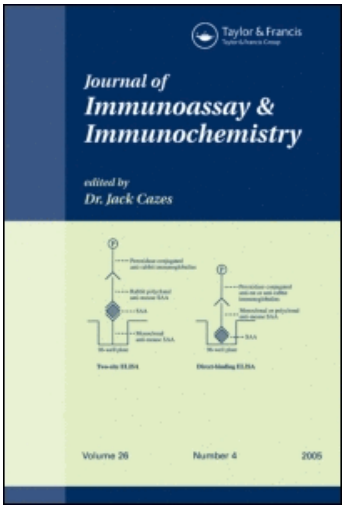

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Sandwich ELISA for Phosphoglycerate Kinase

Weilin Zhao[a]; Seinyone Pao[a]; Fatima Malik[a]; James Soh[b]; Sonalis Fernandez[c]; William J. Chirico[ab]
[a] Department of Anatomy and Cell Biology, [b] Molecular and Cellular Biology Program, [c] Department of Medicine, State University of New York Downstate Medical Center, Brooklyn, New York

Online publication date: 05 November 2010

To cite this Article Zhao, Weilin , Pao, Seinyone , Malik, Fatima , Soh, James , Fernandez, Sonalis and Chirico, William J.(2008) 'A Sandwich ELISA for Phosphoglycerate Kinase', Journal of Immunoassay and Immunochemistry, 29: 3, 220 — 233
To link to this Article: DOI: 10.1080/15321810802119588
URL: http://dx.doi.org/10.1080/15321810802119588

Journal of Immunoassay and Immunochemistry, 29: 220–233, 2008

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810802119588

A Sandwich ELISA for Phosphoglycerate Kinase

Weilin Zhao,[1] Seinyone Pao,[1] Fatima Malik,[1] James Soh,[2] Sonalis Fernandez,[3] and William J. Chirico[1,2]
[1]Department of Anatomy and Cell Biology
[2]Molecular and Cellular Biology Program
[3]Department of Medicine, State University of New York
Downstate Medical Center, Brooklyn, New York

Abstract: Phosphoglycerate kinase (PGK1) is a key enzyme in glycolysis that can also be released from certain cells. In the extracellular milieu, PGK1 reportedly acts as a disulphide reductase to activate plasmin, resulting in the production of angiostatin, a potent angiogenesis inhibitor. Certain cancer cell lines secrete unusually large amounts of PGK1, raising the possibility that serum PGK1 levels can be used to screen for cancer. To facilitate the characterization of the PGK1 secretory pathway and to monitor serum levels of PGK1, we have developed a sensitive sandwich ELISA using an immuno-affinity-purified chicken polyclonal antibody for capturing PGK1 and an immuno-affinity-purified rabbit polyclonal antibody for detecting it. The assay is about 10-fold more sensitive than other reported PGK1 ELISAs. We used the ELISA to quantify the amount of PGK1 released from HeLa cells and PGK1 serum levels in cancer patients. Of 10 cancer patients whose serum was tested, 3 of 4 with pancreatic cancer had 65–900% higher levels of PGK1 than that found in normal serum.

Keywords: Cancer, ELISA, HeLa, PGK1, Secretion, Serum

INTRODUCTION

PGK1 occupies a key position in glycolysis, catalyzing the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate liberating ATP in the

Correspondence: William J. Chirico, Ph.D., Department of Anatomy and Cell Biology, SUNY Downstate Medical Center, 450 Clarkson Ave., Box 5, Brooklyn, NY 11203. E-mail: william.chirico@downstate.edu

process. Interestingly, PGK1 can be released from cells and act as a disulphide reductase to reduce plasmin.[1] Serine- and metallo-proteases subsequently convert reduced plasmin into angiostatin, a potent inhibitor of angiogenesis.

The mechanism by which PGK1 is released from cells is unknown. PGK1, however, lacks a signal sequence rendering entrance into the endoplasmic reticulum and passage through the classical secretory pathway unlikely. Therefore, PGK1 may be released from cells through a nonclassical secretory pathway. At least four non-classical secretory pathways have been identified, but incompletely characterized.[2]

Hogg and coworkers reported that several mammalian cell lines secrete different amounts of PGK1.[1] For example, SU.86.86 cells derived from a pancreatic ductal carcinoma released about 10-fold more PGK1 than HT1080 fibroblasts. These findings raise the possibility that PGK1 levels in serum can be used to screen for cancer. Indeed, Chen et al.[3] showed that the concentration of PGK1 is elevated in human serum obtained from patients with lung carcinoma. Hwang et al.[4] found that PGK1 levels were elevated in serum from patients with pancreatic and other types of cancer.

