Variability in Enzyme-Linked Immunosorbent Assays and Control of Experimental Error by Use of Experimental Designs

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ABSTRACT

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Seven uniformity trials were done on Nunc-Immuno IF and Dynatech Immulon 1 "U" microplates with two monoclonal triple-antibody sandwich enzyme-linked immunosorbent assays to determine if an experimental design was necessary to control plate variability. A single uniform extract of either oat (cv. Clintland 64) tissue infected with barley yellow dwarf luteovirus (BYDV-PAV-IL) or soybean (cv. Williams 82) tissue infected with soybean mosaic potyvirus (SMV-G5) was prepared, and samples were placed in the wells of eight microplates used for each trial. For whole plates, mean absorbances varied within trials and coefficients of variability ranged from 3.8 to 20.3%. Seventeen of the 56 plates tested had one to five wells with absorbances that were outliers from the normal distribution. Nonrandomized designs of two replications of 48 treatments were also assigned to each plate. When replications of a treatment were paired in either rows or columns, differences between treatment means were confounded with row and column differences, indicating the need for an experimental design on microplates. Both the randomized complete block design and the alpha (0,1) one-restrictional resolvable incomplete block design allowed for more precision than the completely randomized design. The smaller, incomplete blocks of the alpha design afforded slightly more precision than the larger, complete blocks of the randomized complete block design.

The enzyme-linked immunosorbent assay (ELISA) is commonly used for detection of plant viruses. The color intensity of the enzyme product produced in the final step of the assay is proportional to the amount of virus present and can be used to quantify virus titer. Titer may be quantified with a calibration curve generated by serial dilution of a standard preparation. Absorbances of test samples are compared with absorbances of the calibration curve, and antigen content is estimated. Methods are well established (13), and the theory and development of calibration curves have received attention in recent years (1,19,20,24). Although calibration curves can be and are used to equate absorbances on several plates (14), they do not reduce the effect of well-to-well variation on a single plate.

When determining differences in antigen concentration of selected preparations, investigators may be concerned more with tests for significant differences between treatments than with estimation of antigen. Therefore, once a procedure has been established to detect differences in antigen content within the expected ranges, absorbance values are compared and no attempt is made to quantify the virus itself (10,14,15).

Regardless of the approach used, variability in the absorbances of microplate wells treated in the same manner has been observed both within and among microplates (2,7,16,17). Variability within microplates was not random, and individual plates had areas or columns of higher or lower than average absorbances. Clark and Adams (7) suggested that the outer rows and columns not be used, leaving the central 60 wells of the microplate for the assay. Hebert et al (16) proposed pairing duplicates of test samples and duplicates of negative controls so that control absorbances could be subtracted from test sample absorbances. Burrows and coworkers (2) proposed arranging test samples on the plates according to classical experimental designs. They concluded that designs with compact blocking features and two-dimensional control over spatial patterns, such as the Youden square and lattice square, could increase precision substantially. Unfortunately, these designs cannot efficiently accommodate a large number of treatments on a single microplate. Though 7×7 lattice squares were tested, this allowed for only one replication of test samples on each of several plates, leaving 47 wells per plate unused.

One experimental design that can be applied to quantitative assays on microplates is the completely randomized design (CRD) in which replications of each treatment are randomized across the entire microplate. The randomized complete block (RCB) is slightly more complicated. The microplate is divided into blocks into which groups of test samples are randomly arranged such that each block contains all treatments. Experimental precision is increased with RCB designs through error control because variation among treatments within the block should be less than variation among treatments in different blocks. However, randomization across the entire microplate or the use of complete blocks may not control variation when the experiment has a large number of treatments; as the area of the microplate within each complete block increases, intrablock variation also increases.

Incomplete block designs may be preferable to complete block designs. Only a subset of the treatments is present in a single block of an incomplete block design. Alpha (0,1) one-restrictional resolvable incomplete block designs (alpha designs) were originally developed for use in cultivar field trials (3–5, 22) and can be adapted to microplates (Fig. 1). These designs are generalized lattices and include most, but not all, of the square lattices and rectangular lattices described by Federer (11) and Cochran and Cox (8). These are resolvable designs because if all the small incomplete blocks in a single replication are combined, a complete block is made. Smaller blocks result in less intrablock variation. The one restriction on the randomization is that a given pair of treatments will be found together once or not at all in a single block. Sums of squares for the total, replication, treatment, and the error sources of variation are calculated just as in a RCB design. The RCB error sum of squares is then partitioned into two parts: 1) the variability among blocks within replications adjusted for the particular treatments that occur in specific incomplete blocks and 2) the intrablock variability or random variation among experimental units within blocks. Treatment means can be adjusted for block effects by using the interblock information (number 1, above), and use of the average effective error variance allows all comparisons to be made with equal precision. Programs and documentation for alpha designs are available on request from the Agronomy Statistical Lab, W201 Turner Hall, 1102 S.

