Techniques

Rapid Sample Analysis with a Simplified ELISA

L. W. Stobbs and D. Barker

Research scientist, Agriculture Canada, Vineland Research Station, Vineland Station, Ontario L0R 2E0 and graduate assistant, Department of Biology, University of Waterloo, Waterloo, Ontario N1G 2W1.
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ABSTRACT

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Simultaneous incubation of virus samples with conjugate reduced the assay time and was as sensitive and specific as the standard ELISA procedure. To further simplify this test, procedures were investigated in which virus-specific antibody-coated plates could also be pretreated with conjugate and stored over extended periods. Air- or freeze-drying of conjugate in antibody-coated wells prior to the addition of virus did not provide adequate identification of virus-positive samples, possibly because of enzyme-antibody crosslinking during dehydration. Solidification of the conjugate in the sample wells with 5% gelatin permitted easy recognition of virus and, by reducing the healthy baseline reactions, made visual assessment less subjective. By increasing the concentration of conjugate while proportionately reducing the volume applied to each well, equivalent conjugate concentrations were used as in the standard ELISA procedure, while occupying only a minimal volume of each well. Gelatin-matrixed conjugate remained stable for extended periods at −20 C and could be stored under refrigeration for several weeks without significant decrease in sensitivity or specificity. This procedure provides a convenient means of preforming the plates with coating antibody and conjugate, such that only sample addition, incubation, and substrate reaction are required to obtain sample diagnosis.

The enzyme-linked immunosorbent assay (ELISA) is a well established reliable procedure for the serodiagnosis of virus disease (10) and has been applied to a wide range of virus/host combinations (3). The most widely used method is the double-antibody sandwich (3) in which virus in the test sample is selectively trapped and immobilized by specific antibody adsorbed on polystyrene microtiter plates. Enzyme-labeled antibody is then complexed with the trapped virus and detected colorimetrically by adding a suitable substrate. However, a major constraint to the more widespread use of ELISA is the time required to form the sandwich, with tests normally requiring 1–2 days to complete.

Several more expeditious approaches have been described that reduce incubation times (7). Simultaneous incubation of virus-containing samples with the conjugate, while reducing the time necessary for test completion, has been shown to permit diagnosis of viruses otherwise undetected by the standard ELISA procedure (1,6). The present investigation was made to evaluate the efficacy of adding conjugate to specific antibody-coated wells, immobilizing the conjugate by either air-drying, freeze-drying, or matrixing in semisolid gelatin and storing the plates until required. Plates, prepared in this fashion, required only the addition of the test samples, incubation and completion of the substrate reaction, greatly reducing the time and manipulations necessary for sample evaluation.

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MATERIALS AND METHODS

Viruses and antiserum. Peach rosette mosaic virus (PRMV) was maintained on *Chenopodium quinoa* Willd. Virus was purified by the chloroform-pH procedure (4) followed by rate-zonal density gradient centrifugation in 10, 15, 25, and 30% sucrose buffered in 0.01 M potassium phosphate buffer, pH 7.0. Antiserum against PRMV was prepared by injecting New Zealand white rabbits intramuscularly with virus preparations emulsified with an equal volume of Freund's complete adjuvant. Three 2-mg intramuscular injections were given at 10-day intervals, followed by one 2-mg intravenous injection. The rabbits were bled 2 wk after the last injection. The titer of the antiserum, as determined by double diffusion in agar gel (9) was 1/1,024.

Virus-infected and healthy plants were prepared by grinding 1 g of foliar tissue in 100 ml of ELISA extraction buffer (3). Six twofold dilutions of virus-infected leaf macerates in extraction buffer were used in subsequent tests while healthy plant macerates were assayed without further dilution. The concentration of PRMV in leaves of *C. quinoa* was estimated at 1–2 mg/100 g of infected material, assuming an extinction coefficient ($E_{1\ cm}^{0.1\%}$) of 10 at 260 nm for combined virus components. Tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), and tomato ringspot virus (TomRSV) were maintained as previously described (8). Purified virus diluted in extraction buffer (1 μg virus per milliliter) was used for specificity tests.