To facilitate the analysis of the pathway by which PGK1 is released from certain cells and to monitor PGK1 levels in serum, we raised rabbit and chicken polyclonal antibodies against purified human recombinant PGK1 and used them to develop a sandwich ELISA. The assay is sensitive, quantitative, and reproducible.

EXPERIMENTAL

Materials

AffiGel 10 and Bradford Reagent were obtained from BioRad Laboratories. Polyvinylidene fluoride (PVDF) membranes were obtained from MilliPore. Black polystyrene 96-well assay plates (#3925) were obtained from Costar. Affinity purified donkey anti-chicken IgY and horseradish peroxidase (HRP) conjugated anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. SuperSignal ELISA Femto Maximum Sensitivity Substrate, SuperSignal West Pico Chemiluminescent Substrate, ImmunoPure TMB Substrate Kit, and Protein-Free (PBS) Blocking Buffer were obtained from Pierce. Minimal Essential Medium (MEM) and Newborn Calf Serum were obtained from Invitrogen. HeLa cells (CCL2) were obtained from American Type Culture Collection.

Buffers

The following buffers were used: RIPA (50 mM Tris, pH 8, 150 mM NaCl, 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate (DOC), 0.1% (w/v) sodium dodecyl sulfate (SDS)), Tris-buffered saline Tween 20 (TBST) (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20), PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl), PGK1 ELISA Diluent (1% (w/v) NP40, 0.5% (w/v) DOC, 0.1% (w/v) SDS, and 1% (w/v) bovine serum albumin (BSA) in PBS), and lysis buffer (9% (v/v) Triton X100 in PBS).

Purification of Human PGK1

Human recombinant PGK1 was expressed in and purified from *E. coli* as described previously.[1] Affinity purified human recombinant PGK1 was dialyzed against 100 mM HEPES, pH 7.5.

PGK1 Antibody Production

Affinity purified human recombinant PGK1 was sent to Pocono Rabbit Farm & Laboratory, Inc., where it was injected into two rabbits and two chickens according to the facility's standard protocols.

Immuno-Affinity Purification of PGK1 Antibodies

AffiGel-10 (0.5 mL) was washed with water according to the manufacturer's instructions and then incubated with 1 mL of purified human recombinant PGK1 (3.3 mg/mL) in 100 mM HEPES, pH 7.5, overnight at 4°C. The resin was washed with the same buffer and then incubated with 100 mM ethanolamine, pH 8, for 1 h at 4°C to block unreacted sites. After extensive washing with high and low pH buffers as previously described,[5] the resin was rotated overnight at 4°C with human PGK1 rabbit antiserum, which had been diluted 10-fold with 10 mM Tris, pH 8. The resin was transferred into a 1 mL column, washed, and then PGK1 antibodies were eluted with high and low pH buffers as described.[5] Fractions containing high concentrations of protein were pooled. Immuno-affinity purified antibodies were concentrated using 95% ammonium sulfate, dissolved in a small volume of PBS, and then dialyzed overnight against PBS. Glycerol was added to a final concentration of 50% and the antibody stored at –20°C. In a similar fashion, PGK1 chicken antibody was purified separately using a fresh aliquot

of PGK1 immobilized on AffiGel-10. The final concentrations of the purified PGK1 rabbit and chicken antibodies were 1.16 and 0.416 mg/mL, respectively.

Protein Assay

Protein was assayed using the method of Bradford with bovine gamma globulin as the standard.[6]

SDS PAGE and Western Blotting

Proteins were separated on 12% SDS-PAGE gels[7] and transferred to PVDF membranes.[8] Proteins in SDS-PAGE gels were stained with 1% amido black in 7% acetic acid and destained with 3% acetic acid.