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ELISA has become a widely used technique for detection and quantification of viruses, other pathogens, and proteins. There is a need to ensure that microplate uniformity is addressed. In addition, an experimental design that can accommodate a large number of treatments is needed. The objectives of this study were to characterize the variability among and within microplates, to determine if an experimental design was necessary to control the variation within microplates, and to determine if the alpha design could control within-microplate variation more effectively than the CRD and RCB designs.

MATERIALS AND METHODS

To study variation within and among ELISA microplates, a single large extract of virus-infected tissue was prepared and samples of the extract were placed in all wells of several microplates. All wells and all microplates were processed in the same manner, so that variability in absorbances is a measure of experimental error (σ_e) of microplates. The effects of experimental designs on experimental error were studied by assigning a treatment to each well on each plate as dictated by individual designs. Different designs were compared by reassigning each well to a different treatment, again as each experimental design dictated. Experimental errors produced by the designs on microplates were compared, and designs producing the lowest experimental errors were identified.

Tissue extracts. Oat (*Avena sativa* L.) cv. Clintland 64 tissue infected with barley yellow dwarf luteovirus strain BYDV-PAV-IL (9) was harvested 14 days after inoculation. The tissue was ground in liquid nitrogen. After the powdered tissue thawed, phosphate-buffered saline (PBS: 0.137 M NaCl, 0.0015 M KH_2PO_4, 0.008 M Na_2HPO_4, 0.003 M KCl, 0.003 M NaN_3, pH 7.4) containing 0.5% Tween 20 was added (1 g/5 ml). The slurry was mixed thoroughly, filtered through cheesecloth, and partially clarified by centrifugation at 3,900 rpm for 25 min in a Beckman GPR centrifuge. Noninoculated oat tissue prepared in the same manner was used as a negative control. Soybean (*Glycine max* (L.) Merr.) cv. Williams 82 trifoliolates infected with soybean mosaic potyvirus strain SMV-G5 (6) were ground in PBS-Tween; as described above, the sample was filtered and partially clarified. Noninoculated Williams 82 tissue prepared in the same way was used as a negative control.

Triple-antibody sandwich ELISA (TAS-ELISA). Methods developed by D'Arcy et al (9) for luteoviruses and by Hill et al (18) for potyviruses were used. Reagents and test samples were applied with a Titertech eight-channel pipette to Dynatech microplates at 50 µl per well and to Nunc microplates at 100 µl per well. Blocking agents were applied at double the volume. Incubations, except for blocking and substrate, were overnight at 4 C. Between steps, each plate was washed four times with distilled tap water. Plates were coated with polyclonal immunoglobulin (1 µg/ml) diluted in carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, 0.003 M NaN_3, pH 9.6). BYDV-PAV-IL microplates were blocked with 0.1% nonfat dry milk diluted in PBS for 1 hr and SMV-G5 microplates were blocked with 0.1% bovine serum albumin for 1 hr, both at room temperature. After application and incubation of tissue extracts, monoclonal antibodies (MAbs) were added. BYDV-PAV-IL MAbs were diluted to 1 µg/ml and SMV MAbs were diluted 0.5 µg/ml in PBS-Tween and their respective blocking agents. Conjugate (rabbit anti-mouse monoclonal antibodies A1902, Sigma Chemical Co., St. Louis, MO) and, finally, substrate (*p*-nitrophenyl phosphate, Sigma 104-105) were added. In general, absorbances were read on a Dynatech MR 700 ELISA Plate Reader 30–45 min after the addition of substrate.

Uniformity trials. Seven uniformity trials were done, each comprising eight microplates (Table 1). Within each trial all plates received the same sample of infected tissue extract. An additional microplate containing 48 wells of infected extracts and 48 wells of noninfected extracts was added to each trial to assure that the TAS-ELISA was functioning correctly, but this microplate was not included in the analysis. In trials 2–7, the substrate reaction rates were slowed by the addition of 50 µl of 3 M NaOH before absorbances (A_{410}) were determined. Two brands of microplates, flat-bottomed Nunc-Immuno IF microplates (VWR Scientific, Chicago, IL) and round-bottomed Immulon 1 "U" microplates (Dynatech Laboratories, Inc., Chantilly, VA), both with wells arranged in an 8 × 12 rectangular grid, were tested. All plates were from one lot from each manufacturer. Although there were slight differences in procedures among trials (operators, incubation times of the substrates, and the application of NaOH), all statistical comparisons were done on the eight microplates within a single trial in which all procedures were constant. Mean absorbances and variances were not compared among trials.

