

Dot-ELISA on Nitrocellulose Membranes for Detection of Potato Leafroll Virus

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ABSTRACT

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Potato leafroll virus (PLRV) was consistently detected by dot-ELISA in buffer extracts of PLRV-infected potato foliage sap diluted 1:2,048 with buffer extracts of healthy foliage sap. The assay procedure used a double-antibody sandwich, alkaline phosphatase and naphthol ASMX phosphate, and fast red TR salt substrate on Millipore 0.45- μ m nitrocellulose membranes (NCM). Potato sap extracts heated at 70 C for 10 min improved sap drainage through the NCM, decreased nonspecific staining, and increased sensitivity. Membranes precoated with anti-PLRV IgG and stored for 1 wk at 4 C retained reaction sensitivity and permitted assay completion within one working day. Dot-ELISA for PLRV in potato leaves was eight times more sensitive than double-antibody sandwich (DAS) ELISA in polystyrene cuvette paks and twice as sensitive as DAS-ELISA in microtiter plates.

Potato leafroll virus (PLRV) is a major potato pathogen worldwide, and control of the disease has depended primarily on use of PLRV-free seed and control of aphid vectors. Early development of accurate, diagnostic enzyme-linked immunosorbent assays (ELISA) for detection of PLRV in potato tubers and foliage used polystyrene plastic solid phase for antibody attachment and provided the first effective serological assay for this virus (6,10,12).

The advantages of nitrocellulose membranes (NCMs) for ELISA have been their high affinity for proteins coupled with a large surface area, permitting the detection of small amounts of protein with appropriate methodology. The first use of NCMs for double-antibody sandwich (DAS) ELISA was made by Banttari and Goodwin, termed dot-ELISA, for detection of potato viruses S, X, and Y (3). Rybicki and von Wechmar (17) earlier electroblotted plant extracts onto NCMs and then used indirect ELISA to detect plant viruses. Berger et al (4) used indirect ELISA to detect 1-5 pg of purified potato virus Y applied to NCM. Similarly, Powell (16) was able to detect 30 pg of purified tobacco mosaic virus and 100 pg of purified tobacco ringspot virus with a dot-blot immunobinding assay on NCMs. Zutra et al used a dot-ELISA technique to detect NCM-bound *Erwinia amylovora* cells (19).

Assaying plant extracts with ELISA in which plant sap is applied directly on NCMs requires special procedures because the NCMs become stained with chlorophyll and oxidized plant material. Parent et al (15) overcame problems inherent in the application of sap extracts directly to NCMs by removing chlorophyll with dichloromethane and heavier plant cell components by centrifugation. Oxidation of plant extracts was prevented by adding 0.2% sodium sulfite or 1% 2-mercaptoethanol. They reported detection of 2 pg of tomato mosaic virus or potato virus X.

The objectives of this study were to develop dot-ELISA methodology on NCMs that would detect PLRV in infected potato foliage and could be completed within one working day. A comparison was made between dot-ELISA and two polystyrene-based ELISA systems.

MATERIALS AND METHODS

Sources of PLRV-infected and virus-free potatoes. The potato (*Solanum tuberosum* L.) cultivars Kennebec, Norland, and Russet Burbank, obtained originally as elite seedstocks from the Agriculture Canada Research Station, Vancouver, BC, were used throughout this study. PLRV-free and PLRV-infected potato plants were grown in the greenhouse or in the field at St. Paul, MN. Only young, fully expanded leaves were used for dot-ELISA experiments, and sap was extracted with a roller press (2).

Processing NCMs for dot-ELISA. The dot-ELISA procedure was modified for work performed by Banttari and Goodwin (3) for detection of potato viruses S, X, and Y. The NCMs used were 47-mm-diameter, 0.45- μ m HAWP disks (Millipore Corp., Bedford, MA). The orientation of the surface of the NCM to antigen application affected sensitivity; therefore, it was necessary to make a test

comparison of each shipment of Millipore NCMs.