PGK1 ELISA Assay

After optimizing experimental parameters, a standard procedure for the PGK1 sandwich ELISA was established as follows. Affinity purified donkey anti-chicken IgY (100 μL, 4 μg/mL in PBS) was added to each well of a black 96-well plate and incubated overnight at 4°C. The wells were washed 3 times with PBS and then incubated with 300 μL Protein-Free (PBS) Blocking Buffer for 1 h at room temperature. The wells were washed once with RIPA buffer, and then incubated with 100 μL of immuno-affinity purified PGK1 chicken antibody (80 ng/mL in diluent) for 1 h at room temperature with gentle shaking. After washing the wells 3 times with RIPA buffer, 100 μL of either unknown sample or purified PGK1 in diluent was added to the wells and the plate was incubated overnight at 4°C with gentle shaking. The wells were washed 3 times with RIPA buffer. Immuno-affinity purified PGK1 rabbit antibody (100 μL, 80 ng/mL in diluent) was added to each well and the plate was incubated for 1 h at room temperature with gentle shaking. The wells were washed 3 times with RIPA buffer and then incubated with 100 μL of donkey anti-rabbit HRP (1/50,000 in diluent) for 1 h at room temperature with gentle shaking. The wells were washed 3 times with RIPA buffer and the plate was placed on ice for 10 min before removing the last wash. Pierce Femto Reagent (100 μL) was added at 4°C. The plate was incubated for 1 min at room temperature with gentle shaking, incubated at 37°C for 2 min, and then luminescence was immediately measured in a Victor^3 1420 Multicolor Fluorescence Plate Reader by Perkin Elmer. Data were expressed as relative light units (RLU).

Cell Culture

HeLa cells were grown in MEM with 10% newborn calf serum in 10 cm plates at 37°C, 5% CO_2. When the cells approached confluence, the media was replaced with 5 mL of serum-free MEM and incubated for 24 h. The conditioned medium was collected, centrifuged at 3,000 rpm in a clinical centrifuge for 5 min, and the supernatant was assayed for PGK1 using the sandwich ELISA described above.

Collection of Serum Samples

Human serum samples were obtained from the National Cancer Institute's Cooperative Human Tissue Network (CHTN). This protocol was approved by the Institutional Review Board of the SUNY Downstate Medical Center. Serum was diluted 5- to 10-fold with PBS and then adjusted with diluent before assaying.

Statistics

Results were expressed as the mean $\pm$ standard deviation (SD) or standard error of the mean (SEM). The coefficient of variation (CV) was obtained by dividing the SEM by the mean and expressing it as a percent. PGK1 levels in sera from cancer patients and the control were compared using the Student's *t* test.

RESULTS

The specificity of the immuno-affinity purified PGK1 rabbit and chicken antibodies was examined using Western blotting. Purified PGK1 and HeLa extract were separated on SDS-PAGE gels (Fig. 1a, lanes 1 and 2) and then transferred to PVDF membranes. Each antibody recognized purified human recombinant PGK1 (Fig. 1b, lanes 1 and 3) and only one band in HeLa cell extracts (Fig. 1b, lanes 2 and 4). These results indicate that the immuno-affinity purified rabbit and chicken antibodies are specific for PGK1.

To facilitate the analysis of the PGK1 export pathway, we developed a sandwich ELISA using the specific immuno-affinity purified PGK1 chicken and rabbit antibodies for capturing and detecting PGK1, respectively. The immuno-affinity purified PGK1 chicken antibody was chosen as the capture antibody because we had limited quantities of it. We first determined whether a coating antibody would be advantageous by comparing standard curves of recombinant human PGK1 in the absence