Analysis. The grand mean absorbances and coefficients of variation (CV) were calculated for each microplate. The assumption that the data were normally distributed was evaluated by regressing the observed data on the corresponding expected values of ranked normal deviates. The Blom plotting position ($p_i = (i - 0.375)/(n + 0.25)$) and the correlation coefficient test was used (21).

	1	2	3	4	5	6	7	8	9	10	11	12
A	38	10	44	22	26	7	6	20	12	19	1	25
B	34	31	35	19	32	18	21	7	5	45	41	17
C	33	16	2	11	21	28	33	27	31	16	43	44
D	4	24	6	23	8	37	39	9	14	42	8	29
E	40	29	30	41	14	15	15	4	24	26	2	47
F	5	36	12	13	39	25	18	38	10	11	34	32
G	48	43	42	17	9	45	28	23	36	37	46	40
H	47	20	27	46	1	3	48	22	30	35	13	3

Fig. 1. A microplate with 48 treatments arranged in an alpha design with two replications. The replications are enclosed in bold lines and the resolvable, incomplete blocks are enclosed in dashed lines.

Table 1. Antigen, microplate brand, and operator tested in each uniformity trial

Trial	Antigen	Microplate brand	Substrate incubation time (min)	Operator
1	BYDV-PAV-IL	Dynatech	30	1
2	BYDV-PAV-IL	Dynatech	40	1
3	SMV-G5	Nunc	120	1
4	SMV-G5	Nunc	40	1
5	SMV-G5	Dynatech	45	1
6	BYDV-PAV-IL	Nunc	45	1
7	BYDV-PAV-IL	Nunc	45	2

Table 2. Absorbance means (A_{410}) and coefficients of variation for each microplate in seven uniformity trials

	Trial 1		Trial 2		Trial 3		Trial 4		Trial 5		Trial 6		Trial 7	
Plate	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
1	1.3406	9.4	1.0418	11.3	1.3804	9.5	1.4780	8.5	1.1882	4.2	1.1327	8.3	1.1628	13.2
2	1.2369	11.6	0.8842	11.0	1.1808	10.1	1.2505	8.3	1.0757	3.8	1.1163	7.2	1.0367	11.2
3	1.1029	16.9	0.8481	9.2	1.0401	11.4	1.0995	8.8	1.0539	4.9	0.9702	12.8	0.9646	10.4
4	1.3024	8.4	0.8623	10.7	0.9726	10.9	0.9818	7.0	1.0223	5.0	0.7793	15.8	0.9465	11.8
5	1.2228	11.5	0.8616	6.9	1.0160	8.0	1.4702	9.3	1.1703	5.0	1.1653	8.9	1.0353	6.8
6	1.1520	8.9	0.7685	8.5	1.4676	12.8	1.1919	11.8	1.0612	5.0	0.8678	11.7	1.0233	9.1
7	1.2210	13.6	0.9066	10.6	1.2213	13.6	1.0060	14.5	1.0561	5.5	0.8103	9.1	1.0328	7.7
8	1.1447	13.8	0.8022	8.4	1.0382	13.2	0.8901	18.1	0.9404	5.7	0.6262	20.3	1.0498	7.9

Table 3. F_{max} values produced by 12 randomizations of the alpha design among randomizations within a single microplate in seven uniformity trials

	F_{max} values[a]						
Plate	Trial 1	Tiral 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7
1	1.97	1.67	1.70	2.29	1.81	1.44	1.48
2	1.48	1.90	1.80	2.19	1.98	1.59	2.27
3	1.68	1.72	1.48	1.67	1.47	2.07	1.30
4	1.86	2.10	2.01	1.83	2.08	2.29	2.08
5	1.97	1.76	2.03	1.64	1.60	1.61	1.70
6	1.48	1.92	1.27	2.02	1.79	1.75	1.84
7	1.59	1.55	1.44	1.58	2.10	1.26	1.64
8	1.77	1.69	1.91	1.46	1.74	1.91	1.75

[a] Largest alpha design average effective error variance produced by 12 randomizations on a single microplate divided by the smallest average effective error variance produced by the 12 randomizations on the same microplate.