Anti-PLRV immunoglobulin (IgG), alkaline phosphatase-conjugated IgG, and enzyme-conjugate buffer were obtained from Agdia, Inc. (Mishawaka, IN). Anti-PLRV IgG was diluted 1:1,000 in a bicarbonate buffer, pH 9.6, containing 0.02% NaN_3 to form the coating IgG solution, and 4 ml of the coating solution was pipetted onto each NCM. The covered petri dish was incubated at 37 C for 4 hr. Unbound antibody was removed by washing the NCM three times during a 10-min period with 0.01 M Tris-HCl buffer, pH 7.4, with 0.9% NaCl and 0.05% Tween 80 (TBS+T).

To saturate any remaining unoccupied sites after IgG treatment of the NCM, they were blocked with a 3% bovine serum albumin (BSA), fraction IV powder (Sigma Chemical Co., St. Louis, MO), in TBS+T. For NCMs to be used immediately, the BSA solution was pipetted on the washed NCM for 10 min at 37 C. All precoated NCMs were blocked for 2 hr and then stored for 1 wk in the refrigerator at 4 C or freezer at -15 C in petri dishes overlaid with a thin film of TBS+T. Air-dried NCMs were also stored for 1 wk in a paper envelope at room temperature. After a 10-min blocking period or storage for 1 wk, the NCMs were assembled into plastic templates without further washing.

The template consisted of two identical pieces of Plexiglas (62 \times 62 \times 18 mm) with 28 holes (5 mm in diameter) drilled in alignment through the two halves of the Plexiglas. The NCM was placed on a precut piece of Whatman no. 1 filter paper wetted with TBS+T seated on top of the lower half of the template. The template was bolted together and fastened tightly with wing nuts. Firm fastening of the template was important to prevent lateral migration of plant sap across the surface of the NCM. The 5-mm-diameter holes in the upper part of the template formed wells that held 0.4 ml of the assay sample. Care was taken when depositing plant sap extracts in wells to avoid trapping air bubbles in the upper template.

After assay samples were pipetted into wells, the template was placed in a moist plastic box to permit drainage of samples through the NCM. After plant extracts had drained, the template was disassembled and the pellicles of debris were removed from the NCM under a stream of deionized water. Any remaining plant

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debris and unbound antigen were removed by washing with several rinses of TBS+T within 10 min.

The NCM was placed in a plastic petri dish, and anti-PLRV IgG enzyme-conjugate (4 ml of 1:1,000 dilution) in the Agdia conjugate buffer solution was added. The dishes containing the incubating NCMs were gently agitated in a TekTator-V serological rotator (American Hospital Supply Corp., Evanston, IL) at 60 rpm at room temperature for 4 hr. The NCM was washed to remove unbound enzyme conjugate.

The substrate consisted of 1 ml of 0.25% naphthol ASMX phosphate (Sigma) in 5.25 ml of 0.2 M Tris-HCl, pH 8.2. Then 60 mg of fast red TR salt (4-chloro-2-methylbenzenediazonium chloride, hemizinc chloride salt) (Sigma) was dissolved in 10 ml of the Tris buffer and filtered through a Whatman no. 1 filter paper. The two solutions were mixed 1:1 (v/v) and poured over NCMs in petri dishes. About 4 ml of substrate solution was used per NCM. NCMs were agitated on the TekTator for 30 min, the time that produced maximum coloration of PLRV-positive dots without staining the white background of the NCM. Reactions were terminated by rinsing the NCM in deionized water. The NCM was then air-dried on a paper towel.

Treatment of plant extracts for dot-ELISA. In dilution end-point experiments, twofold dilutions of PLRV-infected potato foliage sap were made by diluting infected sap with known healthy potato foliage sap. Each dilution of the series of twofold dilutions was then

mixed with an equal amount of TBS+T containing 0.01 M sodium diethyldithiocarbamate (NaDIECA) and 0.01 M sodium ethylenediaminetetraacetate (NaEDTA). Final dilutions ranged from 1:1 to 1:4,096 (parts PLRV-infected foliage sap in buffer to parts total sap in buffer). Therefore, healthy sap was a 0:1 dilution and sap from only PLRV-infected foliage was a 1:1 dilution. Each sap dilution was individually pipetted into disposable (10 × 75 mm) glass culture tubes. The tubes were placed in a test tube rack, lowered into a water bath, usually at 70 C for 10 min, and removed and cooled to room temperature. Because the heating process coagulated plant proteins, the tubes were briefly agitated with a vortex mixer, then large particulates were allowed to settle briefly before sap was deposited into templates with a Pasteur pipette.