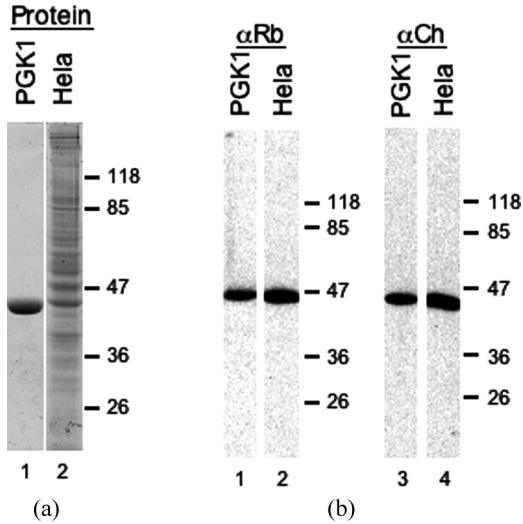

Figure 1. Immuno-affinity purified rabbit and chicken anti-human PGK1 antibodies are specific. (a) Purified human recombinant PGK1 (2 μg, lane 1) and total cellular extracts from HeLa cells (10 μg, lane 2) were separated on SDS-PAGE gels and stained for protein with amido black. (b) Purified human recombinant PGK1 (1 ng, lane 1; 10 ng, lane 3) and total cellular extracts from HeLa cells (80 ng, lane 2; 800 ng, lane 4) were separated on SDS-PAGE gels, transferred to PVDF membranes, and probed with affinity-purified PGK1 rabbit (lanes 1 and 2) or chicken (lanes 3 and 4) antibodies. The primary antibodies (1/10,000 dilution) were detected with the corresponding HRP conjugated antibody (1/50,000 dilution). The secondary antibodies were detected as described in Materials and Methods. The numbers on the right correspond to the migration of molecular mass (kDa) markers.

or presence of donkey anti-chicken IgY bound to the surface of a 96-well plate. The sensitivity of the assay was 8-fold greater in the presence than in the absence of the coating antibody (Fig. 2). The sandwich ELISA also worked well when donkey anti-rabbit IgG was used for coating, the immuno-affinity purified PGK1 rabbit antibody was used for capturing, and the immuno-affinity purified PGK1 chicken antibody was used for detecting (data not shown).

We determined the optimal incubation time for capturing PGK1. The response was linear throughout the course of the 24 h assay (Fig. 3). The results of the time course indicated that a convenient overnight (16 h) incubation would yield excellent sensitivity.

The concentrations of the capture (immuno-affinity purified PGK1 chicken antibody) and detection (immuno-affinity purified PGK1 rabbit antibody) antibodies were also optimized. First, we established standard

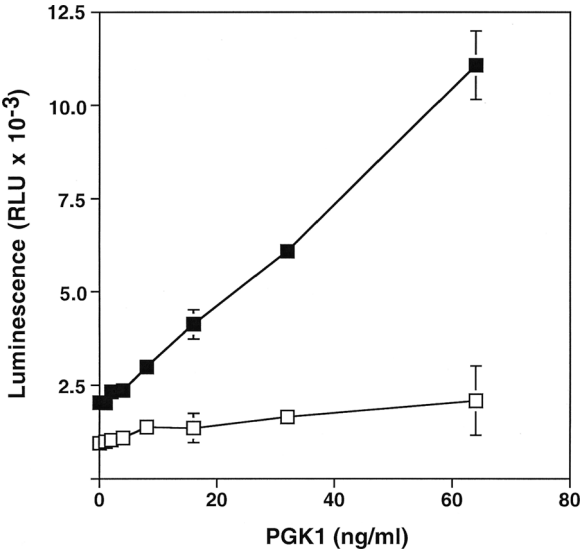

Figure 2. Coating plates with anti-chicken IgY improves sensitivity. Standard curves of PGK1 were generated using 96-well plates that were treated with 100 μL of either PBS (□) or (■) donkey anti-chicken IgY (4 μg/mL in PBS). The other steps of the procedure were performed using the optimized standard ELISA described in Experimental, except that PGK1 was captured for only 1 h. Symbols represent the mean (n = 3) ± SD.

curves of PGK1 at different concentrations of chicken antibody while maintaining standard conditions for all other parameters (Fig. 4). Although sensitivity increased in the presence of increasing concentrations of the chicken antibody, the response began to saturate above 80 ng/mL. Therefore, we chose 80 ng/mL chicken antibody for our experiments because, at this concentration, the standard curve was linear and highly reproducible. In a similar fashion, we optimized the concentration of the immuno-affinity purified PGK1 rabbit antibody (Fig. 5). Interestingly, the optimal concentration of the rabbit antibody (80 ng/mL) was identical to that of the chicken antibody.

We optimized the concentration HRP-conjugated anti-rabbit antibody (Fig. 6). Although higher concentrations of the secondary antibody improved sensitivity, they began to saturate, as expected, at lower concentrations of antigen. Thus, we found that sufficient sensitivity and excellent reproducibility was obtained at 1/50,000 dilution of the secondary antibody.

