Promega Notes **Number 70, 1999, p. 14**

Development of a Rapid Capture ELISA Using PCR Products and the PinPoint™ System

By Ingrid G. Winkler, Ph.D., School of Pharmacy and Medical Science, University of South Australia, Adelaide, Australia
Jean-Pierre Levesque, Ph.D., Haematology Division, Hanson Centre for Cancer Research, Adelaide, Australia
Robert L.P. Flower, Ph.D., Pacific Laboratory Medicine Services, Royal North Shore Hospital, St. Leonards, Australia

Corresponding author: e-mail to Ingrid.Winkler@unisa.edu.au

A simple procedure for the development of an ELISA is presented. The procedure uses the PinPoint™ Xa-1 T-Vector System(a,b) *to express a protein antigen encoded by a PCR product. Because the PinPoint™ System adds a biotin tag to the protein, a single-step purification by affinity for streptavidin allows direct use in ELISA. We report the use of this procedure to express a recombinant protein from the nucleocapsid domain of the feline foamy virus (FeFV) gag gene, fused with a biotin tag. This fusion protein was applied directly to streptavidin-coated ELISA wells. Antibody to FeFV was detected in this ELISA with a 100% correlation to other detection methods, including immunoblot, serum neutralization and virus isolation.*

Introduction

Enzyme-linked immunosorbent assay (ELISA) has considerable advantages over other techniques for detection of specific antibodies to protein antigens. These include ease-of-use, speed, sensitivity, reproducibility and a quantifiable result. A major factor limiting the use of ELISA as a diagnostic and basic research tool has been difficulty encountered in purifying an appropriate antigen. This report describes a simple procedure utilizing the specific binding of a biotinylated recombinant fusion protein to streptavidin-coated ELISA plates as an easy, single-step purification (1). The advantage of this method is the rapid development of an ELISA with wide potential application to any defined protein antigen.

The PinPoint™ Xa-1 T-Vector (Cat.# V2610) carries a gene segment encoding a peptide that becomes biotinylated in *E. coli* and can subsequently function as a tag for single-step purification by affinity for streptavidin (2,3). In our experiments, a PCR product encoding a portion of the feline foamy virus (FeFV) gag protein was directly cloned into the PinPoint™ Xa-1 T-Vector (see Figure 1). The protein encoded by the PCR product was expressed as a fusion protein containing a site naturally biotinylated by biotin ligase in *E. coli* (4) and applied directly to streptavidin-coated ELISA plates to allow single-step purification of the antigen through its biotinylated moiety (1). Routine ELISA was then performed to investigate the prevalence of antibodies to FeFV in a domestic cat population.

