation can sharply reduce reader bias, but this is not possible for many microplate procedures. For example, most immunoassays use detergent in their wash buffers, ensuring significant meniscus formation because of pre-wetting of the well sides.

Comparison of Single and Dual Wavelengths

A comparison of reverse plate wet test results for a Molecular Devices VMax reader in single and dual wavelength modes showed only minor differences in the magnitude of the observed errors. The normal repeatability, as coefficient of variation and maximum individual well N2 − N1 differences, was slightly improved (respectively, 0.1 and 0.6% at 405 nm; 0.1 and 0.3% at 405–650 nm). The reverse repeatability, as coefficient of variation and maximum of individual well N2 − R1 differences, was slightly decreased by reading in dual wavelength mode (respectively, 0.4 and 1.3% at 405 nm; 0.3 and 0.9% at 405–650 nm). Similarly, a comparison of single and dual wavelength readings for a Dynatech MR650 reader showed only slight improvement in reverse repeatability (respectively, 2.3 and 9.5% at 410 nm; 2.1 and 8.1% at 410–490 nm). These comparisons demonstrate that the liquid in the well rather than plate bottom defects or debris is the primary source of the observed errors for these plates.

Volume Dependence

The effect of well volume on reader error was investigated by performing the reverse plate wet test with 50 μL/well of dye solution and then by repeating the test with additional 50 μL/well increments of buffer. This maintained a constant total amount of dye per well while allowing changes in path length and meniscus curvature due to volume changes. Results obtained for the Flow Titertek Multiskan reader at volumes of 50, 100, 150, 200, and 250 μL/well showed that reader errors vary with well volume (Table 6). The error patterns were qualitatively the same at all 5 volumes and are illustrated by the 100 μL volume of Figure 1. Visual inspection of the meniscus formed at each volume showed that the meniscus curvature was greater at lower volumes, especially at 50 and 100 μL. This appears to have led to a non-linearity of average path length at 50 and 100 μL, resulting in decreased absorbance means 95 shown in Table 6. The greater meniscus curvature and shorter path length at the lower volumes also led to significantly greater reader errors at 50 and 100 μL as also shown in Table 6. Both surface scatter and path length differences can cause meniscus dependent reader errors, and this experiment does not distinguish between the two. However, these data suggest that the use of larger volumes at the reading step may be helpful in reducing reader errors.

Consistency of Error Patterns

Two microplate readers were tested by using the reverse plate wet test several times over 4–10 months to verify the consistency of error patterns. Patterns of inaccuracies can be visualized by graphing the rowwise and columnwise means of individual well differences. The error patterns were qualitatively and quantitatively consistent for both instruments (Figures 1 and 2). The Flow Titertek Multiskan reader of Figure 1 had previously been used for microplate-adapted protein assays, but results were equivocal due to intraplate inconsistencies. These problems could have been due to the location dependent reader bias shown in Figure 1. For older instruments especially, previously undetected biases may be a serious impediment to the adaptation of quantitative procedures such as protein assays (12) or enzyme assays (9, 10) to microplates.

Plate Effects

Error patterns for the Flow Titertek Multiskan reader of Figure 1 were examined by using 11 different plates. Flow Titertek readings of dry plates after air blanking showed low interwell variability. The only standard deviations above 0.005 absorbance were those for 2 round-bottom and one V-bottom polystyrene plates, ranging from 0.013 to 0.021. Mean absorbances against air ranged from 0.039 to 0.138. The same plates were then wetted and loaded with dye for the reverse plate wet test (Table 7; Figures 3 and 4). Table 7 shows that errors are substantially reduced for the round-bottom polystyrene plates compared with the flat-bottom polystyrene plates. This observation is consistent with decreased path length differences in the round-bottom plates due to the similarity in curvature between meniscus and well-bottom shape but could also be due to a lensing effect of the curved well-bottom shape. This difference suggests that use of round-bottom plates may be helpful for the attenuation of reader errors. For solid-phase immunoassays, the effect of surface properties on protein binding is likely the most important cause of error. In this case, well-bottom shape is a secondary consideration but may still be important for assay error reduction. However, for procedures such as protein or enzyme assays which are entirely liquid-phase, this is an especially important observation because reader error may be larger than that from any other source. The flat-bottom PVC plate stands apart from the flat-bottom polystyrene plates because of its reduced error (Table 7; Figures 3a and 4a). This is likely due to its narrower wells which provide for a