Polystyrene-based ELISA. Dot-ELISA was compared with two DAS-ELISA procedures that used polystyrene as the solid phase. New polystyrene EIA cuvette-paks (Gilford Instruments Laboratories, Inc., Oberlin, OH) were used with the Gilford PR-50 EIA automatic analyzer. Round-bottom microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were read with a Micro ELISA Minireader MR 590 (Dynatech).

For the polystyrene-based ELISA systems, dilutions of PLRV-infected potato foliage were mixed with an equal volume of 0.01 M phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.05% Tween 20, 2% polyvinylpyrrolidone (mol wt 40,000), and 0.2% egg albumin (7).

TBS+T performed poorly* when polystyrene was used as the solid phase; therefore, TBS+T was not used. Heating the dilutions of plant extracts was not done for polystyrene-based ELISA because no increase in end-point sensitivity was obtained. After deposition into the wells, plant extracts were incubated overnight at 5 C. Otherwise, the methodology paralleled dot-ELISA.

Data evaluation. For dot-ELISA, the intensity of the color reactions was quantified with a TR-524 reflectance densitometer (MacBeth Color and Photometry Division, Kollmorgen Corp., Newburgh, NY). Red dots, produced by reaction of fast red TR salt, were read with a magenta filter. A value of 1.00 was an intense reaction. For the polystyrene-based systems, the Gilford system produced a maximum value of 2.75 for strong serological reactions; the Dynatech system maximum value was 2.00.

Data for all the serological experiments were analyzed by averaging results of at least three comparable assays performed at different times. End-point dilutions were determined by establishing a 95% confidence interval above the mean of the healthy checks, defined as the healthy mean + 2 standard deviations (SD) of the healthy mean. The highest sap dilution exceeding the value, healthy mean + 2 SD of the healthy mean was the dilution end point for the experiment (3,7).

RESULTS

Diluents. Phosphate-buffered saline (PBS) with additives to reduce nonspecific reactions has been used primarily for polystyrene-based ELISA for detection of PLRV (6,7,12,18). However, PBS performed poorly in preliminary experiments when tested as a diluent and washing agent for dot-ELISA, and TBS was used instead.

The concentration of the detergent Tween 80 in the TBS diluent also affected dot intensities and end-point dilutions. Increasing the concentration of Tween 80 from 0.05 to 0.1% improved dot reflectance values for low dilutions of PLRV-containing foliage sap but reduced reaction intensities occurring at high dilutions. Detergent concentrations higher than 0.1% resulted in green staining of the NCM. Without added detergent, dot intensities were weak throughout all dilution values. Therefore, a concentration of 0.05% Tween 80 was used throughout the reported experiments.

Use of several additives (9) to TBS+T when diluting foliage sap statistically increased PLRV-positive dot intensity compared with TBS+T alone. The addition of 0.01 M NaDIECA increased PLRV reaction by 23% at a 1:1 sap solution. Highest end-point dilutions were obtained with addition of 0.01 M NaDIECA and 0.01 M NaEDTA to TBS+T. Antioxidants, such as 0.5% sodium sulfite and 1% sodium thio-

