2. Crystal screen HT, Index HT, Grid screening PEG6000 for the first screening of the crystallization conditions were purchased from Hampton research.
3. Wizard I and II purchased from Emerald BioStructure were used for the first screening of the crystallization condition.
4. Computer programs: HKL2000 (12) for integration and scaling of X-ray diffraction data; SnB (13) for determination of the selenium substructure of the Se-Met variant; SHARP (14) for refinement of the selenium sites and calculation of the initial phase; RESOLVE (15) for automated initial model building and refinement; MOLREP (16) for molecular replacement; Refmac5 (17) and CNS (18) for refinement of the model; XtalView (19) for manual model building.

3. Methods

Cell-free translation of proteins can be achieved mainly by three different modes, batch mode translation (20, 21), bilayer system (22), and CFCF protein synthesis method (10, 23). In the cell-free protein production, mRNA purification represents the most time-consuming step. Recently, based on the bilayer system, a sequential transcription–translation method was developed by directly mixing a transcribed mixture and the wheat germ extract (24). This method provides a very simple system to scale up translational mixture for large-scale protein production because it directly uses unpurified transcriptional mixture without mRNA purification. We used the method for mass production of PabI protein with Met (native) or SeMet (SeMet-substituted) (Fig. 13.1). After purification, native and SeMet-substituted proteins were used for crystallization (Fig. 13.2) and structure determination by X-ray crystallography (8).

3.1. Production and Purification of the Native and SeMet-Substituted Forms of the Protein

3.1.1. mRNA Synthesis

1. Prepare, in a 15-ml tube, 150 µg of high-quality circular plasmid molecules as templates, 1.2 mL of 5× TB, 0.6 mL of 25 mM NTP mix, 70 µL of SP6 RNA polymerase, 70 µL of RNasin, and Milli-Q water (to 6 mL).
2. Incubate the 15-mL tube at 37°C for 4 h (termed "mRNA solution").

3.1.2. Synthesis of Native Protein

1. Prepare four plates each with 6 wells (24 wells in all) (see Note 2).
2. Add 5.5 ml of TSB to each well.
3. Resuspend the white pellet present in the mRNA solution (Subheading 3.1.1) by gently mixing up and down without bubbling.

4. Prepare the translation mixture: 6 mL of the above mRNA solution, 6 mL of wheat-germ extract, and 24 µL of 20 mg/mL creatine kinase.
5. Mix carefully the translational mixture without bubbling.
6. Transfer carefully 500 µL of the translational mixture into the bottom of each well containing the substrate mixture.
7. Incubate the four plates at 26°C for 16 h (see Note 3).

3.1.3. Preparation of SeMet-Substituted Wheat Germ Extract

1. Gel-filtrate 6 mL of the wheat germ extract through 25-mL Sephadex G-25 (fine) column, equilibrated with 50 mL of SeMet buffer.
2. Collect the void fraction.
3. Store at −80°C until use.

3.1.4. Synthesis of SeMet-Substituted Protein

1. Prepare four plates (24 wells in all) (see Note 2).
2. Add 5.5 ml of SeMet buffer to each well.
3. Resuspend the white pellet present in the mRNA solution (Subheading 3.1.1) by gently mixing up and down without bubbling.
4. Prepare SeMet-substituted translation mixture: 6 mL of the above mRNA solution, 6 mL of the SeMet-substituted wheat-germ extract (Subheading 3.1.3), and 24 µL of 20 mg/mL creatine kinase.
5. Mix carefully the SeMet-substituted translational mixture without bubbling.
6. Transfer carefully 500 µL of the SeMet-substituted translational mixture into the bottom of each well containing the SeMet buffer.
7. Incubate the four plates at 26°C for 16 h (see Note 3).

3.1.5. Purification of the Synthesized PabI Protein (a Heat-Resistant Protein)

1. Heat the translational mixture at 90°C for 15 min.
2. Remove the denatured proteins and insoluble materials by centrifugation (10,000×*g*; 15 min; 4°C).
3. Purify PabI protein in the supernatant by a Heparin–Sepharose affinity column using ÄKTA purifier chromatography system.
4. Store the purified PabI protein in protein storage buffer at 4°C.

3.2. Crystallization and Structure Determination

In this section, we describe our experience with one protein in the indicative mood instead of the imperative mood.

3.2.1. Crystallization

1. The crystallization of PabI was performed by the sitting drop vapor diffusion method using the INTELI-PLATE (for initial screening of 96 conditions) and the CrysChem plate (for crystallization screening of 24 conditions).

2. Initial screening of crystallization condition was performed by the sparse-matrix screening method using commercially available screening kits of Crystal screen HT (96 conditions), Index HT (96 conditions), Grid screening PEG6000 (24 conditions), and Wizard I and II (96 conditions). Each crystallization drop was made by mixing 1 μL of the protein solution [0.5 mg/mL protein in 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM dithiothreitol (DTT)] and an equal volume of the reservoir solution.
3. Because of the low solubility of PabI, we used a low concentration solution (0.5 mg/ml) for the first screening. Though the concentration of protein solution was quite low, crystals of PabI could be obtained in the reservoir solution containing PEG6000 as precipitant. The best crystal of PabI appeared under the reservoir conditions containing 100 mM MES (pH 6.0) and 5% PEG6000 after 2 weeks. Typical size of these crystals was $50 \times 50 \times 200$ μm.
4. Although crystals of the Se-Met variant of PabI were also obtained under identical protein solution condition and reservoir solution condition, their size and quality were worse than those of the native crystals. For the improvement of crystal quality, the protein solution condition was modified to increase the solubility of the Se-Met variant. Se-Met variant of PabI was dialyzed against 10 mM MES (pH 6.0), 200 mM NaCl, 10 mM $MgCl_2$, and 10 mM DTT and concentrated to 1.9 mg/ml. The best crystal of the Se-Met variant of PabI was grown under the reservoir conditions containing 50 mM MES (pH 6.8) and 1% PEG6000 using the concentrated protein solution after 1 day.