ELISA procedure. The double antibody sandwich ELISA, as described by Clark and Adams (3), was used for controls. All tests were done in polystyrene MicroELISA plates (Immulon 2, Dynatech Laboratories Inc., Alexandria, VA) with 200 μl of liquid used for each of the four steps. Wells were coated with purified PRMV immunoglobulin (Ig) at 1 μg/ml diluted in 0.05 M sodium carbonate buffer, pH 9.6 (1:1,000, v/v) and incubated for 4 hr at 38 C. Virus samples were incubated in wells for 4 hr at 38 C, and following washing, PRMV Ig (1 μg of protein per milliliter), conjugated with alkaline phosphatase (Type 7, Sigma Chemical Co., St. Louis, MO) at an enzyme: ratio of 2.5:1 (w/w) with 0.06% glutaraldehyde (3), was added for 3 hr at 38 C. Substrate reactions were stopped by adding 20 μl of 3 M NaOH and the absorbance was measured at 405 nm in a Beckman DU-8 spectrophotometer fitted with the microplate accessory.

All plates described in subsequent tests were precoated with PRMV Ig as described previously.

Addition of conjugate to antibody precoated wells. *Air-drying and freeze-drying tests.* Twelve microliters of conjugate, diluted 1:25 in conjugate buffer, was added to PRMV Ig-coated plates. In addition, several buffers amended with sucrose or bovine serum albumen (BSA) were substituted for the conjugate buffer (Table 1). When diluted to 200 μl with virus sample, the conjugate concentration was similar to that used in the standard ELISA procedure. Plates were either air-dried at 40 C for 3 hr or freeze-dried, sealed with cellulose tape (Dynatech, Alexandria, VA), and stored at 4 C until required. Virus-infected or healthy plant macerates were each added to one-half of the sample wells for each treatment and the plates were incubated for 3 hr at 38 C. Following washing in PBS-Tween (0.02 M phosphate plus 0.15 M NaCl with 0.05% Tween-20, pH 7.4), substrate was added and the reaction was read after 45 min. The standard ELISA procedure was used as a reference control.

Gelatin-matrix tests. Conjugate, diluted 1:25 in conjugate buffer, was amended with 5% gelatin (Type 3, Sigma, St. Louis, MO). To suspend the conjugate in the gelatin, the conjugate was heated to 38 C, the gelatin was added, and the mixture was stirred until it dissolved. Four treatments were examined: simultaneous addition of conjugate and virus with, or without gelatin, conjugate with gelatin which was stored frozen for 48 hr, and the standard ELISA procedure. Plates containing gelatin were warmed to 38 C in an incubator to liquify the gelatin, and virus-infected plant macerate dilutions were added to three replicate wells per treatment for each plate. Undiluted healthy plant sap and extraction buffer were used as controls. Following sample addition, all plates were sealed with cellulose tape and agitated at low speed for 10 sec on an SMI multi-tube vortexer (Scientific Manufacturing Industries, Emeryville, CA); the tape was removed, and the plates were incubated for 3 hr at 38 C. Following four washes with PBS-Tween (38 C), substrate was added and absorbance readings taken after 45 min of incubation.

To increase the stability of the conjugate, tests were made with higher conjugate concentrations at low volumes, thereby facilitating more uniform mixing with the test samples. Conjugate dilutions in conjugate buffer (1:200, 1:50, and 1:25) were added to wells with a corresponding volume of 100, 25, and 12 μl, respectively, to provide identical conjugate concentrations when adjusted to 200 μl with sample solutions. All conjugate preparations were matrixed with 5% gelatin and dilutions of virus-infected sap, healthy sap, and buffer alone were added for each conjugate treatment. Plates were processed as described.

To assess the usefulness of sample agitation, plates were treated with 12 μl of a 1:25 dilution of conjugate amended with 5% gelatin and adjusted to 200 μl with dilutions of virus-infected plant tissue, healthy sap, or extraction buffer. The plates were then sealed with cellulose tape and the contents were warmed to 38 C. One-half of the plates were agitated as previously described while the remaining plates were undisturbed. The tape was removed, and the plates were incubated and processed as described.