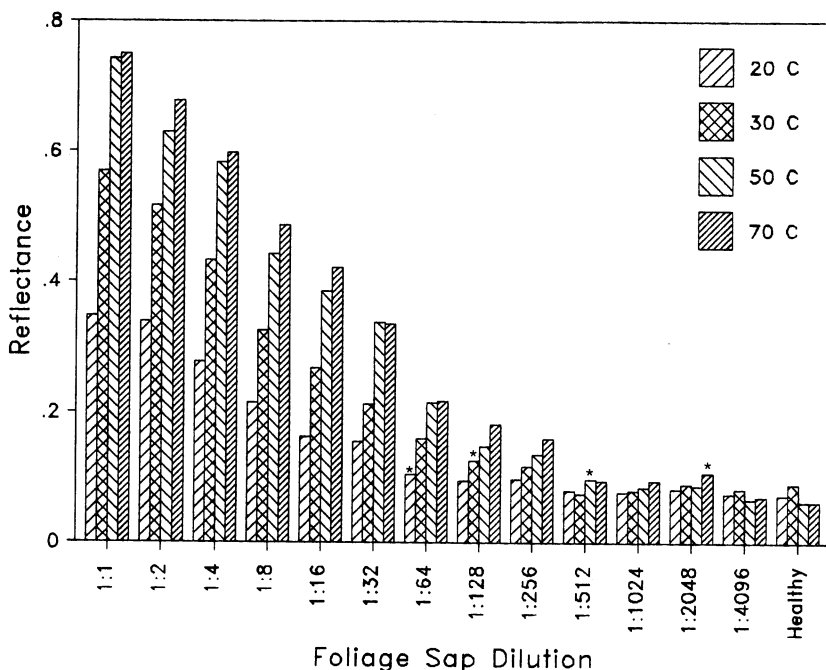


Fig. 1. Effect of a 10-min heat treatment on sap extracts from summer-grown potato foliage on dot-ELISA sensitivity for detection of potato leafroll virus (PLRV). Bars represent the mean reflectance of three experiments, two wells per treatment per nitrocellulose membrane. End-point dilutions for each temperature treatment are identified with an asterisk and are defined as most dilute PLRV-containing sap exceeding the reflectance of healthy mean + 2SD of the healthy mean.

glycollate, also improved serological reactions of PLRV. The virus was stable in mild degrading chemicals, 0.6% pyridine and 0.4 M urea.

Heat treatment of plant extracts. Heating diluted plant sap to 70 C for 10 min resulted in the most successful modification of the dot-ELISA procedure for PLRV (Fig. 1). The end-point dilution was increased from 1:64 with unheated sap (20 C) to 1:2,048 for sap heated at 70 C, and reflectance values, at a 1:1 dilution of PLRV-infected foliage sap, were more than doubled by the 70 C heat treatment. Heat treatments of 50 and 30 C resulted in lower levels of improved sensitivity.

Drainage of foliage sap through the NCM was also improved with heating. At 20 C, about 20% of sap from field-grown foliage passed through the NCM, whereas the 70 C treatment consistently resulted in 100% drainage of foliage sap from summer- or field-grown plants. Heating plant sap to temperatures above 70 C did not improve dot-ELISA reactions. Sap drainage was decreased by temperatures higher than 70 C. A marked drop in reflectance occurred at 90 C as virus proteins lost serological activity (Table 1).

Precoating with IgG and storage of NCMs. Precoated and freshly coated NCMs were compared to determine if IgG-coated NCMs could be stored. If precoating NCMs were effective, it would also facilitate 1-day assays of samples. Reducing times for some dot-ELISA procedures were not effective because IgG coating and enzyme-conjugate incubation each required 4 hr for maximum sensitivity. However, blocking NCMs with TBS+T + 3% BSA for 2 hr was not required; 10 min accomplished the same results for NCMs, which were used immediately.

During storage experiments, it was found that a 4-hr coating incubation of NCMs with anti-PLRV IgG and a 2-hr blocking with BSA can be done up to 1 wk before antigen application (Fig. 2). Air-dried, precoated NCMs were less sensitive (1:128) than NCMs stored under moist conditions. Precoated NCMs stored moist in the refrigerator at 4 C produced reactions with the same end-point dilution (1:2,048) as freshly coated NCMs. NCMs stored moist in the freezer at -15 C were less sensitive (1:256).