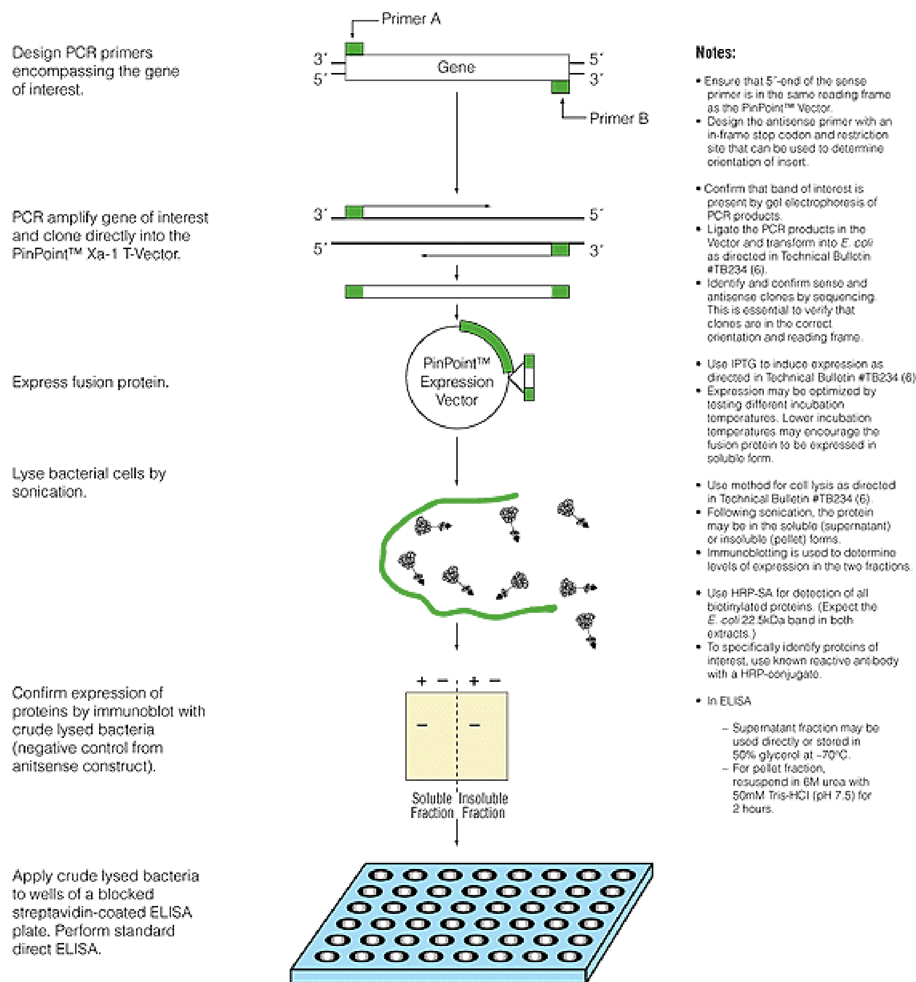

Figure 1. Schematic of the procedure using the PinPoint™ System to produce protein for a rapid capture ELISA. (See reference 1 for more details.)

A clone with the insert in the antisense orientation (PCR products can insert into T-Vectors in the sense or antisense orientation) was used as a negative control. ELISA results were recorded as positive if the average absorbance of duplicate wells with extract from a "sense" clone was at least three times the absorbance of the wells with extract from an "antisense" clone (negative control). Nonspecific reactions (where a positive result is found in the wells from both sense and antisense extracts) were observed in only 1.5% (3/201) of the sera tested. The detection of FeFV antibody by this ELISA was confirmed by immunoblot, serum neutralization and virus isolation (Table 1). Antibody to FeFV, which forms a stable, persistent infection in cats (5), was detected in 53% (107/201) of cats using this ELISA method.

Table 1. Comparison of Immunoblotting, Serum Neutralization, ELISA and Viral Isolation for Detection of FeFV Infection in Four Cats.

	Animal			
	10	**12**	**13**	**14**
Immunoblotting	+	+		+
Serum Neutralization	800	200		25
ELISA	1,600	1,600		400
Isolation of FeFV	+	+		+

A positive immunoblot was identified by the appearance of the gag double band in a protein extract from FeFV-infected cells but not from mock-infected cells. Serum dilutions <25 in the serum neutralization assay or <100 in the ELISA were recorded as negative.

CLONING OF THE FEFV GAG GENE

Molecular cloning and ligation of PCR products into the PinPoint™ Xa-1 T-Vector were performed as specified in the *PinPoint™ Xa-1 T-Vector Systems Technical Bulletin* #TB234 (6). One microliter of the ligation mix was electroporated into 100µl of competent *E. coli* JM109 cells (>10^8 cfu/µg DNA) as described by Dower *et al.* (7). The transformed cells were grown on selective media and recombinant clones were selected after overnight growth.

Orientation of the insert in recombinant clones was determined by restriction digestion and confirmed by sequencing using the PinPoint™ Vector Sequencing Primer (Cat.# V4211, 5´-CGT GAC GCG GTG CAG GGC G-3´). The sequence was compared with that known for the PinPoint™ Xa-1 T-Vector and for the FeFV *gag* gene (8).

EXPRESSION AND DETECTION OF RECOMBINANT PROTEIN

Procedures for expression of recombinant fusion protein were as described in the *PinPoint™ Xa-1 T-Vector Systems Technical Bulletin* #TB234 (6). After disruption of the bacterial pellet by sonification, the supernatant fraction containing soluble bacterial proteins was used for immunoblotting or applied directly to the wells of a blocked streptavidin-coated ELISA plate (or alternatively stored frozen in 50% glycerol until later use). As recombinant proteins may also be expressed in an insoluble form, the pellet fraction was resuspended at 10% w/v in 50mM Tris-HCl (pH 7.5), 6M urea and gently agitated for 2 hours at 4°C before use.