3.2.2. Structure Determination

1. The X-ray diffraction datasets of the crystals of native PabI and its Se-Met variant were collected using high-brilliance X-ray generated by synchrotron radiation in Photon Factory (Tsukuba, Japan).
2. All the measurements were carried out under cryogenic conditions to reduce radiation damage. Each crystal was soaked in a corresponding reservoir solution containing 20% ethylene glycerol (final concentration) as the cryoprotectant, before being picked up and flash-cooled in a dry nitrogen stream at 95 K.
3. The diffraction data of the native crystal were collected at a wavelength of 1.000Å using a Quantum 315 CCD detector (ADSC) at the BL-5A beamline in Photon factory. A native crystal of PabI diffracted X-rays to a resolution of 3.0Å. The X-ray diffraction data were integrated and scaled with the program HKL2000 (23). Analysis of the diffraction date showed that the native PabI crystal belonged to the primitive monoclinic space group $P2_1$ with the unit cell parameters of a= 84.6Å, b=114.0 Å, c=89.2Å, and β=116.3°. Data collection, phasing, and refinement statistics of PabI are summarized in Table 13.1.

Table 13.1
Data collection, phasing, and refinement statistics of R.PabI

	Native crystal	Se-Met labeled crystal
Data collection		
Beamline	Photon Factory BL-5A	Photon Factory NW12
Detector	ADSC Quantum 315	ADSC Quantum 210r
Data collection temperature (K)	95	95
Wave length (Å)	1.0000	0.9792
Unit Cell dimensions *a*, *b*, *c* (Å) β (degree)	84.6, 114.0, 89.2 116.3	84.6, 114.5, 89.4 116.3
Space Group	$P2_1$	$P2_1$
Resolution (Å)	20–3.0 (3.11–3.00)	20–2.9 (3.00–2.90)
Completeness	99.3 (96.7)	100.0 (100.0)
Unique reflection	30687	33650
Averaged redundancy	3.6 (3.4)	7.5 (7.6)
R_{merge} (%)	6.7 (28.3)	7.7 (28.0)
I/sigma	14.8 (4.5)	17.8 (5.1)
Phasing		
R_{cullis}		0.748
Phasing power		1.27
FOM before density modification		0.31
FOM after density modification		0.86
Refinement		
Number of nonhydrogen atoms		
Protein	10,599	
Water	0	
R/R_{free} (%)	24.9/31.8	
RMSD bond length (Å)	0.009	
RMSD bond angle (deg.)	1.5	
Ramachandran plot		
In most favored regions (%)	74.0	
In additional allowed regions (%)	22.3	
In Generously allowed regions (%)	2.3	
In disallowed regions (%)	1.4	

Values in parentheses are for the highest resolution shell

4. To determine the structure of PabI, which was predicted to possess novel protein fold (7), we applied SAD (single-wavelength anomalous dispersion) phasing method using the Se-Met variant crystal. The diffraction data of Se-Met crystal were collected at a wavelength of 0.9792 Å using a Quantum 210r CCD detector (ADSC) at the NW12 beam line in Photon Factory. Diffraction data of the Se-Met variant of PabI were processed in the same way. Analysis of the diffraction date showed that a Se-Met variant crystal diffracted X-rays to a resolution of 2.9 Å and belonged to the same space group as the native crystal, $P2_1$ with the unit cell parameters of a=84.6 Å, b=114.2 Å, c=89.4 Å, and β=116.3°.
5. Consideration of the Matthew's coefficient (25) (V_M) suggests that native and Se-Met variant crystals of PabI have six protein molecules per asymmetric unit (V_M=2.5 Å^3/Da).
6. The crystal structure of PabI was determined by the SAD phasing method using the diffraction data set of the crystal of the Se-Met variant. The selenium substructure was determined by a direct method program, SnB (13). A total of 19 selenium sites was determined in the asymmetric unit. Because each PabI molecule possesses six methionine sites, this result indicated that approximately 80% of the selenium sites were detected by the SnB calculation.
7. Refinement of the coordinates of the selenium sites and calculation of the initial phase were performed using the program SHARP (14). Phase calculation resulted in an overall figure of merit (FOM) of 0.31 for the resolution range of 20–2.9 Å. After that, density modification and initial model building were performed with the program RESOLVE (15). Molecular models of 759 residues (56% of the total) were automatically built with this calculation.
8. The initial model of the Se-Met variant of PabI was refined and manually rebuilt with the programs CNS (18) and XtalView (19), using 10% (randomly chosen) of the reflections to calculate the R_{free}. The partially built protomer model was transformed into the other five subunits using the program MOLREP (16) in CCP4 (26) and refined with the program REFMAC5 (17) with noncrystallographic symmetry (NCS) restraints.
9. Crystal structure of the native PabI was determined by a molecular replacement method using the coordinates of the partially built structure of the Se-Met variant crystal with the program MOLREP (16). The final structure of PabI was refined and built using the diffraction data set (20–3.0 Å) of native crystal with the program CNS (without NCS restraints) and XtalView (see Note 4).
10. As a result, we were able to determine the structure of PabI at 3.0 Å resolution. The current model has been refined to an

R-factor and R_{free} values of 24.9% and 31.8%, respectively. The paper on the structure of PabI represents the first report of structure determination by X-ray crystallography using the protein overexpressed with the wheat-germ-based cell-free protein expression system (8).

4. Notes

1. RNase contamination is dramatically decreased by protein production in the cell-free system. Unfortunately, however, it is difficult to control the contamination even though commercially available kits are used. To prepare high-quality circular plasmid molecules, we use extraction with phenol/chloroform (phenol:chloroform:isoamyl alcohol=24:24:1, pH 7.9) and with chloroform after plasmid purification by commercially available kits such as those by Qiagen.
2. To obtain a sufficient amount of PabI, we used four plates (24 wells in all). Twenty-four wells of the reaction gave approximately 3 mg of native and SeMet-substituted PabI.
3. For unstable proteins, incubation at 17°C for 18 h is better.
4. We did not use NCS refinement in the final step of refinement because R-factor and R_{free} values became worse when refined with NCS restraint. We could not build coordinates of water molecules and other nonprotein atoms in this structure.

