Improvement of Proteomic Profile of *Plasmodium falciparum* by Two-step Protein Extraction in Two-dimensional Gel Electrophoresis

Patcharee Panpumthong
Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand
National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand Science Park, Pathum Thani 12120, Thailand

Phantip Vattanaviboon
Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand
corresponding author: mtpvt@mahidol.ac.th

Abstract

Here, we demonstrate a modified two-step protein extraction technique to improve proteomic profile of *P. falciparum* in two-dimensional gel electrophoresis (2-DE). An experiment was initiated by resuspending the trophozoite in 40 mM Tris buffer followed by a freeze-thawing process. The cytosolic fraction was collected by centrifugation. The pellet was subsequently solubilized in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributyl phosphine (TBP), 2% IPG buffer pI 3-10 and 1x protease inhibitor. These two fractions were individually applied onto the 2-DE and the protein spots were then detected by silver staining. A total of 239 protein spots were detected, while using the single-step protein extraction, only 165 visible proteins could be distinguished. More importantly, the two-step extraction provided a significant recovery of approximately 10 protein spots of surface antigens and membrane proteins as identified by their molecular weights and isoelectric points. These findings open up a potential approach for construction of a complete proteome map of *P. falciparum*, which can provide for a better opportunity of drug and vaccine development.

Keywords: *P. falciparum*, protein extraction, two-dimensional electrophoresis, proteomics, malaria

1. Introduction

Plasmodium falciparum is a protozoan parasite accountable for the most life threatening form of human malaria, in which over millions of death are reported annually, especially in sub-Sahara African countries [1]. The symptoms of this disease progress to once the asexual intraerythrocytic of the parasite has been developed. This development comprises several decisive steps involving membrane proteins. Thus, attempts have been geared towards a better understanding of the basic parasite biology, to aid in drug and vaccine development. Analysis of malarial proteome via multidimensional protein identification technology (MudPIT) employing classical two-dimensional gel electrophoresis (2-DE) in combination with MALDI-TOF-MS, liquid chromatography–tandem mass spectrometry (LC-MS/MS) or nanoLC-MS/MS, has extensively been carried out [2-4].

Much attention has been focused on the 2-DE profiling of *P. falciparum* [5-8], however, little is known on the pertinent information especially on the subcellular fractionation prior to 2-DE [8]. This leads to the difficulty on the discovery of proteomic master maps as well as on the protein identification [2,3]. The major

obstacles to 2-DE for *P. falciparum* are attributable to the cross-contamination by abundant human host proteins and the low solubility of parasite membrane proteins. The latter is particularly important since several of which are required for host-parasite interaction and are targets of antimalarial drugs development and vaccine candidates.

Although, many standard protein extraction protocols have widely been established, it is worth stating that none of these methods can universally be relevant to all kinds of samples analyzed by 2-DE [9]. Visualization of proteins from total cell or tissue homogenates on the 2-DE gel remains problematic due to the high dynamic range of protein abundance and the disparity in their molecular weight (M.W.), isoelectric point (pI) and solubility. Therefore, maximization of the extraction protocol based on the type of sample (e.g. prokaryotic or eukaryotic cells) and the type of target proteins (e.g. cytosolic protein or membrane protein) is required. Sequential extraction is one example that has successfully been applied for discovery of plant and microbial proteins [10-12]. In this report, we demonstrate that using two-step protein extraction (prefractionation step) can give rise to an enrichment of membrane proteins and finally lead to improved proteomic maps of the *P. falciparum* trophozoite stage.