The production of recombinant biotinylated proteins was assessed by immunoblotting with either HRP-conjugated streptavidin (HRP-SA) or with sera from a FeFV-positive cat and a FeFV-negative cat revealed by HRP-conjugated protein A/G (Figure 2). The sense extract contained a unique 32kDa apparent molecular weight band (the recombinant FeFV protein) detected by both HRP-streptavidin (lane 1) and FeFV-positive serum (lane 3), which was absent in antisense extracts (lanes 2 and 4). The 22.5kDa apparent molecular weight band detected by HRP-streptavidin in extracts from both sense and antisense clones corresponded to biotin carboxyl carrier protein, the only biotinylated protein produced naturally by *E. coli* (4). No naturally occurring antibodies to biotin carboxyl carrier protein were observed in cat sera following immunoblotting. The 13.5kDa band detected by HRP-streptavidin is the vector portion of the fusion protein as described in the *PinPoint™ Xa-1 T-Vector Technical Bulletin* #TB234 (6).

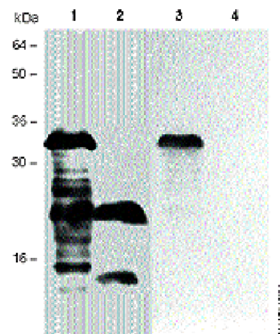

Figure 2. Detection of biotinylated, recombinant nucleocapsid domain of FeFV gag expressed by sense and antisense clones. Recombinant sense (lanes 1 and 3) and antisense (lanes 2 and 4) protein pellet fractions expressed at 37°C were immunoblotted against HRP-conjugated streptavidin for detection of biotinylated proteins (lanes 1 and 2) or against FeFV positive sera (lanes 3 and 4) and HRP-conjugated protein A/G (lanes 3 and 4). Following expression of recombinant fusion proteins and lysis of the bacterial pellet by sonication, 10µl of the lysate were separated on an 18% polyacrylamide gel and transferred to a PVDF membrane by blotting overnight at 24V. The membrane was then blocked using PBST with 5% dried skim milk (blocking buffer), before a one-hour incubation with cat serum diluted 1/100 in blocking buffer followed by three washes in PBST and a second one-hour incubation with 7µg/ml horseradish peroxidase-conjugated protein A/G in PBST (Pierce) or, alternatively, a portion of the membrane was incubated directly with horseradish peroxidase-conjugated streptavidin (Vector Labs) in blocking buffer. Following incubation with conjugates, the membranes were washed five times in PBST before detection by enhanced chemiluminescence (ECL™, Amersham) detection. Reprinted from the *Journal of Immunological Methods* **207**, 6977, 1997, with permission from Elsevier Science and the author.

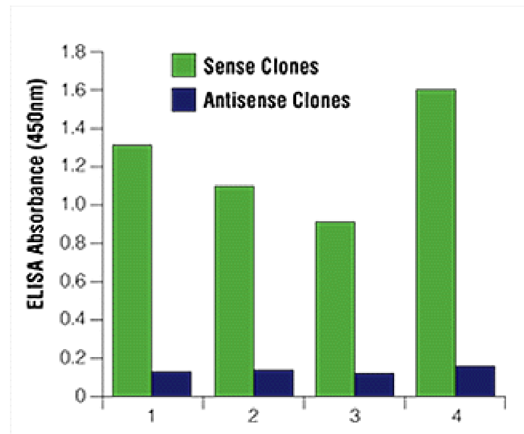

Figure 3. Distribution of recombinant FeFV NC domain proteins in supernatant and pellet fractions after bacterial expression. Duplicate ELISA wells were coated with the supernatant (lanes 1 and 3) and pellet (lanes 2 and 4) fractions after induction of biotinylated recombinant fusion protein at either 25°C for 17 hours (lanes 1 and 2) or at 37°C for 5 hours (lanes 3 and 4). Green columns are absorbance for sera with recombinant protein from sense clones, and blue columns represent absorbance from antisense clones.

Optimizing the ELISA

The expression of recombinant proteins in soluble (supernatant) or insoluble (pellet) bacterial extracts can be influenced by incubation temperature. Therefore, the concentration of antigen in extracts from both supernatant and pellet fractions of sense and antisense clones expressed at two different temperatures were assessed by ELISA using serum from a FeFV-positive cat. FeFV-positive cat serum detected recombinant protein following expression at either 25°C (lanes 1 and 2) or 37°C (lanes 3 and 4), in both soluble fraction (lanes 1 and 3) and insoluble pellet fractions (lanes 2 and 4). However, when incubated at 25°C, a greater proportion of soluble recombinant protein was formed than when at 37°C. Subsequent ELISAs were performed using recombinant FeFV antigen supernatant fraction expressed at 25°C.