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References

1. Zhang, Q. and Wang, Y. (2008) High mobility group proteins and their post-translational modifications. *Biochim. Biophys. Acta* **1784,** 1159–1166.
2. Hay, R.T. (2005) SUMO: a history of modification. *Mol. Cell* **18,** 1–12.
3. Kobayashi, I. (2001) Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Res.* **29,** 3742–3756.
4. Kobayashi, I. (2004) Restriction – modification systems as minimal forms of life. In

Restriction Endonucleases (Pingoud, A., ed.), Berlin, Springer. pp. 19–62.

5. Orlowski, J. and Bujnicki, J.M. (2008) Structural and evolutionary classification of Type II restriction enzymes based on theoretical and experimental analyses. *Nucleic Acids Res.* **36,** 3552–3569.
6. Chinen, A., Uchiyama, I. and Kobayashi, I. (2000) Comparison between Pyrococcus horikoshii and Pyrococcus abyssi genome sequences reveals linkage of restriction-modification genes with large genome polymorphisms. *Gene* **259,** 109–121.
7. Ishikawa, K., Watanabe, M., Kuroita, T., Uchiyama, I., Bujnicki, J.M., Kawakami, B., Tanokura, M. and Kobayashi, I. (2005) Discovery of a novel restriction endonuclease by genome comparison and application of a wheat-germ-based cell-free translation assay: PabI (5′-GTA/C) from the hyperthermophilic archaeon *Pyrococcus abyssi. Nucleic Acids Res.* **33,** e112.
8. Miyazono, K., Watanabe, M., Kosinski, J., Ishikawa, K., Kamo, M., Sawasaki, T., Nagata, K., Bujnicki, J.M., Endo, Y., Tanokura, M. and Kobayashi, I. (2007) Novel protein fold discovered in the PabI family of restriction enzymes. *Nucleic Acids Res.* **35,** 1908–1918.
9. Roberts, R.J., Belfort, M., Bestor, T., Bhagwat, A.S., Bickle, T.A., Bitinaite, J., Blumenthal, R.M., Degtyarev, S.Kh., Dryden, D.T., Dybvig, K., Firman, K., Gromova, E.S., Gumport, R.I., Halford, S.E., Hattman, S., Heitman, J., Hornby, D.P., Janulaitis, A., Jeltsch, A., Josephsen, J., Kiss, A., Klaenhammer, T.R., Kobayashi, I., Kong, H., Krüger, D.H., Lacks, S., Marinus, M.G., Miyahara, M., Morgan, R.D., Murray, N.E., Nagaraja, V., Piekarowicz, A., Pingoud, A., Raleigh, E., Rao, D.N., Reich, N., Repin, V.E., Selker, E.U., Shaw, P.C., Stein, D.C., Stoddard, B.L., Szybalski, W., Trautner, T.A., Van Etten, J.L., Vitor, J.M., Wilson, G.G. and Xu, S.Y. (2003) A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res.* **31,** 1805–1812.
10. Sawasaki, T., Ogasawara, T, Morishita, R. and Endo, Y. (2002) A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 14652–14657.
11. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33,** 103–119.
12. Otwinowski, Z. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276,** 307-326.
13. Weeks, C. and Miller, R. (1999) The design and implementation of SnB version 2.0. *J. Appl. Cryst.* **32,** 120–124.
14. Bricogne, G., Vonrhein, C., Flensburg, C., Schiltz, M. and Paciorek, W. (2003) Generation, representation and flow of phase information in structure determination: recent developments in and around SHARP 2.0. *Acta Crystallogr. D Biol. Crystallogr.* **59,** 2023–2030.
15. Terwilliger, T. (2003) Automated main-chain model building by template matching and iterative fragment extension. *Acta Crystallogr. D Biol. Crystallogr.* **59,** 38–44.
16. Vagin, A. and Teplyakov, A. (1997) MOLREP: an automated program for molecular replacement. *J. Appl. Cryst.* **30,** 1022-1025.
17. Murshudov, G. N., Vagin, A. A. and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D Biol. Crystallogr.* **53,** 240–255.
18. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* **54,** 905–921.
19. McRee, D. E. (1999) XtalView/Xfit – a versatile program for manipulating atomic coordinates and electron density. *J. Struct. Biol.* **125,** 156–165.
20. Madin, K., Sawasaki, T., Ogasawara, T. and Endo, Y. (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 559-564
21. Hanes, J. and Pluckthun, A. (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 4937–4942.
22. Sawasaki, T., Hasegawa, Y., Tsuchimochi, M., Kamura, N., Ogasawara, T. and Endo Y. (2002) A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett.* **514,** 102–105.
23. Spirin, A. S., Baranov, V. I., Ryabova, L. A., Ovodov, S. Y. and Alakhov, Y. B. (1988) A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **242,** 1162–1164.

24. Sawasaki, T., Gouda, M.D., Kawasaki, T., Tsuboi, T., Tozawa, Y., Takai, K. and Endo, Y. (2005) The wheat germ cell-free expression system: methods for high-throughput materialization of genetic information. *Methods Mol. Biol.* **310,** 131–144.
25. Matthews, B. W. (1968) Solvent content of protein crystals. *J. Mol. Biol.* **33,** 491–497.
26. Collaborative, Computational, Project, Number, 4. (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr.* **50,** 760–763.

Chapter 8

An Efficient Approach to the Production of Vaccines Against the Malaria Parasite

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Abstract

In malaria vaccine research, one of the major obstacles has been the difficulty of expressing recombinant malarial proteins and it is mainly due to the lack of an efficient methodology for the synthesis of sufficient quantity of quality proteins. We demonstrate that the wheat germ cell-free protein synthesis system can be applied for the successful production of leading malaria vaccine candidate antigens and, thus, prove that it may be a key tool for malaria vaccine research.