Many ELISAs use 3,3′,5,5′-tetramethylbenzidine (TMB) as a colorimetric substrate and often achieve high sensitivity with prolonged incubations. The luminol/enhancer combination in Pierce's SuperSignal ELISA

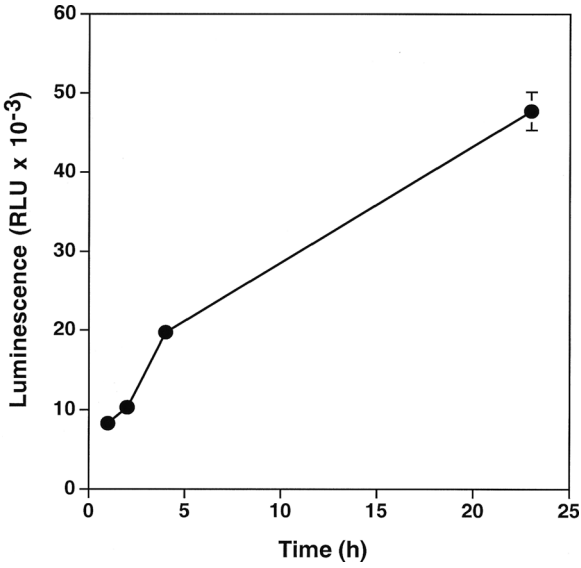

Figure 3. Time course of capturing PGK1. The time course of capturing purified human recombinant PGK1 (20 ng/mL) by immuno-affinity purified PGK1 chicken antibody during a 24 h incubation was determined. With the exception of the length of this incubation, the standard ELISA was used (see Experimental). Symbols represent the mean (n = 3) ± SD.

Femto Maximum Sensitivity Substrate (FEMTO) yields excellent sensitivity, but the product is labile and samples must be measured within minutes of adding the substrate. We, therefore, compared the sensitivity of TMB and FEMTO (Fig. 7). We found that FEMTO was much more sensitive than TMB under the conditions of our standard assay. In fact, the TMB response at the highest concentration of PGK1 was negligible even with prolonged incubation.

One of the primary reasons for developing the PGK1 sandwich ELISA was to quantify the amount of PGK1 secreted from cells. To test the utility of the assay for secretion studies and to determine its intra- and inter-assay reproducibility, we enlisted four independent researchers to measure the amount of PGK1 released from HeLa cells. After 24 h, HeLa cells released 10–12 ng/mL of PGK1 into the conditioned media (Table 1). The intra-assay variation was 2.8–4.1%, whereas the inter-assay variation was 4.4–7.3% (Table 1). These results demonstrate that HeLa cells release PGK1 and that our sandwich ELISA can be used reliably to quantify it.

Hogg's group reported that a pancreatic cancer cell line released large amounts of PGK1.[1] We hypothesized, therefore, that levels of PGK1 in serum might be used to screen for cancer. To test whether our PGK1 ELISA could be used to quantify PGK1 in human serum,

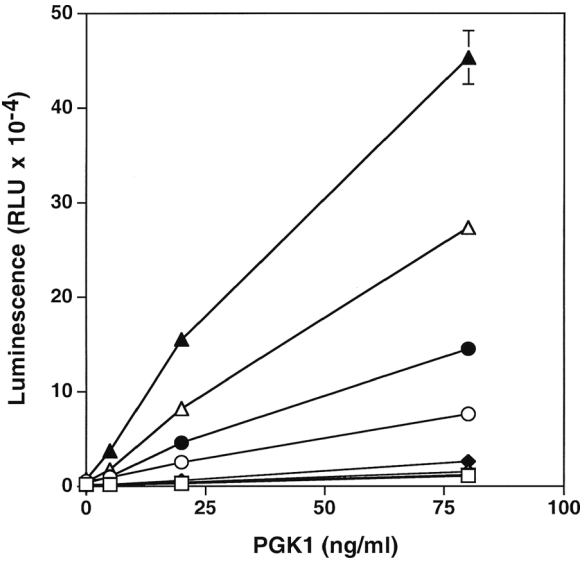

Figure 4. Optimal concentration of the capture antibody. Standard curves of purified human recombinant PGK1 were generated in the presence of different concentrations of the immuno-affinity purified PGK1 chicken antibody used for capturing. The concentrations of this antibody were 0 (□), 10 (■), 20 (◇), 40 (◆), 80 (○), 160 (●), 320 (Δ), and 640 (▲) ng/mL. All other steps were performed according to the standard PGK1 ELISA (see Experimental). Symbols represent the mean (n = 3) ± SD.

we examined serum from breast, colon, and pancreatic cancer patients and from a patient with leiomyoma, a benign neoplasm of smooth muscle (Fig. 8). PGK1 levels were 65–900% higher in the serum of 3 of the 4 pancreatic cancer patients. In contrast, no pattern emerged from serum PGK1 levels among the colon cancer patients. One patient's PGK1 level was 70% lower and another's 90% higher than the control. A third colon cancer patient had a PGK1 serum level indistinguishable from the control. PGK1 serum levels in the breast and leiomyoma patients were essentially the same as the control. These results show that our PGK1 sandwich ELISA can be used to monitor serum levels of PGK1. Although the results suggest that pancreatic cancer patients have elevated PGK1 levels, more patients must be examined to determine whether PGK1 serum levels can be used to screen for cancer.