To provide maximum sensitivity, a comparison was made between four different blocking agents commonly used in ELISA. The absorbance for the FeFV-negative serum was similar for all four blocking agents. Absorbances, however, varied greatly with the blocking agent used. With FeFV-positive sera, the absorbance was highest with dried skim milk as a blocking agent (4.3-fold greater than the antisense control), followed by casein (3.6-fold), gelatin (3.0-fold), and the lowest absorbances for the positive control sera were observed when BSA was used as a blocking agent (only 2.6-fold greater than the antisense control). Subsequent ELISAs were performed using PBST with 0.5% dried skim milk as a blocking agent.

Conclusion

As for any immunoassay, selection of an appropriate antigen is important in development of a sensitive, specific test. In the ELISA described, the nucleocapsid domain of FeFV gag was selected, as this is antigenically different from similar proteins in other retroviruses and can be specifically detected by immunoblotting using sera from naturally infected cats (8). Apart from the detection of antibodies to infectious agents, this method has potential application for the screening of proteins for which the immunogenic regions have not already been defined, or as a rapid tool for screening hybridoma supernatants for production of specific antibodies to a defined antigen.

Sequences for coding regions of many proteins are now widely available. Primers for PCR can be designed to encompass the exact region or epitope(s) of interest. The size of proteins that can be expressed in this system would theoretically only be limited by the length of PCR products that can be successfully cloned into the PinPoint™ Xa-1 T-Vector. Any incorrect coding sequences or inserts that are not in-frame with the vector fusion protein can be detected by sequencing of the insert. Confirmation by sequencing prior to investigation of reactions with sera is important as prematurely truncated peptides are unlikely to be detected by antisera.

We describe a procedure for rapid development of an ELISA that is ideal for use as a research tool. To be effective, this procedure requires knowledge of DNA sequence and antisera to confirm detection of the antigen in the recombinant protein. An ELISA for FeFV, an agent that stimulates sustained antibody responses, was established using this technique and was found to be specific and reliable. Serology, based on production of antigen by recombinant DNA techniques, has many advantages over preparation of antigen using traditional purification procedures, in that establishment of ELISA is rapid and requires no special equipment. For these reasons, techniques of this type are likely to become more widely used.

Acknowledgement

Sera from 201 Australian cats were kindly donated by Veterinary Pathology Services and Vetlab, both of Adelaide, Australia.

References

1. Winkler, I.G. *et al.* (1997) *J. Immunol. Meth*. **207**, 69.
2. Lesley, S.A. and Groskreutz, D.J. (1997) *J. Immunol. Meth*. **207**, 147.
3. Lesley, S.A., *et al.* (1995) *Promega Notes* **54**, 8.
4. Cronan, J.E. (1990) *J. Biol. Chem*. **265**, 10327.
5. Shroyer, E.L. and Shalaby, M.R. (1978) *Am. J. Vet. Res.* **39***,* 555.
6. *PinPoint™ Xa-1 T-Vector Systems Technical Bulletin* #TB234, Promega Corporation.
7. Dower, W.J. , Miller, J.F. and Ragsdale, C.W. (1988) *Nucl. Acids. Res*. **16**, 6127.
8. Winkler, I.G. *et al.* (1997) *J. Virol.* **71**, 6727.

Ordering Information

Product	Size	Cat.#
PinPoint™ Xa-1 T-Vector System I	20 reactions	V2610
PinPoint™ Xa-1 T-Vector System II plus Competent Cells	20 reactions	V2850
PinPoint™ Xa Protein Purification System[b]		V2020
PinPoint™ Xa-1 Vector[b]	10µg	V2031
PinPoint™ Xa-2 Vector[b]	10µg	V2051
PinPoint™ Xa-3 Vector[b]	10µg	V2061
PinPoint™ Vector Sequencing Primer	2µg	V4211
JM109 Competent Cells, High Efficiency (>10^8cfu/µg)	1ml	L2001
Wizard® PCR Preps DNA Purification System[c]	50 preps	A7170

[a]Licensed under one or both of U.S. Pat. No. 5,487,993 and European Pat. No. 0 550 693.

[b]For research purposes only. Not for diagnostic or therapeutic use. For nonresearch uses of the portion of the PinPoint™ Vector encoding the biotinylation sequence, please contact Promega Corporation for licensing information.

[c]Licensed under U.S. Pat. No. 5,075,430.

© 1999 Promega Corporation. All Rights Reserved.

Wizard is a trademark of Promega Corporation and is registered with the U.S. Patent and Trademark Office. PinPoint is a trademark of Promega Corporation.

ECL is a trademark of Amersham Pharmacia Biotech Ltd.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.