Key words: Cell-free protein synthesis, Parasite, Malaria, Vaccine

1. Introduction

Malaria, a serious infectious disease that challenges the global health, causes millions of deaths annually, as well as illness in hundreds of millions of people. The most deadly form of the disease is caused by the inoculation of the malaria parasite, *Plasmodium falciparum*, by infected mosquito bites. The disease is re-emerging mainly due to the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes (1). Therefore development of malaria vaccine has been considered as one of the essential components for the malaria eradication (2). However, efforts to develop a successful vaccine have not yet accomplished (3). Since we need multiple vaccine candidate antigens to succeed in controlling malaria, post-genome malaria vaccine candidate discovery is necessary. One of the obstacles in this process is at the malaria protein production step and is mainly due to the lack of an efficient methodology to prepare quality proteins. *P. falciparum* genes have a very high A/T content (average 76% per coding

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sequence throughout the genome) and a number of them encode repeated stretches of amino acid sequences (4), and these features have been proposed as the major factors limiting *P. falciparum* protein expression in conventional cell-based systems (5–7). Moreover, the presence of glycosylation machinery in eukaryotic cell-based protein expression systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses (8–10). There are also constraints such as requirement of disulfide bond formation if the target protein requires it for its bioactivity and requirement of preparation of large quantities of antigen for immunization. The above limiting factors are impediments completely insurmountable in the case of eukaryotic cell-based expression systems such as yeast, baculovirus, or Chinese hamster ovary cell. But fortunately we found that the wheat germ cell-free system can surmount most of the above impediments in the way of finding and developing malaria vaccine candidates (11).

In this chapter, we describe how the wheat germ cell-free system is effective (1) in producing properly folded good quality protein (2) in sufficient quantities (3) that too without the need for codon optimization, using the leading malaria vaccine candidate Pfs25 as an example. And we also describe that (1) proteins produced by the wheat germ cell-free system can be easily purified using simple affinity chromatography, (2) they can be directly used for immunization, and (3) the antibody raised against the proteins are functional in our biochemical, immunocytochemical, and biological analyses. Therefore we hope the wheat germ cell-free system may have dramatic impact on malaria vaccine research.

2. Materials

2.1. Parasite cDNA Preparation, PCR Amplification, and Construction of the DNA Template for Transcription

1. Malaria parasite pellet, *P. falciparum* 3D7 strain (available from Malaria Research and Reference Reagent Resource Center managed by ATCC, Manassas, VA, see Note 1) stored at −80°C in the presence of Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland).
2. RNeasy Micro Kit (QIAGEN, Valencia, CA).
3. SuperScript III RT First-Strand Synthesis System (Invitrogen, Carlsbad, CA).
4. Oligonucleotide primers (see **Note 2**). Design the 5′ primers for the target to be amplified as follows: desired restriction site followed by a 30-mer of unique sequence covering the 5′ region of the open reading frame containing the start codon.

Design the 3′ primers, desired restriction site followed by a 30-mer of unique sequences covering the 3′ region of the open reading frame upstream of the termination codon. Either 5′ or 3′ primer may contain nucleotide sequence coding hexa-histidine tag for Nickel affinity purification.

5. Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA).
6. Thermal cycler (MJ Research, Waltham, MA) (see Note 3).
7. Plasmid of Ehime University (pEU)-E01 protein expression vector specialized for the wheat germ cell-free system.
8. Ligation high ligation reagent (Toyobo, Osaka, Japan).

2.2. Antigen Scale Cell-Free Protein Synthesis and Affinity Purification

1. pEU-E01 plasmid that contains target cDNA.
2. 5× transcription buffer (TB): 400 mM HEPES-KOH (pH 7.8), 80 mM magnesium acetate, 10 mM spermidine, and 50 mM DTT.
3. Nucleotide tri-phosphates (NTPs) mix: a solution containing 25 mM each of ATP, GTP, CTP, and UTP.
4. SP6 RNA polymerase and RNasin (80 U/mL, Promega, Madison, WI).
5. 40 mg/mL creatine kinase.
6. 240 OD/mL WEPRO®1240H (CellFree Sciences, Matsuyama, Japan).
7. 1× Translational substrate buffer (SUB-AMIX): 30 mM HEPES-KOH (pH 7.8), 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 4.0 mM DTT, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate.
8. 6-well microplate (Greiner, Frickenhausen, Germany).
9. Nickel-nitrilotriacetic acid agarose beads (Qiagen).
10. Poly-Prep chromatography column (Bio-Rad, Hercules, CA, USA).
11. Phosphate buffered saline (PBS): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by diluting one part with nine parts of water.
12. 1× Wash buffer: 1× PBS supplemented with 30 mM of imidazole and 300 mM NaCl.
13. 1× Elution buffer: 1× PBS supplemented with 500 mM of imidazole and 300 mM NaCl.

2.3. Characterization of the Target Molecules by Confocal Immunofluorescence Microscopy

1. Female BALB/c mice 6–8 weeks of age (Kitayama Labes, Ina, Japan).
2. Freund complete and incomplete adjuvant (Wako Pure Chemical, Osaka, Japan)
3. CF11 cellulose powder (Whatman, Maidstone, UK)
4. 50% Percoll: Percoll (GE Healthcare Bio-Sciences, Piscataway, NJ) diluted 1:1 (v/v) with 2× PBS
5. Ookinete culture medium: RPMI1640 medium (Invitrogen) supplemented with 50 μg of hypoxanthine per mL, 25 mM HEPES, 20% heat-inactivated fetal calf serum (Invitrogen), 24 mM NaHCO3, %U of penicillin per mL, and 5 μg of streptomycin per mL (pH 8.4).
6. Eight-well Multitest slides (Flow Laboratories, McLean, VA).
7. Blocking and dilution buffer: 5% (w/v) nonfat dry milk in PBS prepared freshly.
8. Secondary antibody: Antimouse IgG conjugated to Alexa488 (Invitrogen) (see Note 4).
9. Nuclear stain: 2 mg/mL 1,000× DAPI (4,6-diamidino-2-phenylindole) stock solution in 100% methanol.
10. Mounting medium: ProLong Gold Antifade Reagent (Invitrogen) (see Note 5).