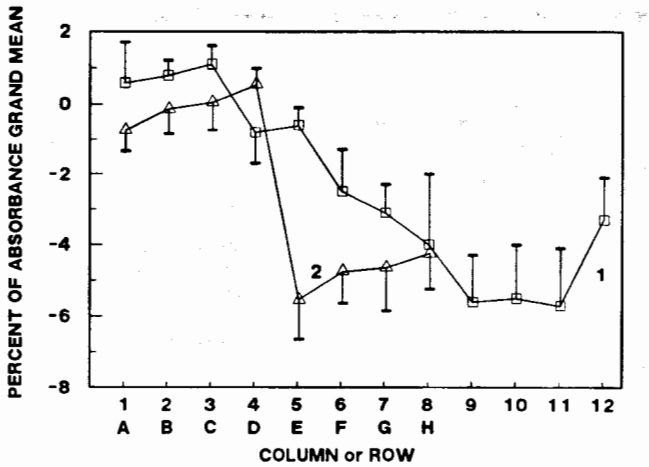

Figure 1. Results of reverse plate wet test for Flow Titertek Multiskan reader using 100 μL/well in Flow Linbro EIA plate. Columnwise (1) and rowwise (2) means of N2 − R1 individual well differences as percent of absorbance grand mean. Data points represent means and standard deviations of 4 tests over 8 months.

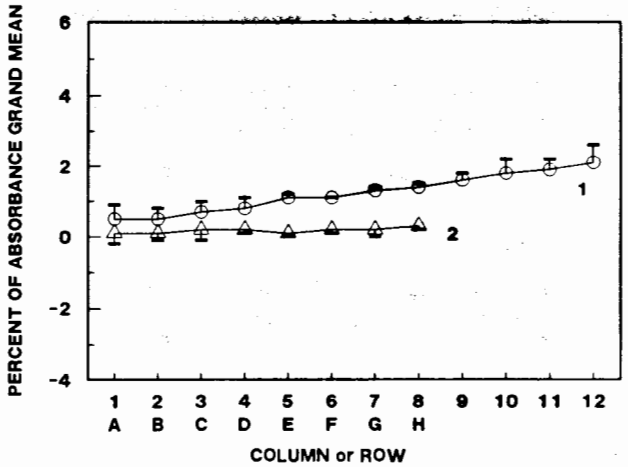

Figure 2. Results of reverse plate wet test for Molecular Devices VMax reader using 100 μL/well in Flow Linbro EIA plate. Columnwise (1) and rowwise (2) means of N2 − R1 individual well differences as percent of absorbance grand mean. Data points represent means and standard deviations of 4 tests over 4 months.

longer total path length and reduced error as shown in Table 6.

Figure 3 indicates that rowwise errors for the Flow Titertek reader are not qualitatively different for plates from different manufacturers, except for the inversion of the curves for the non-flat-bottom plates (Figure 3b) compared with flat-bottom plates (Figure 3a). This inversion suggests that the reader errors shown are due to path length differences and that the well centers of the non-flat bottom plates represent path length maxima rather than minima, as this is the case for the flat-bottom plates. However, the qualitative pattern of columnwise errors varies greatly for all 11 plates (Figure 4). This apparent discrepancy between rowwise and columnwise patterns is not inconsistent with reader dependence of the observed errors since 2 independent parts of the reader are responsible for errors propagated in 2 directions. With the Flow Titertek reader rowwise reading errors depend on the fixed alignment of the optic fibers, lenses, and detectors for each channel while columnwise reading errors are dependent on the indexing of the plate carrier during each reading.