DISCUSSION

To facilitate the study of the mechanism by which PGK1 is released from certain cells and to determine whether serum levels of PGK1 were

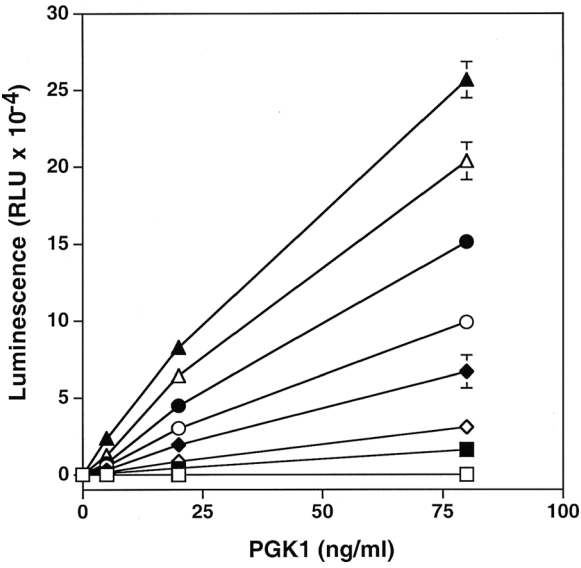

Figure 5. Optimal concentration of the detection antibody. Standard curves of purified human recombinant PGK1 were generated in the presence of different concentrations of the immuno-affinity purified PGK1 rabbit antibody used for detection. The concentrations of this antibody were 0 (□), 40 (■), 80 (◇), 160 (◆), 320 (○), 640 (●), 1280 (Δ), 2560 (▲) ng/mL. All other steps were performed according to the standard PGK1 ELISA (see Experimental). Symbols represent the mean (n = 3) ± SD.

correlated with different types or severity of cancer, we developed a sandwich ELISA. We generated rabbit and chicken polyclonal antibodies against human PGK1 because none was commercially available when we started this project. We immuno-affinity purified the polyclonal antibodies and used them to obtain a sensitive, specific, and reliable PGK1 sandwich ELISA.

Of the several parameters optimized during the development of the PGK1 ELISA, coating the plate with donkey anti-chicken antibody was the most important and dramatically improved the sensitivity of the ELISA. We suspect that either the coating antibody optimally displays the PGK1 chicken antibody for recognizing PGK1 or that the immuno-affinity purified chicken antibody becomes inactive upon binding to the surface of the 96-well plate. We also found that the optimal concentration of both the capture and detection antibody was 80 ng/mL under standard conditions. We speculate that this reflects a stoichiometric balance in the assay resulting from steric hindrance of closely aligned epitopes.

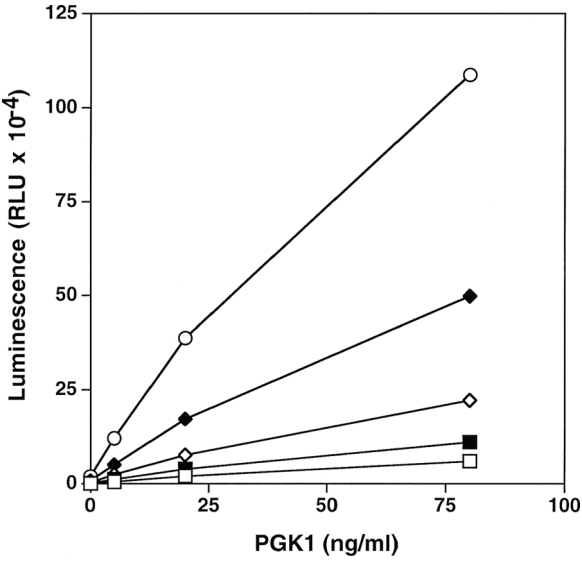

Figure 6. Optimization of HRP-conjugated donkey anti-rabbit antibody. Serial dilutions (1/200,000) (□), 1/100,000 (■), 1/50,000 (◇), 1/25,000 (◆), and 1/12,500 (○)) of HRP-conjugated donkey anti-rabbit IgG were used to detect immuno-affinity purified PGK1 rabbit antibody bound to captured PGK1. With the exception of the concentration of the HRP secondary antibody, all other steps were performed according to the standard ELISA procedure (see Experimental). Symbols represent the mean ($n = 3$) ± SD.