To determine the long-term storage characteristics of gelatin-matrixed plates, 20 plates loaded with 12 μl of a 1:25 dilution of conjugate in gelatin were stored at −20 C. At 14-day intervals, four plates were removed from storage and 12 wells of each plate were loaded with virus-infected plant dilutions, healthy sap, or buffer.

TABLE 1. Effect of air-drying and freeze-drying of conjugate in wells precoated with specific antibody on the efficacy of ELISA

	Averaged absorbances[a] at 405 nm ($\bar{X}$ ± S.D.) with conjugate:					
	Air-dried		Freeze-dried		Normal ELISA procedure	
Conjugate buffers	PRMV[b]-infected plant macerate	Healthy plant macerate	Virus-infected plant macerate	Healthy plant macerate	Virus-infected plant macerate	Healthy plant macerate
Extraction buffer	0.58 ± 0.05	0.56 ± 0.03	0.67 ± 0.07	0.60 ± 0.01	1.18 ± 0.05	0.24 ± 0.01
Extraction buffer + 20% sucrose	0.54 ± 0.02	0.55 ± 0.03	0.60 ± 0.09	0.48 ± 0.01	–	–
Extraction buffer + 1% BSA[c]	0.39 ± 0.02	0.42 ± 0.04	0.49 ± 0.05	0.45 ± 0.06	–	–
PBS-Tween[d]	0.79 ± 0.02	0.81 ± 0.01	0.44 ± 0.01	0.48 ± 0.01	–	–
PBS-Tween + 20% sucrose	0.77 ± 0.03	0.89 ± 0.01	0.52 ± 0.02	0.44 + 0.01	–	–
PBS-Tween + 1% BSA	0.73 ± 0.03	0.88 ± 0.01	0.66 ± 0.01	0.53 ± 0.03	–	–
Distilled water	0.66 ± 0.04	0.69 + 0.03	0.72 ± 0.06	0.59 ± 0.03	–	–

[a] Twelve wells were used in each of 24 plates for each conjugate treatment. Eight plates were either air-dried, freeze-dried, or tested by the normal ELISA procedure.
[b] PRMV = peach rosette mosaic virus in maintenance host, *Chenopodium quinoa*.
[c] BSA = bovine serum albumin.
[d] PBS-Tween = 0.02 M phosphate plus 0.15 M NaCl with 0.05% Tween-20 at pH 7.4.

An equivalent run was made with refrigerated plates (4 C) sampled at 1-day intervals. Replicate plates were checked against 200 μl of the purified preparations of CMV, TMV, and TomBSV for nonspecific reactions.

RESULTS

Air-dried/freeze-dried conjugate tests. Air-drying or freeze-drying of conjugate in sample wells precoated with PRMV Ig failed to provide adequate distinction between virus-infected and healthy plant macerates over a range of buffers amended with sucrose or BSA (Table 1). Prewashing plates with BSA prior to conjugate addition did not result in a significant improvement.

Gelatin-matrix tests. Simultaneous addition of conjugate and virus to specific antibody-coated plates produced significantly higher substrate absorbances than obtained through the standard ELISA procedure (Table 2). Optimal virus detection was obtained with 2–3 hr of incubation of the conjugate-virus mixture at 38 C, compared to 3–4 hr for the standard ELISA procedure. While addition of 5% gelatin to the conjugate reduced the absorbance values for virus-positive samples, a similar reduction in the baseline for healthy tissue samples was also obtained, making visual assessment of plates less subjective. On gelatin solidification, plates could be easily handled at room temperature without mixing or loss of conjugate in the sample wells.

Optimal absorbance readings were obtained with higher concentrations of conjugate in low volumes of buffer (Table 3). By minimizing this volume, there was ample space in each well following sample addition to facilitate mixing. Maximum absorbances with reduced baselines were attained when the plates were agitated following virus addition (Table 4). Inadequate mixing of the well contents or incomplete gel liquifaction resulted in stratification of the conjugate and the plant extracts.