2.4. Malaria Transmission-Blocking Vaccine Efficacy Assay

1. Malaria parasite-infected blood specimen collected from malaria patient, under informed consent, at the clinics in Thailand.
2. *Anopheles dirus* mosquitoes reared in the insectary, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand (see Note 6).
3. Anti-Pfs25 sera produced in BALB/c mice.
4. Malaria naïve human AB-type serum obtained from volunteers lived in Bangkok.
5. Custom made water-jacketed membrane-feeding apparatus.
6. Parafilm M (Alcan Packaging, Neenah, WI)

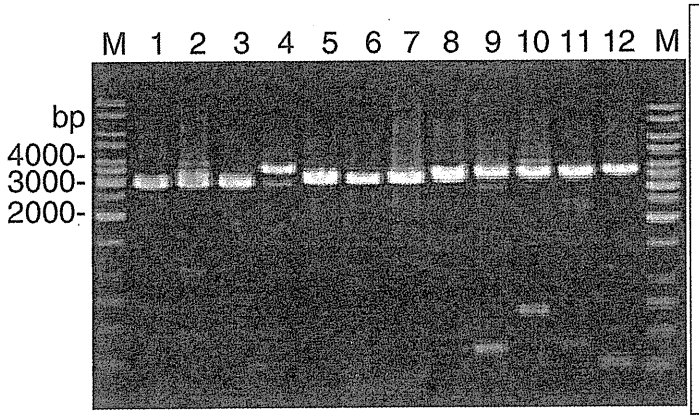
3. Methods

One of the well-known difficulties in malaria research is the PCR amplification of the target sequence because nucleotide sequences of *P. falciparum* genes are highly A/T rich. This feature requires longer primers than those for the G/C rich organism to amplify

the target sequences by PCR. Moreover, there are a number of malaria proteins that contain stretches of amino acid repeat motifs and also a number of malaria genes that contain multiple adenine-rich nucleotide sequences (A-islands) in the coding region. This makes the cDNA cloning dramatically difficult, because these A-islands sometimes cause different number of A-nucleotides in each plasmid clone that causes frameshift. Therefore, the initial hurdle for the malaria protein production is the cDNA-cloning; these factors also require consideration on the plasmid construct design. The following methods explain (1) PCR amplification and cloning of the parasite ORF into the pEU expression vector, (2) synthesis of mRNA using expression vector, (3) translation of prepared mRNA in bilayer system, and (4) biological qualification of the synthesized proteins, which includes the recognition of the parasite molecule with the antibody raised against the recombinant malaria protein. This can be accomplished through indirect immunofluorescence microscopy and vaccine efficacy assay.

3.1. Parasite cDNA Preparation and PCR amplification of DNA-Template Construction for Transcription

1. Extract total RNA from cultured *P. falciparum* 3D7 parasites, then reverse transcribe the mRNA into cDNA by using SuperScript III RT First-Strand Synthesis System.
2. PCR amplify the desired target using Phusion™ High-Fidelity DNA Polymerase.
3. Clone the PCR products (see Fig. 8.1) into the pEU-E01 plasmid using Ligation high ligation reagent.
4. Confirm the sequence of the cloned insert (see Note 7).
5. Purify the plasmid.



	Size (bp)	A/T(%)
M:	Marker	
1:	3018	76.3
2:	3048	78.8
3:	3102	81.1
4:	3222	79.4
5:	3342	78.9
6:	3345	75.3
7:	3393	73.0
8:	3540	75.7
9:	3663	78.6
10:	3678	78.9
11:	3696	79.7
12:	3771	75.0

Fig. 8.1. Efficient PCR amplification of the *Plasmodium falciparum* cDNAs. Twelve cDNA targets were selected from *P. falciparum* 3D7, randomly. The cDNA targets were PCR amplified using Phusion™ High-Fidelity DNA Polymerase, under the following conditions: 98°C for 2 min and then 40 cycles at 98°C for 15 s, 55°C for 20 s, and 68°C for 2 min, followed by a final extension at 68°C for 10 min. The amplified products were visualized in a 0.8% agarose gel stained with ethidium bromide. All of the targets ranging from 3,018 bp (*lane 1*) to 3,771 bp (*lane 12*) were successfully amplified even in the presence of very high A/T contents (i.e., 73.0–81.1%).

3.2. Antigen Scale Cell-Free Protein Syntheses of Malaria Protein and Affinity Purification

An example of the methods and the results for Pfs25 is described.

1. Prepare a plasmid clone which has an insert as shown in Fig. 8.2A.
2. Incubate 200 µL of transcription mixture containing 20 µg of the plasmid DNA, 1× TB, 2.5 mM each of NTPs, 200 U of SP6 RNA polymerase, and 200 U of RNasin for 6 h at 37°C.