2. Materials and methods

2.1 Cultivation of *Plasmodium falciparum*

A multidrug resistant *P. falciparum* strain K1CB1, a generous gift from S. Thaithong, Department of Biology, Faculty of Science, Chulalongkorn University, was used throughout the study. Cultivation of *P. falciparum* was performed according to the standard protocol described by Trager and Jensen [13]. The parasite was cultured in type O-positive human erythrocytes at 3% (v/v) in RPMI 1640 medium supplemented with 0.65 mM hypoxanthine, 25 mM HEPES, 50 mM glucose, 0.2% $NaHCO_3$, 40 mg/l gentamycin, 8% heat inactivated pooled human serum, under an atmospheric condition of 3% CO_2. Synchronization of the parasite at the early ring stage was performed using sorbitol lysis [14]. The synchronized culture was further maintained until approximately 20-30% parasitemia of the late trophozoite stage was obtained.

2.2 Isolation of parasites from human erythrocytes

Harvesting of the infected erythrocytes was carried out by centrifugation at 500 x g for 10 minutes. The pellet was then resuspended and washed three times using Trager's solution (57 mM NaCl, 58 mM KCl, 11 mM Na_2HPO_4, 7 mM K_2HPO_4, 1 mM $NaHCO_3$, 14 mM glucose). Isolation of the parasites from human erythrocytes was performed by addition of Saponin solution (0.01% w/v) containing 1 mM PMSF and 1 mM Phenantonin. The parasite was then pelleted and washed three times with Trager's solution until the supernatant became colorless. The parasite pellets were lysed immediately or stored at -80°C for later processing.

2.3 Extraction of parasite protein

Extraction of parasite protein was performed using either one-step or two-step protein extraction (prefractionation) technique as follows. The single step parasite protein extraction was initiated by freeze-thawing (4 cycles) of the parasite pellet in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributyl phosphine (TBP), 2% IPG buffer pI 3-10 and 1x protease inhibitor (GE Health Care, USA). The lysate was further incubated at room temperature for 30 minutes and the undissolvable pellet was removed by centrifugation at 8,000 x g for 30 minutes at 20°C. A clear supernatant was collected for further analysis. In the two-step protein extraction, the frozen pellet was firstly resuspended in 40 mM Tris buffer (denoted as solution 1) before subjecting to the freeze-thawing process. The sample was then centrifuged and the soluble fraction was collected. The insoluble portion was extensively washed with 40 mM Tris buffer, solubilized in lysis buffer (ingredients as mentioned above, denoted as solution 2), and further processed in a similar manner as described above. Quantitation of protein concentration was performed by using Bradford reagent (Bio-Rad Laboratories, USA).

2.4 Two-dimensional gel electrophoresis (2-DE)

2-DE was carried out using a 2-D Electrophoresis System (GE Health care, USA) according to the manufacturer's recommen-

dations with some modifications. One hundred fifty micrograms of parasite protein extract were mixed with 250 µl of rehydration buffer (8 M urea, 4% CHAPS, 2 mM TBP, 0.001% bromphenol blue and 65 mM dithiothreitol) containing 1% 3-10 IPG buffer. The mixture was allowed to stand for 15 minutes at room temperature and the insoluble materials was removed by centrifugation at 13,000 x g for 10 minutes at 20°C. The supernatant was applied onto 13-cm IPG strips with a pH range of 3-10 in an isoelectric focusing system (IPGphore™). Samples were run through steps of strip rehydration (20°C, 12 hours) and isoelectric focusing (500 volts for 1 hour, 1,000 volts for 1 hour, and 8,000 volts to reach 16,000 volt-hours). The maximum current was maintained at 50 mA per strip. After the complete process was reached, the strip was equilibrated two times (15 minutes each) in equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.03% bromphenol blue) in the presence of 65 mM DTT and 135 mM iodoacetamide prior to applying the strip for the second dimensional separation using a SDS-polyacrylamide gel (12.5%). Protein separation was conducted under the applied voltage of 20 volts per gel at 15°C. The electrophoresis was stopped once the bromphenol blue dye front reached 0.5 cm from the bottom of the gel.