Two ELISAs have been developed previously to measure PGK1.[3,4] Hogg and coworkers[3] developed a sandwich ELISA using a mouse monoclonal antibody to capture PGK1 and a rabbit antibody to detect it. They reportedly detected as little as 25 ng/mL of PGK1. Hwang et al.[4] developed a PGK1 antibody capture ELISA in which serum proteins were added to a 96-well plate and goat polyclonal antibody was used to detect bound PGK1. Neither the purity of the antibody nor the sensitivity of the assay was reported.[4] In contrast, our sandwich PGK1 ELISA is composed of immuno-affinity purified PGK1 chicken polyclonal antibody for capturing and immuno-affinity purified PGK1 rabbit polyclonal antibody for detecting PGK1. The assay can detect as little as 2.5 ng/mL PGK1, about 10-fold more sensitive than other reported PGK1 ELISAs.

PGK1 serum levels in a variety of cancer subjects have been investigated by others.[3,4] Chen et al.[3] showed that PGK1 serum levels are negatively correlated with survivability in lung cancer. Hwang et al.[4] showed that the PGK1 concentration was elevated in serum from patients with different types of cancer. We found that 3 of the 4 pancreatic cancer

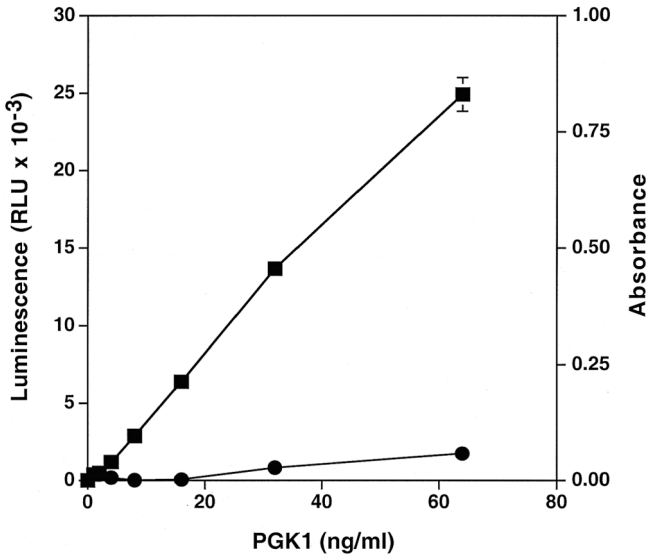

Figure 7. Comparison of TMB and FEMTO detection substrates. The relative sensitivity of the TMB (●) and FEMTO (■) substrates were compared by generating standard curves of PGK1 using the standard PGK1 ELISA described in Experimental. The absorbance of TMB at 490 nm and the luminescence of the FEMTO substrate were measured. Symbols represent the mean (n = 3) ± SD.

patients tested had 65–900% higher serum PGK1 levels than control serum. No clear pattern emerged from the serum PGK1 levels among the colon cancer patients examined. Serum PGK1 levels in the breast cancer patient and in the patient diagnosed with leiomyoma were essentially the same as the control. Although the small number of patient sera tested limits our ability to draw conclusions, the results from the pancreatic cancer patients suggest that a large scale study should be undertaken to determine whether PGK1 serum levels can be used to screen for cancer.