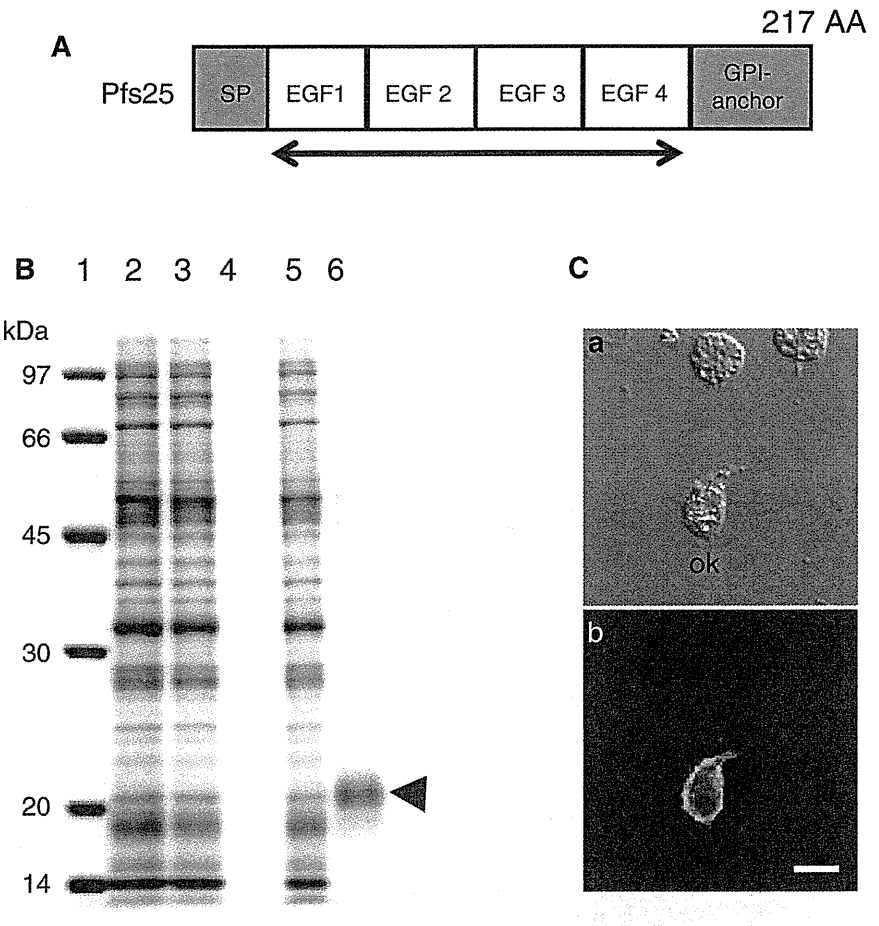


Fig. 8.2. Cloning, expression, and immunolocalization of Pfs25, a leading malaria transmission-blocking vaccine candidate. (**A**) Schematic representation of Pfs25. Pfs25 was expressed without the nucleotide sequences coding for the signal peptide (SP) and the GPI anchor (*arrow*). This Pfs25 gene was amplified by PCR from the *P. falciparum* 3D7 strain using antisense primer that contains nucleotide sequence encoding for hexa-histidine tag at the C terminus and subcloned into pEU-E01 plasmid at the EcoRV site. The A/T content of the *pfs25* insert was 70%. (**B**) The expression, affinity purification, and SDS-PAGE of recombinant Pfs25. Recombinant Pfs25 was expressed using the wheat germ cell-free method, affinity purified by Nickel affinity chromatography, and size-fractionated by 12.5% SDS-PAGE under reducing condition and stained with CBB. *Lane 1*, molecular mass markers in kDa; *lane 2*, total translation mixture; *lane 3*, supernatant fraction of the translation mixture; *lane 4*, pellet fraction of the translation mixture; *lane 5*, flow-through fraction after the affinity purification, *lane 6*; affinity-purified recombinant protein (*arrow head*). (**C**) The immune serum raised against recombinant Pfs25 specifically recognized native Pfs25 proteins expressed on the surface of *P. falciparum* immature ookinetes (ok). (**a**) Differential interference contrast and (**b**) fluorescence confocal images were obtained on a LSM5 PASCAL microscopy (Carl Zeiss MicroImaging, Thornwood, NY) using a 63× oil-immersion lens. *Bar*=5 µm.

3. Mix the transcription solution containing transcribed mRNA (see Subheading 3.2, step 1) with 200 µL of WEPRO®1240H (240 OD/mL) supplemented with 0.4 µL of creatine kinase (40 mg/mL). Add 4.4-mL of 1× SUB-AMIX into a single well of a 6-well plate and then underlay the above transcription mixture and incubate at 17°C for 16 h.
4. Add imidazole (pH 8.0) in the translation reaction mixture (final concentration, 20 mM) and then add 80 µL of 50% slurry of Ni-NTA beads.
5. Incubate the tube for 16 h on a continuous rotator, at 4°C, for the binding of proteins on to the beads.
6. Transfer the solution with the beads into a Poly-Prep column.
7. Wash the beads by 0.4 mL of wash buffer three times and then elute the recombinant protein with 80 µL of elution buffer five times.
8. Analyze the purified protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition, and the bands were visualized with Coomassie brilliant blue (see Fig. 8.2B).

3.3. Preparation of Antiserum and Parasite Antigen for Indirect Immunofluorescence Assay

3.3.1. Antiserum Preparation

1. Immunize female BALB/c mice (6–8 weeks of age) by subcutaneous injection with affinity purified recombinant proteins emulsified with the same volume of Freund's complete adjuvant as a priming dose and then administer two additional booster doses with Freund's incomplete adjuvant at 3-weeks interval.
2. Collect blood by cardiac puncture a week after the final boost under anesthesia and then separate sera after the coagulation of the blood (see Note 8).

3.3.2. Preparation of Parasite Antigens from the Cultured Ookinetes of Plasmodium falciparum

1. Collect peripheral blood with heparinized syringes from malaria patients under written informed consent. Purify gametocytes by passing 5–10 mL of blood through CF-11 column to remove leukocytes followed by 50% Percoll density gradient centrifugation (350 × *g* for 25 min at room temperature).
2. Collect the interface rich in gametocytes on the Percoll cushion into a new tube. Wash them twice with PBS and then culture this parasite pellet to ookinete in 1 mL of ookinete medium for 24 h at 24°C in air.
3. Wash the cultured parasite preparations rich in ookinetes twice with PBS and then spot them on 8-well Multitest slides and fix them with ice-cold acetone for 5 min.
4. Store the slides at −80°C until use.

3.3.3. Staining Procedure for Immunofluorescence Assay

1. Take the desired number of antigen slides out from the freezer and then place them quickly in a desiccator until they are brought to room temperature.
2. Block the slides with blocking buffer for 30 min at 37°C in the humidified chamber.
3. Incubate with anti-Pfs25 immune sera (1:100 dilution with blocking buffer) for 1 h at 37°C (see Note 9).
4. Wash the slides with ice-cold PBS for 5 min and incubate with secondary antibody (1:500) and DAPI (1:1,000) diluted with blocking buffer for 30 min at 37°C, followed by washing with ice-cold PBS for 5 min. Mount the slides with Prolong Gold Antifade Reagent and incubate the slides for overnight at room temperature to allow complete solidification of the mounting medium.
5. View the slides under confocal microscopy. Excitation at 488 nm induces the Alexa Fluor 488 fluorescence (green emission) for the Pfs25 (Fig. 8.2Cb), while the differential interference contrast image (Fig. 8.2Ca) is also captured. For example, the fluorescent signal for Pfs25 on the surface of ookinete is shown in Fig. 8.2Cb.