Comparison of dot-ELISA on NCM with DAS-ELISA on polystyrene for detection of PLRV. Use of dot-ELISA for detection of PLRV resulted in an eightfold increase in dilution end point (1:2,048) compared with DAS-ELISA (7) in polystyrene cuvettes read with the Gilford PR-50 EIA processor analyzer (1:256) and a twofold increase compared with microtiter plates read with a Dynatech Micro ELISA Minireader (1:1,024) (Table 2). Visual evaluation was

accurate to a maximum dilution of 1:32 with both polystyrene-based systems (Table 2). The dot-ELISA technique was sensitive to a dilution end point of 1:2,048 according to reflectance readings. There was also complete agreement between visual (positive or negative) evaluations of dot reactions and quantitative data obtained from reflectance measurements by the MacBeth reflectance densitometer.

Finally, the red color of dot-ELISA on NCMs is stable and can be used as a long-term record of assay results. After 6 mo of storage in the dark under normal room conditions, NCMs processed with fast red TR salt and ASMX phosphate lost only 11% of their original intensity.

DISCUSSION

PLRV, a luteovirus infecting potato plants, was accurately assayed by dot-ELISA in this study. The thermal inactivation temperature of the virus, based on aphid transmission studies, is between 70 and 80 C (13); however, the nucleocapsid retained serological activity after a 10-min heat treatment at 85 C. Because most plant proteins are denatured between 50 and 60 C, heat treatment of expressed sap accomplished two things: 1) the coagulation of host proteins prevented blockage of the 0.45- μ m pores of the NCM, and 2) denaturation of host proteins may have helped to reduce "background" reactions on the NCMs.

Table 1. Effect of high temperatures on serological activity of potato leafroll virus (PLRV) in potato foliage sap^w

Water bath temperature (C)	Reflectance ^{x,y}		Drainage (%) through NCM ^z
	PLRV-infected sap	Healthy sap	
98 (boiling)	2.8 c	2.0 a	10
95	3.2 c	2.3 a	20
90	13.2 b	3.7 ab	20
85	39.6 a	5.0 bc	30
80	43.2 a	6.8 c	30
75	45.3 a	5.7 c	50
70	48.9 a	3.0 ab	100

^wPLRV-infected or healthy potato foliage sap was mixed 1:2 with Tris-buffered saline + Tween 80 containing 0.01 M NaDIECA + 0.01 M NaEDTA, placed in test tubes, and heat-treated in a water bath for 10 min.

^xEach number represents the mean reflectance of four experiments, four wells per treatment per nitrocellulose membrane (NCM).

^yMeans within a column and followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

^zPercent drainage represents the approximate amount of sap passing through NCMs after 2 hr of antigen incubation time.