The same 11 plates were read on the Molecular Devices VMax reader of Figure 2, and reverse plate wet test results were calculated (Table 8). With the VMax reader, like the Flow Titertek reader, the lowest errors were seen with the round-bottom polystyrene plates and the highest with the V-bottom plates. These results illustrate that a properly aligned reader will read a variety of plates properly. The sole exception among the 11 plates tested was a V-bottom polystyrene plate which exhibited high variability when read dry (SD = 0.018). This plate was read in both single and dual wavelength modes using the same VMax reader, but it still showed higher errors in dual wavelength mode than any of the other 10 plates in single wavelength mode (Table 8). Plate differences, including well-bottom shape, appear to be a major problem only if the reader has significant bias already.

Despite the qualitative differences shown in Figure 4, these tests clearly show that the errors observed for the Flow Titertek reader are due to the reader and that the different plates merely present these errors differently. However the interaction between plates (including manufacturer, well-bottom shape, material, and well dimensions) and reader biases is a potentially serious source of systematic error in poorly aligned instruments. It is important when applying the reverse plate wet test to use the same type of plate as used routinely for quantitative microplate procedures.

Precise Repetition of Severe Bias

The severest bias of any instrument tested was shown by a BioTek EL310 reader which exhibited excellent repeatability. This reader was a demonstration unit that passed all of the built-in diagnostic tests upon start-up each time. The normal repeatability was excellent (Table 9). The individual well coefficient of variation was 0.2%, and the maximum individual well difference was 0.4% and within original specifications (13). But, the location dependent bias was large. For repeated reversed readings, the range of columnwise difference means was 27.7%, and the range of rowwise difference means was 7.1%. The individual well coefficient of variation was 9.8%, and the maximum individual well difference was 26.1%. This instrument's worst bias was channel dependent (each column is read by a different channel). The extensive movement and handling of this demonstration instrument likely contributed to its defects and would not be duplicated in most instruments in laboratory use. Since these defects are not detected by the routine diagnostics built into the reader, the reverse plate wet test should be routinely used to test for them. This is recommended by Bio-Tek (13).

Table 6. Results of reverse plate wet test; effect of volume on repeatability of Flow Titertek using Flow plate (buffer was added in 50 μL increments to initial dye volume of 50 μL)

Volume/well, μL	Mean OD405	Normal repeat Well[a] CV	Normal repeat Well[b] max.	Reverse repeat Well[a] CV	Reverse repeat Well[b] max.	Reverse repeat Col.[c] range	Reverse repeat Row[d] range
50	0.785	1.0	3.6	5.1	10.4	11.5	7.1
100	0.955	0.6	1.4	3.4	7.6	9.2	4.5
150	1.007	0.6	2.3	2.6	6.6	6.9	3.1
200	1.007	0.5	1.2	2.2	5.2	5.7	2.3
250	1.007	1.0	4.2	2.4	5.6	6.1	1.6

[a] CV of individual well differences (% of absorbance grand mean).
[b] Maximum individual well difference (% of absorbance grand mean).
[c] Range of columnwise means of individual well differences (% of absorbance grand mean).
[d] Range of rowwise means of individual well differences (% of absorbance grand mean).