3.4. Malaria Transmission-Blocking Vaccine Efficacy Assay

1. Collect peripheral blood into heparinized syringe from a volunteer patient.
2. Aliquot the collected blood into tubes (300 µL/tube) and remove plasma by a brief centrifugation.
3. Dilute mouse immune sera into 1:2, 1:8, and 1:32 (v/v) with heat-inactivated normal human AB serum prepared from Thai malaria naïve donors.
4. Mix each diluted test serum with *P. falciparum*-infected blood cells (1:1, v/v) and incubate for 15 min at room temperature.
5. Place the mixture into a water-jacketed membrane feeding apparatus (see Fig. 8.3a) whose bottom is sealed by Parafilm M kept at 37°C with circulating water outside of the feeder to allow starved *A. dirus* mosquitoes (Armed Forces Research Institute of Medical Sciences) to feed on the blood meals for 30 min (see Fig. 8.3b).
6. Remove unfed mosquitoes by manual aspiration and maintain only fully engorged mosquitoes for a week by giving 10% sucrose water in the insectary.
7. Dissect 20 mosquitoes for each mouse test immune serum and count the number of oocysts developed within the mosquito midgut under the microscope by staining with 0.5% mercurochrome. The transmission-blocking vaccine efficacy is accessed by the number of oocysts per mosquito (see Fig. 8.3c).

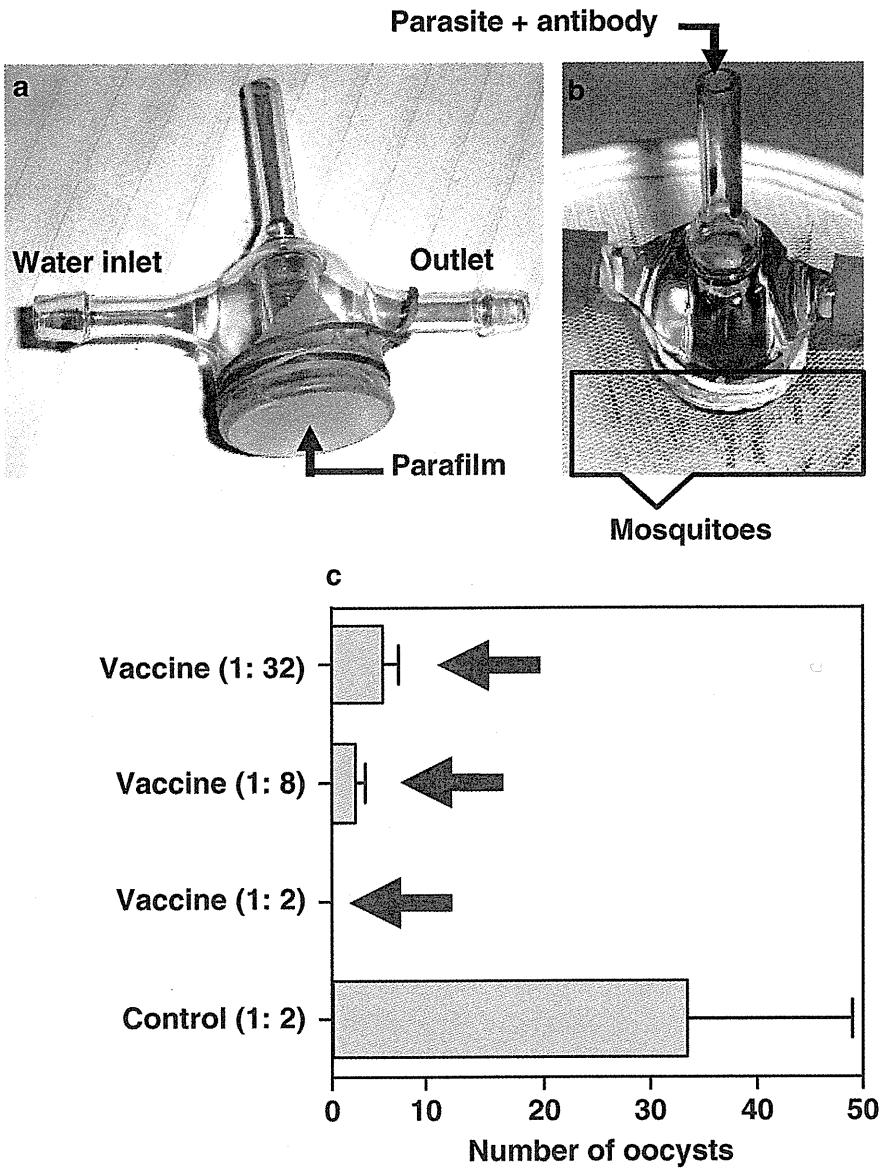


Fig. 8.3. Transmission-blocking efficacy of antibodies against *Plasmodium falciparum* parasites. (**a**) A membrane feeding apparatus with its bottom sealed by Parafilm M. (**b**) The apparatus is kept at 37°C with circulating water outside of the feeder to allow mosquitoes to feed on the infected blood mixed with test and control sera. (**c**) The median numbers of oocysts per mosquito ($n=20$) (*bars*) with interquartile ranges (*lines on top of the bars*) were compared among groups of mosquitoes fed on either anti-Pfs25 serum serially diluted or control mouse serum. Dilution range of test immune serum used is shown as 1:2 to 1:32. *Arrows* indicate statistically significant differences compared to the control group analyzed using Kruskal–Wallis test ($P<0.05$).