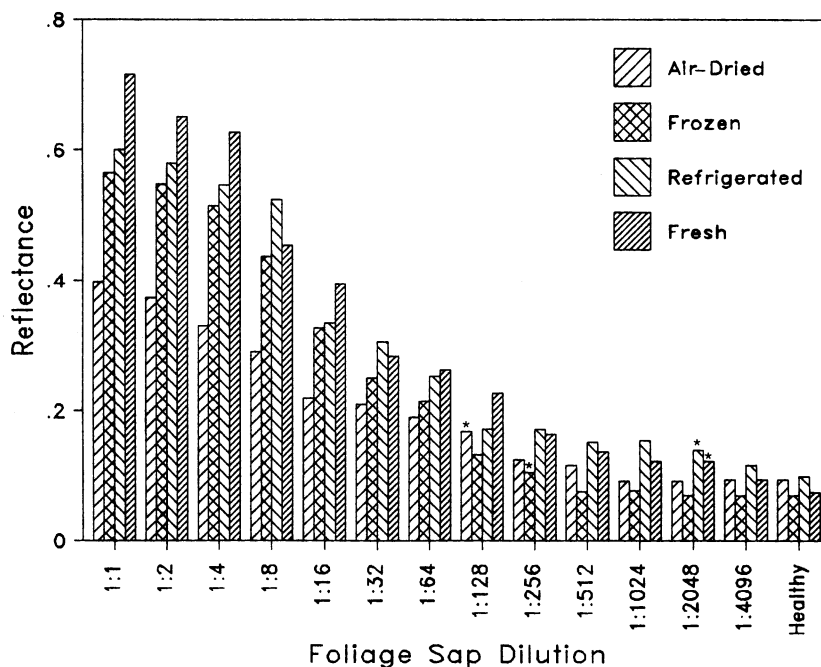


Fig. 2. Effect of precoated nitrocellulose membrane storage conditions on dot-ELISA sensitivity for detection of potato leafroll virus (PLRV). Air-dried nitrocellulose membranes (NCMs) were stored in a paper envelope at room temperature for 1 wk. Refrigerated (4 C) and frozen (-15 C) NCMs were stored for 1 wk in a petri dish containing a thin layer of buffer solution. Fresh NCMs were not stored and were used directly. Bars represent the mean reflectance of three experiments, two wells per treatment per NCM. End-point dilutions for each temperature treatment are identified with an asterisk and are defined as most dilute PLRV-containing sap exceeding the reflectance of healthy mean + 2 SD of the healthy mean.

Tamada and Harrison (18) noted that aging tuber extracts for 3–4 hr before assaying improved DAS-ELISA in microtiter plates. PLRV-infected potato foliage sap that was permitted to stand for several hours at room temperature before it was deposited into template wells also improved drainage and dot-ELISA reactions for PLRV but not as substantially, consistently, or quickly as did heat treatment.

The use of additives to TBS+T diluent also enhanced sensitivity of dot-ELISA. A combination of 0.01 M NaDIECA and 0.01 M NaEDTA consistently gave the highest dilution end-point assays for the virus from foliage. NaDIECA chelates Cu^{2+} ions, which are essential for activity of oxidase enzymes. Inhibition of oxidative enzymatic reactions prevents formation of *o*-quinones, which are virus inactivators (8). NaDIECA also functions as a nucleocapsid stabilizing agent. NaEDTA has been shown to release virions from host organelles, enhancing assay sensitivity. NaEDTA is helpful in promoting drainage of plant sap through the NCM (3). Reducing agents improved sensitivity of dot-ELISA, indicating that PLRV is sensitive to oxidation products.

The literature is not clear regarding the benefit of detergents in a diluent. It was reported that despite virtually instantaneous protein adsorption to nitrocellulose, excessive concentrations of Tween or similar surfactants may cause bound proteins to be released from membranes (1). Palfree and Elliott (14) found that detergents were useful to solubilize cell membrane proteins but that protein binding was poor when

samples contained Triton X-100 or Tween 80 at concentrations of 0.05% or more. At the assay concentration, Tween 80 was beneficial to dot-ELISA for PLRV. The detergent may have released virions from cell membranes and allowed for internal binding of the virions in the NCM by reducing surface tension.

The use of blocking agents was not required for dot-ELISA if the NCMs were used immediately after coating with IgG. Apparently, most binding sites on the NCM were occupied by anti-PLRV IgG. A 10-min treatment of IgG-coated NCMs in blocking agent prevented the occurrence of a trace amount of green staining. However, 2 hr of incubation of IgG-coated NCMs in BSA was useful to protect IgG from storage degradation when the NCM was stored for 1 wk before antigen application.

TBS+T performed substantially better than PBS when used as a diluent and washing solution for detection of PLRV on nitrocellulose, whereas the opposite occurred when polystyrene was the solid-phase carrier. The buffer effect was probably due to a reaction between phosphate-treated PLRV and NCM. Lockhart et al (11) detected barley yellow striate mosaic virus (BYSMV) at a 1:25,600 dilution of infected leaf extracts in phosphate buffer with a NCM-based assay. Salt concentration or pH of a buffer was not a major factor in binding ability of anti-PLRV IgG because nitrocellulose adsorbs protein molecules efficiently under a wide range of pH (4.0–10.0) and salt concentrations (0.1–0.5 M) (1).