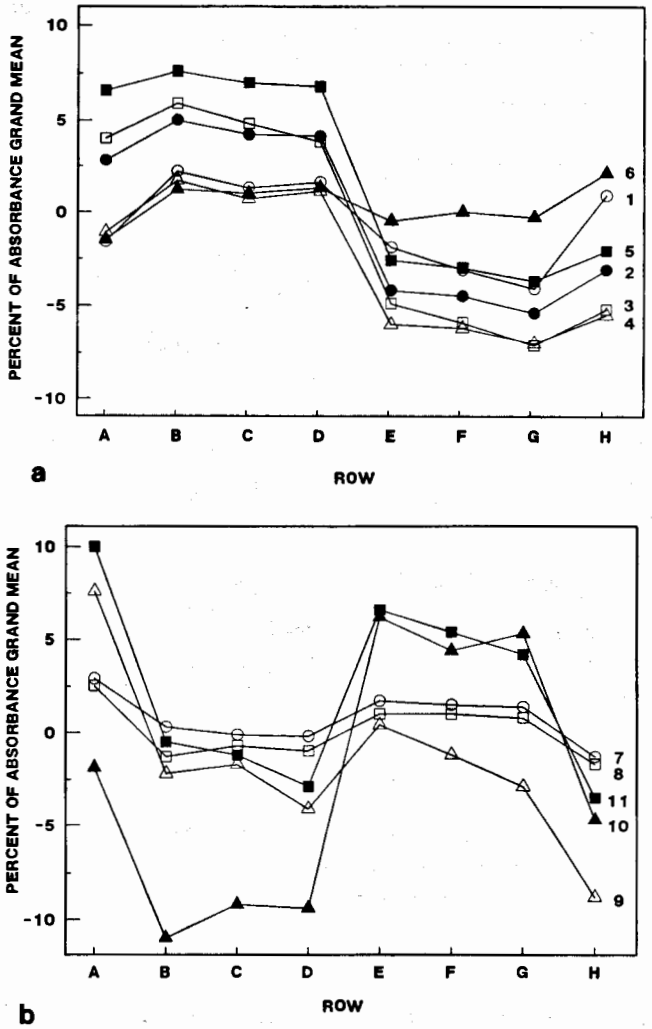

Figure 3. Results of reverse plate wet test for Flow Titertek Multiskan reader using 100 μL/well in 11 different plates. Rowwise means of N2 − R1 individual well differences as percent of absorbance grand mean for (a) 6 flat-bottom and (b) 5 round- or V-bottom plates. Plate numbers correspond to full plate information given in Table 4.

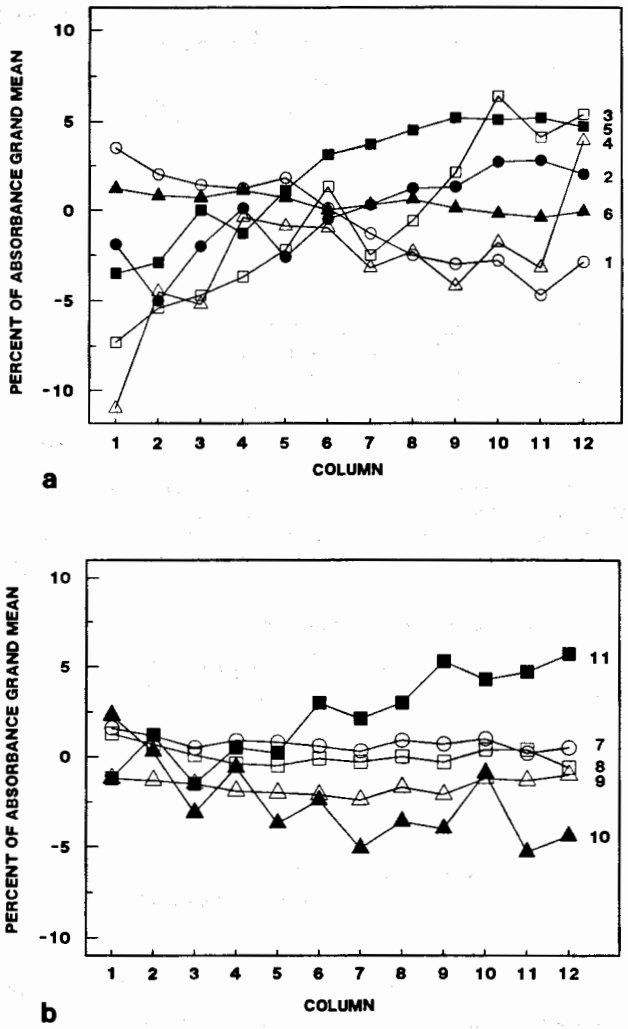

Figure 4. Results of reverse plate wet test for Flow Titertek Multiskan reader using 100 μL/well in 11 different plates. Columnwise means of N2 − R1 individual well differences as percent of absorbance grand mean for (a) 6 flat-bottom and (b) 5 round- or V-bottom plates. Plate numbers correspond to full plate information given in Table 4.