Conjugate plates matrixed in gelatin and stored at −20 C remained stable for over 56 days (Table 5). Repeated thawing and freezing of plates did not affect the sensitivity or specificity of the plates. Plates could be stored at 4 or 25 C for several weeks without significant reductions in plate sensitivity although storage at −20 C

TABLE 2. Effect of simultaneous addition of conjugate and virus with or without gelatin on efficacy of ELISA

Treatment[c]	Average absorbance[a] at 405 nm ($\bar{X}$ ± S.D.) of PRMV-infected plant macerate[b] diluted: 2×	4×	8×	16×	32×	64×	Healthy sap	Buffer only
Standard ELISA procedure	1.08 ± 0.02	1.11 ± 0.03	1.07 ± 0.02	0.99 ± 0.02	0.49 + 0.01	0.40 + 0.01	0.29 ± 0.03	0.34 ± 0.03
Simultaneous addition of conjugate and virus	1.15 ± 0.02	1.13 + 0.01	1.12 ± 0.01	1.09 ± 0.02	0.84 ± 0.04	0.55 ± 0.02	0.33 + 0.02	0.45 + 0.02
Simultaneous addition of conjugate and virus + 5% gelatin	1.17 ± 0.04	1.11 + 0.04	0.94 + 0.04	0.88 + 0.03	0.84 + 0.03	0.72 + 0.01	0.21 + 0.02	0.30 + 0.04
Conjugate in 5% gelatin frozen 48 hr; virus added + tested	1.19 ± 0.02	1.15 ± 0.01	1.14 ± 0.02	1.07 ± 0.01	0.58 ± 0.04	0.31 ± 0.01	0.20 ± 0.01	0.33 ± 0.08

[a] Twenty-four wells ine ach of eight plates were used for each treatment.
[b] Macerate of leaves of peach rosette mosaic virus-infected maintenance host, *Chenopodium quinoa*.
[c] Manipulation of ELISA procedures.

TABLE 3. Effect of conjugate concentration and volume on efficacy of conjugate-matrixed ELISA plates

Volume/dilution of conjugate[a]	Average absorbance at 405 nm ($\bar{X}$ ± S.D.) of PRMV-infected plant macerate diluted: 2×	4×	8×	16×	32×	64×	Healthy sap	Buffer only
100 μl, 1:200	0.96 ± 0.04	0.92 ± 0.04	0.91 ± 0.06	0.90 ± 0.01	0.34 ± 0.01	0.33 ± 0.02	0.37 ± 0.04	0.36 ± 0.01
25 μl, 1:50	1.53 ± 0.06	1.03 ± 0.04	0.73 ± 0.04	0.65 ± 0.05	0.54 ± 0.04	0.35 ± 0.06	0.22 ± 0.01	0.21 ± 0.04
12 μl, 1:25	1.91 ± 0.06	1.36 ± 0.03	0.92 ± 0.04	0.88 ± 0.06	0.62 ± 0.02	0.40 ± 0.04	0.28 ± 0.03	0.24 ± 0.02

[a] Thirty-two wells in each of eight plates was treated with each conjugate dilution and each virus dilution added to four replicate wells per plate for each conjugate treatment.

TABLE 4. Effect of sample agitation on efficacy of conjugate-matrixed ELISA plates

Treatment[a]	Average absorbance at 405 nm ($\bar{X}$ ± S.D.) of PRMV-infected plant macerate diluted: 2×	4×	8×	16×	32×	64×	Healthy sap	Buffer only
Agitation	1.75 + 0.04	1.32 + 0.05	0.82 + 0.04	0.60 + 0.02	0.40 + 0.06	0.39 + 0.07	0.24 ± 0.03	0.25 ± 0.03
No agitation	0.86 ± 0.08	0.84 ± 0.07	0.70 + 0.07	0.56 + 0.04	0.48 + 0.03	0.34 + 0.02	0.27 + 0.06	0.26 + 0.01

[a] Each treatment consisted of 32 wells in each of four plots.