Table 4. F_{max} values produced by 12 randomizations of the alpha design among microplates within a single randomization in seven uniformity trials

	F_{max} values[a]						
Randomization	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7
1	3.46	4.15*[b]	5.78**	3.36	2.08	3.65*	5.71**
2	4.65**	6.62**	7.28**	2.15	3.38	3.02	5.38**
3	4.74**	4.99**	7.48**	4.34*	1.48	3.44	6.76**
4	2.33	3.90*	10.18**	4.54*	1.88	2.71	4.24*
5	4.09*	6.40**	7.01**	3.22	1.82	3.31	6.74**
6	3.47	4.23*	7.03**	4.01*	2.28	3.75*	7.12**
7	4.97**	4.71**	5.42**	2.18	2.14	3.45	6.52**
8	3.82*	4.26*	6.62**	3.16	2.05	2.88	6.63**
9	4.03*	4.77**	5.28**	4.61**	1.95	8.44**	4.86**
10	3.96*	4.73**	6.48**	3.69*	1.59	2.90	5.35**
11	3.41	4.87**	5.65**	2.89	1.86	3.06	6.04**
12	5.61**	4.77**	5.93**	2.29	2.19	3.22	6.47**

[a] Largest alpha design error variance produced by a given randomization on microplates divided by smallest average effective error variance produced by that randomization on the microplates. [b] * = Significant at $P \leq 0.05$ and ** = significant at $P \leq 0.01$.

Analysis of variance and F tests were used to detect statistical differences among rows and columns of all microplates. Two replications of 48 treatments were artificially imposed on microplates in two nonrandom designs. First, two replications of each treatment were paired side by side in rows, and analysis of variance was then used to detect differences between paired treatments. Second, two replications of each treatment were paired side by side in columns, and analysis of variance was used to detect differences between treatments. Next, randomized designs were tested. Twelve randomizations of a CRD, an RCB design, and an alpha design with 48 treatments and two replications were assigned to each of the eight microplates within a trial. The microplate-randomization combinations totaled 96 for each design within each trial. The CRD and RCB error variances and alpha design average effective error variance were calculated for each microplate-randomization combination. To compare the performance of the designs, relative precision ratios of the CRDs to alpha designs and of the RCB designs to alpha designs were determined. These were calculated by dividing CRD and RCB error variances by the average effective error variance of the alpha design (2). Least significant differences at $P = 0.05$ were calculated for all randomized designs, and the mean LSD for each design in each trial was calculated.

The F_{max} statistic was used to determine homogeneity of variances among microplates within a single trial. The largest alpha design average effective error variance produced by a given randomization on the eight microplates within each trial was divided by the smallest error variance produced by that randomization. Similarly, the F_{max} tests for homogeneity of variances within microplates were calculated by dividing the largest alpha design average effective error variance by the smallest error variance produced by the 12 randomizations on a given microplate.

RESULTS

Seventeen of the 56 microplates tested had one to five wells with absorbances that were outliers from the normal distribution. Plate mean absorbances varied from plate to plate within each trial on both brands of microplates (Table 2). The largest range of means occurred in trial 4 (1.4780–0.8901) and the smallest range occurred in trial 7 (1.1628–0.9465). The CVs ranged from a low of 3.8% on microplate 2 in trial 5 to a high of 20.3% on microplate 8 in trial 6.

The F_{max} values resulting from the 12 randomizations of the alpha design on each microplate demonstrate that there were no significant differences in the variances generated by the different randomizations on a given microplate (Table 3). However, there were significant differences among variances of the microplates within a given randomization (Table 4). This indicates that variances on microplates within a trial were not homogeneous.

When analysis of variance and F tests were used to detect differences among the rows on each microplate, the majority of plates (66%) had differences ($P \leq 0.05$) between rows (Table 5). When 48 treatments were assigned on the microplates with the two replications of each treatment paired side by side in rows, significant differences were found among treatment means on 73% of the microplates. Significant differences among columns were detected on most microplates and on all microplates in trials 3–7. When 48 treatments were imposed on the microplates with two replications paired side by side in a single column, significant

differences among treatment means were detected on 86% of the microplates and on all microplates in trials 3, 4, 6, and 7. The type I error rate (rejection of a true null hypothesis) was equal to 0.05. Since there were, in reality, no differences among treatment means (each well on each microplate contained a sample of the same extract), only 5% of the tests for differences among treatments should have been significant. When the alpha design was used and two replications of the 48 treatments were randomized within the restrictions imposed by the design, treatment mean differences were detected in 5.2, 6.3, 3.1, 5.2, 7.3, 3.1, and 5.2% of the tests in trials 1–7, respectively. These values are very close to the expected 5%.