Dynatech MR650 Design Modification

Among the instruments tested was a Dynatech MR650, a single channel reader which reads when the plate is moving (columnwise serpentine fashion) rather than when the plate is still. An earlier test of this model showed significant accuracy and precision problems when compared to other Dynatech readers (Table 9, MR650#1). The results for a second MR650 (Table 9, MR650#2) agreed quantitatively with the previous results and exhibited a consistent pattern of single well errors at opposite ends of alternate rows. Dynatech confirmed that this pattern was typical of the MR650. Because of these test results, Dynatech initiated design modifications for the MR650 relating to synchronization of plate movement and reading, and then provided a modified instrument for testing by the authors. A striking improvement is apparent in the reverse plate wet test data from the modified instrument. For the 2 readers tested before the design change (Table 9), the N2 − N1 well CV values were 1.1 and 1.7% for MR650#1 and MR650#2, respectively, N2 − R1 well CV values were 2.3% for both readers, and the maximum N2 − R1 individual well differences were 9.0 and 9.5% for MR650#1 and MR650#2, respectively. For the modified MR650 (Table 9, MR650#3), the N2 − N1 well CV value was 0.3%, the N2 − R1 well CV value was 0.5%, and the maximum N2 − R1 individual well difference was 1.4%. This example illustrates the necessity of using a realistic quality control test (i.e., meniscus based) such as the reverse plate wet test at all stages of design and manufacture of vertical beam microplate readers, in addition to filter based calibration and precision testing.

Possible Inadequacy of Routine Maintainence Procedures

The Flow Titertek reader of Tables 5–7 was returned to the manufacturer for service and calibration. The reader passed the routine quality control tests before being returned, but its performance was not significantly improved (Table 9). The error patterns of Figure 1 were qualitatively and quantitatively preserved (Table 9). This failure to improve the instrument's performance demonstrates that its problems were not due to poor calibration or optical-electronic matching of channels. It also shows that defects causing meniscus dependent errors may fall outside the scope of some maintainence programs. This was not the case for a Dynatech MR600 reader; its performance was significantly improved by repairing an alignment defect (R. O. Harrison and J. O. Nelson, unpublished data). The difficulty of such repairs will, of course, vary with the instrument design.

Table 7. Results of reverse plate wet test for several plates with one Flow Titertek reader; single test (3 readings) for one plate of each type using 100 μL/well and single wavelength reading at 405 nm (full plate information in Table 4)

No. from Table 4	Plate (well shape)[a]	Normal repeat		Reverse repeat			
		Well[b] CV	Well[c] max.	Well[b] CV	Well[c] max.	Col.[d] range	Row[e] range
1.	Linbro EIA (F)	0.9	2.4	3.4	9.4	8.2	6.3
2.	Dynatech Immulon 4 (F)	1.5	5.9	4.9	10.0	7.9	10.4
3.	Beckman (F)	1.5	5.8	6.8	13.3	13.7	13.0
4.	Nunc Immunoplate II (F)	2.5	10.5	5.1	14.5	14.7	8.7
5.	Dynatech Immulon II (F)	1.2	5.0	5.9	10.5	8.7	11.2
6.	Dynatech (PVC, F)	0.7	2.4	1.3	2.8	1.6	3.6
7.	Nunc Immunoplate II (R)	0.6	2.6	1.3	3.8	1.3	4.2
8.	Dynatech Immulon II (R)	0.4	1.3	1.5	5.2	1.8	4.2
9.	Dynatech (PVC, R)	0.4	1.0	4.4	9.6	1.4	16.4
10.	Dynatech (PS, V)	2.3	6.9	7.2	18.4	7.6	17.2
11.	Dynatech (PVC, V)	0.6	2.8	5.3	11.9	7.2	13.5

[a] Shape of well bottom: F, flat; R, round; V, vee.
[b] CV of individual well differences (% of absorbance grand mean).
[c] Maximum individual well difference (% of absorbance grand mean).
[d] Range of columnwise means of individual well differences (% of absorbance grand mean).
[e] Range of rowwise means of individual well differences (% of absorbance grand mean).