TABLE 5. Effect of storage time on efficacy of conjugate-matrixed ELISA plates

Length of plate storage at −20 C (days)[a]	Average absorbance at 405 nm ($\bar{X}$ ± S.D.) of PRMV-infected plant macerate diluted: 2×	4×	8×	16×	32×	64×	Healthy sap	Buffer only
0	1.86 ± 0.02	1.71 ± 0.10	0.92 ± 0.07	0.89 ± 0.06	0.50 ± 0.07	0.40 ± 0.05	0.30 ± 0.03	0.37 ± 0.05
14	1.91 + 0.05	1.36 ± 0.07	0.92 ± 0.06	0.88 + 0.06	0.62 ± 0.02	0.40 ± 0.04	0.28 ± 0.05	0.24 + 0.02
28	1.75 + 0.07	1.32 + 0.06	0.82 + 0.04	0.60 + 0.02	0.40 + 0.06	0.41 + 0.04	0.24 + 0.03	0.25 + 0.03
42	1.71 + 0.05	1.24 + 0.05	0.81 + 0.10	0.71 + 0.03	0.68 + 0.04	0.41 + 0.01	0.28 + 0.02	0.22 + 0.01
56	1.49 + 0.06	1.27 + 0.03	0.93 + 0.05	0.69 + 0.06	0.59 + 0.04	0.40 + 0.01	0.30 + 0.04	0.25 + 0.01

[a] Each sampling consisted of four replicate plates using 12 wells in each plate for each treatment.

was preferable. Plates prepared against PRMV gave negative readings against TMV, CMV, and GFLV.

DISCUSSION

Several modifications of the ELISA procedure have been described that reduce the time required for sample diagnosis. Coating of polystyrene plates was accomplished within 30 min by using higher concentrations of coating Ig (7) while incubation at elevated temperature during coating has been shown to similarly reduce adsorption time (8). While these procedures permit more expedient testing of samples, they do not represent any significant simplification of the ELISA method, with numerous incubations and washes still being required. To reduce the number of manipulations required, simultaneous incubation of virus and conjugate with solid-phase antibody has been attempted (2), eliminating the requirement for virus incubation and washing. Additionally, this procedure appears to provide protection of the virus against salt-induced breakdown, possibly through the formation of an antigen-antibody complex that protects the virus against the degrading effects of the salt solutions in subsequent rinsing steps (6). As suggested by the authors, virus stabilization may also be assisted by various plant extract constituents during incubation with the conjugate. However, comparisons of the modification with the standard ELISA procedure have not been reported.

To facilitate rapid processing of the test samples, tests were made to determine whether the conjugate could be combined with the coating Ig in ELISA plates and stored until required. In this way, virus assay would require only sample addition, washing, and substrate incubation. Attempts to air-dry or freeze-dry conjugate within microplates were unsuccessful. High baselines associated with both healthy and buffer controls indicated nonspecific binding of the conjugate within the ELISA wells. Since binding did not occur in plates that were not treated with coating Ig, binding was associated with the coating Ig. Drying of the conjugate in wells may have resulted in cross-linkage of enzyme between coating Ig and Ig present in the conjugate.

Conjugate could be added to Ig-coated wells and frozen without significantly increasing nonspecific reactions. While advantageous where refrigeration is available, the contents of the wells became mixed on handling once the conjugate thawed. To facilitate shipment and handling of plates, the conjugate was matrixed in 5% gelatin providing solid-phase conjugate in each well. Upon sample addition, the plates were warmed to liquify the gelatinized conjugate and the contents of the wells were mixed prior to incubation. With adequate mixing, consistently reproducible results were obtained, with healthy baselines generally lower than those obtained by using the standard procedure. By proportionately increasing the concentration of conjugate, a reduced volume could be applied to each well, facilitating mixing of the test samples with the conjugate. Optimal results were obtained by adding 12 μl of conjugate at a 1:25 dilution, stabilized with gelatin, to each well.

Without special precautions, conjugate has been stored for at least 1 yr with little decrease in activity (5). Conjugate matrixed in gelatin and stored at −20 C remained stable during a 56-day test period with only a marginal decline in activity. Short-term exposure to ambient temperatures or refrigeration (4 C) similarly had little effect on reducing test specificity. By preforming plates with specific Ig and conjugate, tests may be completed with a reduced amount of manipulation.

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