2.5 Gel staining

Silver staining of the gels was performed according to the recommendation from the manufacturer (GE Healthcare, USA). Consequently, gel images were taken from a GS-710 Imaging Densitometer (Bio-Rad Laboratories, USA). Spots of protein were counted by ImageMaster 2D Platinum (GE Healthcare, USA) software tool.

2.6 Protein prediction and analysis

Spots of protein on each gel were analysed according to their molecular weight and isoelectric point and compared with those reported in the SWISS-PROT database (http://ca.expasy.com). The types of protein were classified according to the protocols described in www.genedb.org. Prediction of protein in each fraction was performed by comparison of the actual and the predicted values.

3. Results

3.1 Enrichment of insoluble proteins by two-step protein extraction

Enrichment of insoluble protein in 2-DE of *P. falciparum* was successfully achieved by using a sequential extraction approach. Synchronized trophozoite stages were isolated and prepared by the two-step technique. The crude protein extracts from both fractions were individually separated by 2-DE using a pH gradient ranging from 3 to 10, followed by silver staining. As represented in Figures 1A and 1B, a total of 239 protein spots were obtained from these two fractions. More importantly, a significant difference on the protein patterns identified by 2-DE was revealed at each step of the sequential extraction. The proteins represented in the dotted boxes (regions 1-6, Figure 1B) were assumed to be the insoluble proteins of *P. falciparum*, since none of these spots was found in the soluble fractions (Figure 1A).

For comparison, one-step protein extraction using the common lysis buffer was applied. As shown in Figure 2, only 165 visible protein spots were observed. In addition, a marked increase of horizontal streaking was found, especially at the area of medium-to-high molecular weight and low pI. This was in contrast to the findings from the two-step extraction where a high resolution could be detected (Figure 1B).

Figure 3 summarizes the number of protein spots presented in different fractions. Approximately 155 spots of protein solubilized in solution 1 were assumed to be derived predominantly from the cytosolic portion. Proteins in solution 2 were presumed to contain abundant cytosolic proteins as well as membrane proteins. With respect to the total of 84 protein spots found in solution 1 and 2, 74 protein spots appeared in the same position of both extracts, whereas 10 proteins spots were found to be present only in solution 2. Moreover, when compared with extraction in solution 1, solubilization of proteins by solution 2 gave rise to the following improvements:

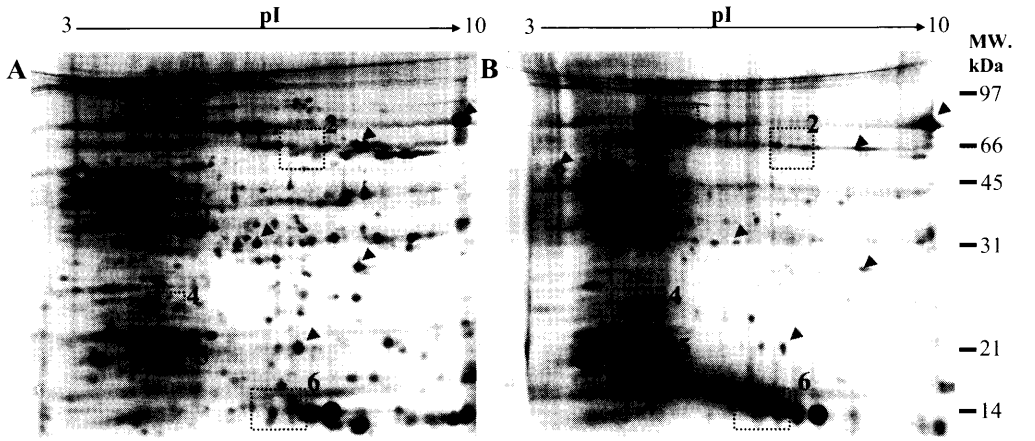

Figure 1 2-DE separation of *P. falciparum* proteins. One hundred fifty micrograms of *P. falciparum* proteins were loaded on the 2-D gels. The first dimension was a 3–10 linear pH gradient, and the second dimension was a 12.5% acrylamide gel. The proteins were extracted in solution 1 containing 40 mM Tris-buffer (A) and solution 2 containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM TBP, and 2% IPG buffer (B). Reference markers were indicated by arrows. Insoluble proteins were indicated by the squares.