Conclusions and Recommendations

While well location dependent biases may be minor in most cases, they appear to occur often enough to be of significant concern, especially in rigorously quantitative microplate procedures. Of greater concern, however, is the insidiousness of these biases, which can be of catastrophic magnitude even in instruments exhibiting excellent repeatability. Even the severest biases observed in this study were unrecognized by the users until revealed by the reverse plate wet test. Furthermore, these errors are systematic rather than random and are compounded with all the other errors accumulated in the procedure. The simplicity of the reverse plate wet test allows routine quality control tests to be run for all microplate readers regardless of design or location and in addition to other tests for linearity, sensitivity, and precision.

The reverse plate wet test should be used for routine manufacturing quality control for all instruments before sale and for routine laboratory or field quality control to maintain instrument performance. This test is employed by at least one reader manufacturer for new instruments and is recommended in the user manual for field quality control (13), but the importance of routine testing is not always appreciated by the user. Instrument service facilities should include this test in their routine repair workup and should develop repair protocols to address the problems identified by the test. Instrument designers would be wise to employ this test on prototype instruments before manufacturing scale-up. This may become increasingly important as interest in faster readings for kinetics applications spurs the use of dynamic reading modes which require extremely precise synchronization of plate movement and reading.

Instrument designers should also address the issue of repairing misalignment. Increasing awareness of potential instrument biases and increasing use of rigorously quantitative microplate-based methods will place an increasing demand on instrument manufacturers for low bias instruments. The importance of bias testing is reinforced by the use of standard plate layout templates in quantitative microplate procedures. Commercial data analysis software and multichannel pipetters allow easy template standardization and decrease the flexibilty of placement of replicates which has been suggested as a method of bias neutralization (14, 15). The interaction between repeatable location dependent reader biases and

Table 8. Results of reverse plate wet test for several plates with one Molecular Devices VMax reader; single test for one plate of each type using 100 μL/well and single wavelength reading at 405 nm (full plate information in Table 4)

No. from Table 4	Plate (well shape)[a]	Normal repeat		Reverse repeat			
		Well[b] CV	Well[c] max.	Well[b] CV	Well[c] max.	Col.[d] range	Row[e] range
1.	Linbro EIA (F)	0.2	0.8	0.5	2.2	0.6	0.6
2.	Dynatech Immulon 4 (F)	0.1	0.3	0.2	0.8	0.5	0.5
3.	Beckman (F)	0.1	0.5	0.4	0.9	0.5	0.6
4.	Nunc Immunoplate II (F)	0.1	0.3	0.4	1.2	1.0	0.7
5.	Dynatech Immulon II (F)	0.3	2.4	0.4	2.0	0.8	0.5
6.	Dynatech (PVC, F)	0.2	0.7	0.4	1.2	0.8	0.9
7.	Nunc Immunoplate II (R)	0.1	0.4	0.2	0.6	0.9	0.4
8.	Dynatech Immulon II (R)	0.1	0.2	0.2	0.6	0.3	0.3
9.	Dynatech (PVC, R)	0.1	0.4	0.5	2.1	0.7	0.6
10.	Dynatech (PS, V)	0.1	0.3	1.6	8.0	3.4	2.9
10.	Dynatech (PS, V)[f]	0.1	0.2	0.8	2.8	1.7	0.7
11.	Dynatech (PVC, V)	0.1	0.2	0.7	2.9	1.0	1.0

[a] Shape of well bottom: F, flat; R, round; V, vee.
[b] CV of individual well differences (% of absorbance grand mean).
[c] Maximum individual well difference (% of absorbance grand mean).
[d] Range of columnwise means of individual well differences (% of absorbance grand mean).
[e] Range of rowwise means of individual well differences (% of absorbance grand mean).
[f] Repeated reading using dual wavelength, 405–650 nm.