Table 1. Reproducibility of the PGK1 Sandwich ELISA

	Mean PGK1 ± SEM (ng/mL)	CV (%)
A. Intra-assay precision (n = 4)		
Sample 1	12.3 ± 0.5	4.1
Sample 2	10.7 ± 0.3	2.8
B. Inter-assay precision (n = 4)		
Sample 1	11.4 ± 0.5	4.4
Sample 2	9.6 ± 0.7	7.3

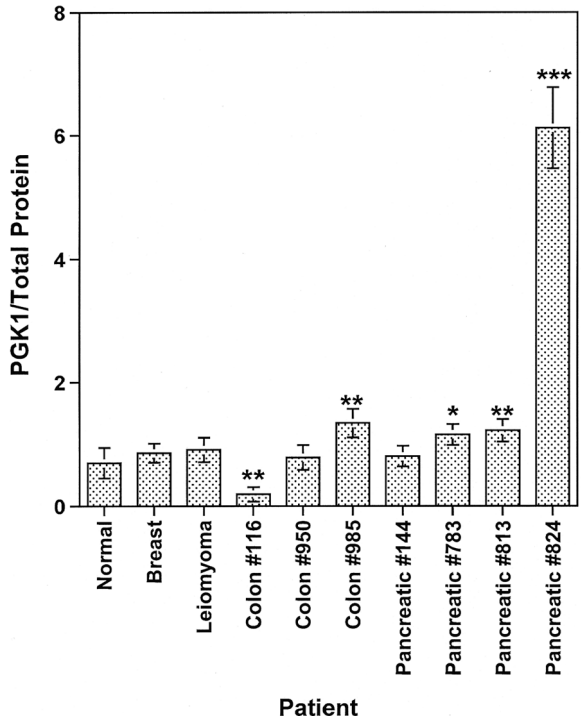

Figure 8. PGK1 serum levels in cancer patients. The sera from the indicated patients were diluted 5- to 10-fold and the concentration of PGK1 was determined using the standard PGK1 ELISA described in Experimental. The PGK1/total protein ratio was determined by dividing the concentration of PGK1 (ng/mL) by the concentration of total protein (mg/mL). The asterisks indicate PGK1 serum levels that are significantly different from control serum (Normal). The P values corresponding to one, two, and three asterisks are <0.05, <0.01, and <0.001, respectively. Symbols represent the mean ($n = 4$) $\pm$ SD.

In conclusion, the sandwich ELISA described here can be used to quantify PGK1 levels in conditioned media of cell cultures for in vitro PGK1 export studies and in human serum for potential use as screen for cancer. The assay is sensitive, quantitative, and reliable.

ABBREVIATIONS

The abbreviations used were: horseradish peroxidase (HRP), minimal essential media (MEM); 3,3′,5,5′-tetramethylbenzidine (TMB); phosphate-buffered saline (PBS); radioimmuno precipitation assay (RIPA); polyvinylidene fluoride (PVDF); sodium dodecyl sulfate (SDS); standard

deviation (SD); standard error of the mean (SEM); Tris-buffered saline Tween 20 (TBST).

ACKNOWLEDGMENTS

We thank Drs. Richard Kream, John Lewis, and Paul Rava for helpful discussions, Dr. Mahmood Hussain and Kezi Dai for the use of the fluorescence plate reader, Jenny Belizare for technical assistance, Dr. Philip Hogg for the generous gift of the human PGK1 expression plasmid, CHTN for providing sera from human cancer patients, and Dr. George Ojakian for critiquing the manuscript. This work was supported by a Dean's Research Investment Award from the SUNY Downstate Medical Center.

REFERENCES

1. Lay, A.J. Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase. Nature **2000**, *408* (6814), 869–873.
2. Nickel, W. Unconventional secretory routes: Direct protein export across the plasma membrane of mammalian cells. Traffic **2005**, *6* (8), 607–614.
3. Chen, G. Protein profiles associated with survival in lung adenocarcinoma. Proc. Natl. Acad. Sci. U S A **2003**, *100* (23), 13537–13542.
4. Hwang, T.L. Overexpression and elevated serum levels of phosphoglycerate kinase 1 in pancreatic ductal adenocarcinoma. Proteomics **2006**, *6* (7), 2259–2272.
5. Harlow, E.; Lane, D. *Antibodies, a Laboratory Manual*; Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1988.
6. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **1976**, *72*, 248–254.
7. Ngosuwan, J. Roles of cytosolic Hsp70 and Hsp40 molecular chaperones in post-translational translocation of presecretory proteins into the endoplasmic reticulum. J. Biol. Chem. **2003**, *278* (9), 7034–7042.
8. Sheng, Z. Direct regulation of rRNA transcription by fibroblast growth factor 2. Mol. Cell Biol. **2005**, *25* (21), 9419–9426.

Received January 20, 2008
Accepted February 28, 2008
Manuscript 3272