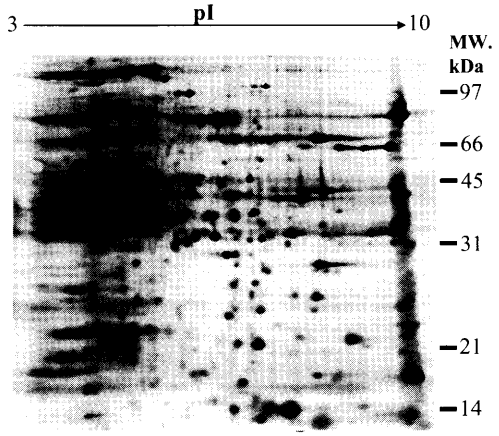

Figure 2 2-DE separation of *P. falciparum* proteins. One hundred fifty micrograms of *P. falciparum* proteins were loaded on the 2-D gels. The first dimension was a 3–10 linear pH gradient, and the second dimension was a 12.5% acrylamide gel. The proteins were extracted in a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM TBP, and 2% IPG buffer.

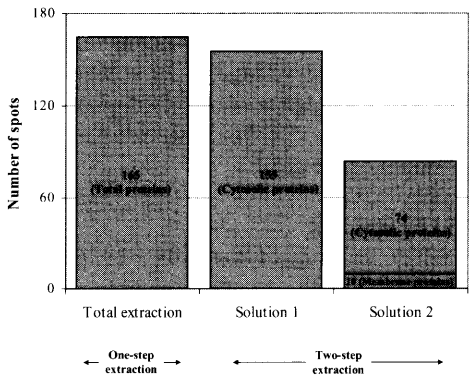

Figure 3 Comparison of the number of protein spots found in different fractions. The total extraction is denoted as a one-step protein extraction in a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM TBP, and 2% IPG buffer. The other is denoted as a sequential extraction where the proteins were extracted in solution 1 containing 40 mM Tris-buffer and solution 2 containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM TBP, and 2% IPG buffer.

i) some of the protein spots were stained more heavily, and ii) distribution of protein spots was easily located with high resolution (Figure 2).

3.2 Protein prediction and analysis

Table 1 summarizes the prediction of proteins in six regions according to their

molecular weight and pI. The actual values of M.W. and pI were measured and compared with those reported in the SWISS-PROT database. Our results demonstrated that the parasite proteins were mainly distributed as cytosolic proteins. The supportive evidence was that the numbers of spots from the cytosolic fraction were nearly equal to those derived from the one-step extraction. However, there were approximately 10 protein spots (e.g. 41-3 antigen, blood stage antigen 41-3 precursor, actin, and integral membrane protein, etc.) that are supposed to be membrane proteins because they were effect-tively extracted by thiourea and TBP, the chemical substances for membrane extraction. Furthermore, these proteins were found only in the membrane protein fractions while neither of them appeared in the total extraction containing both the cytosolic and membrane proteins. Therefore, it was worth noting that some membrane proteins, which were the low-abundant proteins, disappeared when the high abundant proteins were located at the same position. The 74 protein spots were assumed to be high abundant proteins since they still existed even after excess washing was applied.