Table 9. Results of reverse plate wet test for 16 readers; single test (3 readings) for each reader using 100 μL/well in a Flow Linbro plate (No. 76-381-04) and single wavelength reading at 405 or 410 nm, unless noted otherwise

		Normal repeat		Reverse repeat			
Reader (design)[a]	Use[b]	Well[c] CV	Well[d] max.	Well[c] CV	Well[d] max.	Col.[e] range	Row[f] range
BioTek EL309 (12)#1[g]	N	0.2	0.5	0.5	1.5	1.3	1.1
BioTek EL309 (12)#2	R	0.2	0.6	0.7	1.7	2.4	0.2
BioTek EL310 (12)	D	0.2	0.4	9.8	26.1	27.7	7.1
Dynatech MR580 (1)	R	0.2	0.5	1.2	3.5	3.0	2.6
Dynatech MR600 (1)[g]	N	0.2	0.6	0.5	3.3	0.7	0.9
Dynatech MR650#1 (1M)[g]	R	1.7	7.2	2.3	9.0	3.5	2.5
Dynatech MR650#2 (1M)	D	1.1	6.3	2.3	9.5	3.7	5.7
Dynatech MR650#3 (1M)	D	0.3	1.1	0.5	1.4	0.8	0.6
Dynatech MR700 (1)[g]	N	0.4	1.9	0.6	3.2	1.1	0.8
Flow Titertek (8)[h]	R	0.8	3.0	3.6	9.6	7.6	6.2
(SD)		(0.3)	(1.6)	(0.6)	(1.8)	(1.6)	(1.2)
Flow Titertek (8)[h,i]	R	1.1	3.6	3.2	8.3	7.5	5.2
(SD)		(0.3)	(1.3)	(0.5)	(2.0)	(1.7)	(1.3)
Flow/HP-Genenchem (8)	D	0.2	0.6	0.7	2.2	0.9	1.7
VMax#1 (96)	D	0.2	0.6	0.7	4.2	1.1	0.9
VMax#2 (96)[h]	N, R	0.1	0.5	0.5	1.7	0.9	0.4
(SD)		(0.0)	(0.1)	(0.1)	(0.7)	(0.4)	(0.2)
VMAX#3 (96)	N	0.3	1.5	0.7	1.7	1.6	1.2
SLT-EAR400 (8M)	R	0.2	0.8	0.6	1.4	1.1	1.2
SLT-EAR340 (8M)	D	0.2	0.8	0.9	2.3	1.0	2.9

[a] Number of reading channels; reads while plate is stopped unless marked with M to indicate that readings are made during plate movement.
[b] Use abbreviations: N, new; D, demonstration; R, research.
[c] CV of individual well differences (% of absorbance grand mean).
[d] Maximum individual well difference (% of absorbance grand mean).
[e] Range of columnwise means of individual well differences (% of absorbance grand mean).
[f] Range of rowwise means of individual well differences (% of absorbance grand mean).
[g] Data provided by the manufacturer, plate type unknown.
[h] Means and standard deviations (SD) of 4 tests.
[i] After repair and calibration by the manufacturer.

standardized assay templates is a potentially serious source of error which demands more study.

Manufacturers should confront the issue of well location dependent biases by writing instrument specifications which explicitly address the bias problem through use of the reverse plate wet test. Potential purchasers should demand reversed plate readings and discuss them with sales representatives. It is interesting to note the potential rigor of the reverse plate wet tests in specifying repeatability and accuracy. The standard set by Bio-Tek (13) for repeatability (no N2 − N1 individual well differences greater than 0.005 and 0.5%) is met by only 4 of the 16 readers of Table 9. The Bio-Tek specification for accuracy (as reverse repeatability; no N2 − R1 individual well differences greater than 0.010 and 1.0%) is not met by any of the 16 readers of Table 9. However, the VMax#2 reader of Table 9 exceeds the accuracy specification in single wavelength mode with some plates as shown in Table 8 and with the Flow Linbro plate in dual wavelength mode. The use of alternate plates or dual wavelength reading may bring some of the readers examined in this study within the Bio-Tek specifications, but this appears unlikely for many instruments from Table 9.

The problem of location dependent biases is clearly an area where the present sources of error can and should be identified, quantified, and controlled. The viability of quantitative analytical methods based on microplate readers hinges on the availability of reliable instrumentation with minimal inherent bias.

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