The relative precision ratios of CRDs compared with alpha designs (Table 6) ranged from 1.26 (trial 5) to 3.79 (trial 3) and indicated that error variances produced by CRD averaged 26–279% larger than those produced by alpha designs. The relative precision ratios of RCB designs to the alpha designs also indicated that error variances produced by RCB designs were larger than those produced by alpha designs (Table 6), although the range of the ratios was not as great as reported for CRD (1.07–1.44). In all trials, the mean CRD LSD was larger than the mean RCB LSD and the mean RCB LSD was larger than the mean alpha design LSD (Table 7).

Table 5. Number of microplates with statistically significant analysis of variance F tests ($P \leq 0.05$) for row, column, and treatment effects when treatments were imposed on the plates paired side by side in both rows and columns

Trial	Row effects[a]	Treatments paired in rows[b]	Column effects[c]	Treatments paired in columns[d]
1	3	3	7	7
2	4	5	7	5
3	7	8	8	8
4	5	8	8	8
5	7	5	8	4
6	5	7	8	8
7	6	5	8	8
Percentage of all microplates	66	73	96	86

[a] Number of plates with significant row effects.
[b] Number of plates with significant F tests when two replications of each of 48 treatments were imposed on the microplates paired side by side in a single row.
[c] Number of plates with significant F tests for column effects.
[d] Number of plates with significant F tests when two replications of each of 48 treatments were imposed on the microplates paired side by side in a single column.

Table 6. Mean relative precision ratios for CRD and RCB designs to alpha designs for 12 randomizations of each design on eight microplates in each trial

Design	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7
CRD to alpha[a]	1.27	1.43	3.79	3.48	1.26	2.34	1.40
RCB to alpha[b]	1.07	1.08	1.40	1.44	1.15	1.23	1.28

[a] CRD error variance divided by the alpha design average effective error variance.
[b] RCB error variance divided by the alpha design average effective error variance.

Table 7. Mean LSD ($P = 0.05$) produced by CRD, RCB, and alpha designs for 12 randomizations of each design on eight microplates in each trial

Design	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7
CRD LSD	0.2884	0.1760	0.3091	0.2906	0.1063	0.2327	0.2068
RCB LSD	0.2686	0.1567	0.1964	0.1886	0.1017	0.1732	0.1996
Alpha LSD	0.2668	0.1549	0.1739	0.1634	0.0978	0.1612	0.1854

DISCUSSION

Randomizing samples on microplates is important when using ELISA quantitatively, since significant row and column effects were detected in all trials in this study. Row and column differences on microplates resulted in unacceptably high type I error rates (rejection of a true null hypothesis).

Both block designs (RCB and alpha) allowed for more precision than the CRD. The smaller incomplete blocks of the alpha design were 7–44% more effective than the larger complete blocks of the RCB design (Table 6). As a result of the increased precision, alpha design LSDs were smaller than RCB LSDs and RCB LSDs were smaller than CRD LSDs (Table 7).

Seventeen of the 56 microplates tested had one to five outliers from the normal distribution, but outliers were not unique to a plate brand. The consequences of this type of nonnormality are not very substantial. Only a very skewed distribution would have a marked effect on the significance level of the F test or on the efficiency of an experimental design (23).

Fenlon and Sopp (12) suggested combining variance estimates from several microplates and using the combined variance estimate to determine the positive-negative threshold in ELISA tests. The results of F_{max} tests between microplates within a single randomization indicated that variances produced by the plates within the trials are not homogeneous and, therefore, should not be combined. The variability of some plates was considerably greater than the variability of others.

To control plate variability in experiments that are too large to fit on a single microplate, all samples from a single replication of an experiment with a RCB design could be placed on a single microplate with subsamples arranged in an alpha design. Microplate differences would then be confounded with block differences and the portion of the variability attributed to the error term by the use of several microplates would be excluded. Since use of the alpha design allows for adjustment of treatment means for block effects, benefit from the small incomplete blocks could be derived by using the adjusted treatment means instead of the unadjusted treatment means.

It is judicious to test microplates, procedures, and techniques with a uniformity trial prior to attempting a quantitative ELISA. When precision is lower than desired, it can be increased by reducing experimental error through the use of experimental designs. Burrows et al (2) first suggested the use of experimental designs on microplates and recommended Youden square and lattice square designs for control of two-dimensional local variations within plates. The alpha design also provides effective control of within-microplate variability. In addition, it can accommodate two replications of 48 treatments on a single plate and can be used to reduce the effects of within-microplate variability in experiments that are too large to fit on a single plate.

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