4. Notes

1. The Malaria Research and Reference Reagent Resource Center (MR4) is a central source of quality reagents to the malaria research community. Materials available to registered users include parasites, mosquito vectors, antibodies, antigens, gene

libraries, etc. MR4 Web site (http://www.mr4.org/Home/tabid/93/Default.aspx) also provides a lot of useful information such as online protocol book named *Methods In Malaria Research* (version 5.2). This book includes protocols that are useful to malaria research, for example, parasite culture methods, immunological assays, or molecular biological techniques.

2. For the efficient PCR amplification, the melting temperature of oligonucleotide primer should be higher than 70°C and it can be roughly calculated by the following formula; 2°C×(A+T)+4°C×(G+C). This is one of the essential factors for the efficient PCR amplification of *P. falciparum* because the *P. falciparum* genome is A/T rich.
3. Thermal cycler, which has gradient temperature function, is useful when optimizing the PCR condition, such as testing a lot of different annealing temperatures.
4. For the multiple labeling experiment, check the quality of the secondary antibodies in advance. For example, some of the commercially available secondary antibodies recognizing mouse IgG are highly cross-adsorbed using bovine, goat, human, rabbit, and rat IgG and also human serum. This secondary antibody can be useful for the double labeling with both mouse and rat primary antibodies.
5. This antifade reagent will solidify, and the sample can be saved for months after mounting, and it also offers enhanced resistance to photobleaching. This criterion is very important for the high-quality imaging because this reagent allows the repeated scanning with the confocal laser microscope until the satisfactory images are obtained.
6. *A. dirus* is a major malaria vector mosquito species in Thailand. This mosquito line is established and adapted as one of the laboratory lines.
7. Pay special attention to confirm the number of adenine nucleotides in the A-islands present in the target region. Otherwise wrong number of adenine nucleotides cause frameshift. If a plasmid clone contains wrong number of adenine nucleotides, verify another plasmid clone which sometimes might have a correct number of adenine nucleotides in the A-island.
8. The antisera for immunofluorescence assay can be saved by addition of sodium azide (final concentration, 0.02%) (Caution highly toxic) and stored at 4°C to prevent repeated freeze thaw cycles which damage the antibodies. However, the antisera for the biological assay such as membrane feeding should not contain any preservatives. In this case, all the antisera are kept frozen at –80°C.

9. One well in the 8-well Multitest slide can hold up to 20μL of the blocking or antibody solution. In the case of 24-well Multitest slide, one well can hold up to 10μL of the solution.

Acknowledgments

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References

1. Greenwood, B. and Mutabingwa T. (2002) Malaria in 2002. *Nature* **415,** 670-672.
2. Greenwood B. (2009) Can malaria be eliminated? *Trans. R. Soc. Trop. Med. Hyg.* **103S,** S2-S5.
3. Richie, T. L. and Saul, A. (2002) Progress and challenges for malaria vaccines. *Nature* **415,** 694-701.
4. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W. et al., (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419,** 498-511.
5. Aguiar, J. C., LaBaer, J., Blair P. L., Shamailova, V. Y., Koundinya, M., Russell, J. A., et al. (2004) High-throughput generation of P. *falciparum* functional molecules by recombinational cloning. *Genome Res.* **14,** 2076-2082.
6. Mehlin, C., Boni, E., Buckner, F. S., Engel, L., Feist, T., Gelb M. H., et al. (2006) Heterologous expression of proteins from *Plasmodium falciparum*: results from 1000 genes. *Mol. Biochem. Parasitol.* **148,** 144-160.
7. Vedadi, M., Lew, J., Artz, J., Amani M., Zhao, Y., Dong, A., et al. (2007) Genome-scale protein expression and structural biology of *Plasmodium falciparum* and related Apicomplexan organisms. *Mol. Biochem. Parasitol.* **151,** 100-110.
8. Gowda, D. C., and Davidson, E. A. (1999) Protein glycosylation in the malaria parasite. *Parasitol. Today* **15,** 147-152.
9. Kedees, M. H., Azzouz, N., Gerold, P., Shams-Eldin, H., Iqbal, J., Eckert, V., et al. (2002) *Plasmodium falciparum*: glycosylation status of *Plasmodium falciparum* circumsporozoite protein expressed in the baculovirus system. *Exp. Parasitol.* **101,** 64-68.
10. Samuelson, J., Banerjee, S., Magnelli, P., Cui, J., Kelleher, D. J., Gilmore, R., et al. (2005) The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. *Proc. Natl. Acad. Sci. USA* **102,** 1548-1553.
11. Tsuboi, T., Takeo, S., Iriko, H., Jin, L., Tsuchimochi, M., Matsuda, S., et al. (2008) Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. *Infect. Immun.* **76,** 1702-1708.

Chapter 7

Cell-Free-Based Protein Microarray Technology Using Agarose/DNA Microplate

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Abstract

Protein microarray is considered to be one of the key analytical tools for high-throughput protein function analysis. We found that Arabidopsis HY5 protein functions as a novel DNA-binding tag (DBtag), and DBtagged proteins are immobilized and purified on a newly designed agarose/DNA microplate. In this chapter, we demonstrate a protocol for making the DBtag-based protein microarray and will provide protocols for two applications using the microarray: (1) detection of autophosphorylation activity of DBtagged human protein kinases and inhibition of their activity by staurosporine, and (2) detection of a protein–protein interaction between the DBtagged UBE2N and UBE2v1.

Key words: Protein microarray, High-throughput functional analysis, DNA-binding tag, Protein kinase, Agarose/DNA microplate

1. Introduction

Currently available protein microarray technology has allowed large-scale screening of biomarker proteins recognized by serum antibodies (1). However, this method is yet to become a commonly used biochemical tool for the analysis of proteins (2). Certainly there is room for further improvement before this technology could become a routinely used laboratory tool. For example, one of the problems is the difficulty in immobilizing a variety of proteins in their functionally active forms. Many proteins needed to be appropriately oriented for proper functioning (3). However, it is not easy to control the orientation of the protein during its mobilization on the surface of the microplate. Another problem is that the high-throughput functional analysis requires freshly produced and purified proteins; however, unlike DNA, many purified proteins are not stable and thus, these cannot

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