Dot-ELISA for PLRV was performed

within 8 hr with the precoated NCMs. About 100 samples were extracted, mixed with TBS + T and additives, heated, and deposited in 2 hr. Incubation and drainage of sap for 2 hr, enzyme-conjugate incubation period of 3.5 hr, and substrate reaction of 0.5 hr resulted in adequate color development of positive assays. Treating sap samples with centrifugation or organic solvents was not needed. Between dot-ELISA steps, three washes during 10 min were sufficient.

There are several advantages of dot-ELISA over standard DAS-ELISA with polystyrene solid-phase carriers. The high binding affinity of NCMs for protein, uniform surface, and diminished “leakage” of antibody-antigen complexes improved sensitivity of the test. Compared with DAS-ELISA using the Gilford system, dot-ELISA was eight times more sensitive for detection of potato virus X and four times more sensitive for detection of potato viruses S and Y (3). Bode et al (5) reported that the sensitivity of nitrocellulose-based ELISA for detection of adenovirus antigen was eightfold to 10-fold greater than conventional ELISA with microtiter plates. Lockhart et al reported a 30-fold improvement in sensitivity to detect PBS-diluted BYSMV, a plant rhabdovirus, by using NCMs as the solid phase instead of polystyrene plates (11). Low background reflectance values and small standard deviations associated with healthy readings of dot-ELISA also helped improve the sensitivity of the assay by reducing the value: healthy mean + 2 SD of the healthy mean. Usually, the spectrophotometric absorbance values ($A_{405\text{nm}}$) of healthy controls in the cuvette paks of the polystyrene-based Gilford DAS-ELISA tests were more variable. A lower variability of healthy checks in the Dynatech microtiter plates resulted in a better performance than the Gilford cuvette paks.

Table 2. Comparison of dot-ELISA on nitrocellulose membranes (NCM) with two types of DAS-ELISA on polystyrene solid-phase carriers for detection of potato leafroll virus (PLRV) in potato foliage

Sap dilution ^a	Polystyrene cuvettes ^b (Gilford)		Polystyrene plates ^b (Dynatech)		NCMs ^c (Millipore)	
	Absorbance	Visual ^d	Absorbance	Visual ^d	Reflectance	Visual ^d
1:1	2.368	+,+,+	1.08	+,+,+	0.693	+,+,+
1:2	2.364	+,+,+	0.82	+,+,+	0.677	+,+,+
1:4	2.363	+,+,+	0.61	+,+,+	0.610	+,+,+
1:8	2.235	+,+,+	0.42	+,+,+	0.600	+,+,+
1:16	1.198	+,+,+	0.38	+,+,+	0.477	+,+,+
1:32	0.675	+,+,+	0.31	+,+,+	0.365	+,+,+
1:64	0.448	+,,-	0.15	+,,-	0.265	+,+,+
1:128	0.322	-,,-	0.09	-,,-	0.212	+,+,+
1:256	*0.277 ^e	-,,-	0.06	-,,-	0.173	+,+,+
1:512	0.226	-,,-	0.05	-,,-	0.208	+,+,+
1:1,024	0.192	-,,-	*0.04 ^e	-,,-	0.185	+,+,+
1:2,048	0.182	-,,-	0.03	-,,-	*0.153 ^e	+,+,+
1:4,096	0.168	-,,-	0.04	-,,-	0.100	-,,-
Healthy	0.166	-,,-	0.02	-,,-	0.090	-,,-

^a Sap dilution expressed as parts PLRV-infected foliage sap with buffer + additives:parts total sap with buffer.

^b Each number represents the mean absorbance of three experiments, four wells per treatment per plate or cuvette pak. Diluent buffer was phosphate-buffered saline + 2% polyvinylpyrrolidone and 0.2% egg albumin.

^c Each number represents the mean reflectance of three experiments, four wells per treatment per NCM. Diluent buffer was Tris-buffered saline + Tween 80 containing 0.01 M NaDIECA + 0.01 M NaEDTA.

^d For visual analysis, + = positive, - = negative.

^e End-point dilution (*), defined as most dilute PLRV-containing sap exceeding the absorbance/reflectance of the healthy mean + 2 SD of the healthy mean.

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