4. Discussion

One of the major problems of protein purification from *P. falciparum* for proteomic analysis is due to the poor solubility of membrane proteins that can lead to significantly reduced intensity or loss of membrane proteins in two-dimensional gel electrophoresis (2-DE). Herein, a modified two-step extraction of *P. falciparum* proteins has successfully been applied to improve proteomic profiles derived from 2-DE. Detection of up to 239 protein spots is revealed with a high discrimination power compared to those obtained from one-step extraction (Figures 1 and 2). A plausible explanation can be drawn on the potentiation effect of Thiourea (membrane-solubilizing agent) upon removal of cytosolic protein. Reduction of protein complexity in the sample by the two-step extraction resulting in the high resolution power may also be taken into account. In addition, fortification of proteins from the insoluble fractions can significantly reveal more functional and structural proteomics (Table 1). Similar observations have also been reported on the analysis of plant proteins from barley and wheat seeds [10,11]. These plant proteins have simply been extracted with Tris-HCl buffer, aqueous alcohols and urea/NP-40/DTT lysis buffer and further analyzed by 2-DE. In a similar manner, Tris-base has been used to solubilize cytosolic protein from *Escherichia coli* [12]. Furthermore, this technique provides more advantages for protein identification through the PMF, which may not work well when several different proteins are present in the same spot as seen in the total extraction (Figure 2). The enhanced amount of protein loading in the IPG strip in each fraction will be helpful to increase the amount of protein suitable for MS identification.

It is worth noting that the excessive washing step prior to silver staining has experimentally been proven to be an important step for achieving a satisfactory result of 2-DE and also MALDI-TOF identification (Kamchon-wongpaisan S, Manuscript in preparation). Many chemicals in polyacrylamide gel including glycerol and urea have been reported to interfere with the staining reaction [15]. In addition, glycine used in the Tris-glycine electrophoresis buffer is known to form complexes with silver [16]. Soaking the gel in methanol or water for longer periods (at least 24 hours) along with excessive solution change is recommended to eliminate the interfering substances.

5. Conclusion

All our results strongly support the potential usage and applicability of using this two-step extraction for the proteomic analysis of insoluble proteins in particular membrane proteins of malarial parasites and other eukaryotic organisms. Since this technique is believed to be a crucial step in which i) the complexity due to the high dynamic range and diversity of expressed proteins in the sample can be reduced and ii) the enrichment of certain proteins e.g. low-copy number proteins can be obtained [17]. Furthermore, the high resolution of the gel motivates not only the identification of proteomic profiling of malarial parasites but also offers a better chance for vaccine and drug development in the future.

Table 1 A list of *P. falciparum* trophozoite proteins predicted by their molecular weight and isoelectric point

Accession No.	Location/protein name	Calculated pI	Calculated M.W.
	Region 1	5.38	45,120.00
		pI	**M.W.**
O97284	Hypothetical 50.2 kDa protein. *Plasmodium falciparum* (isolate 3d7)	5.3	50,151.34
Q9NHP4	41-3 antigen. *Plasmodium falciparum*	5.34	43,412.11
Q99019	Blood stage antigen 41-3 precursor. *Plasmodium falciparum*	5.34	43,426.14
P50650	Ribonucleoside-diphosphate reductase small chain (e.c. 1.17.4.1) (ribonucleotide reductase r2 subunit).	5.37	40,595.49
O97244	Hypothetical 47.1 kDa protein. *Plasmodium falciparum* (isolate 3d7)	5.42	47,119.37
		Calculated pI	**Calculated M.W.**
	Region 2	5.16	42,530.00
		pI	**M.W.**
Q25838	Circumsporozoite protein. *Plasmodium falciparum*	5.11	45,155.35
Q27325	Circumsporozoite protein. *Plasmodium falciparum*	5.18	46,687.86
Q27425	Circumsporozoite protein. *Plasmodium falciparum.*	5.18	45,592.77
P14883	Actin ii	5.21	42,678.85
P10988	Actin i	5.27	41,842.63
Q9U6U4	Actin i. *Plasmodium falciparum*	5.27	41,820.66
Q9NFU3	Gap protein. *Plasmodium falciparum*	5.28	39,176.39
		Calculated pI	**Calculated M.W.**
	Region 3	7.04	42,010.00
		pI	**M.W.**
O96235	Integral membrane protein. *Plasmodium falciparum*	6.82	48,273.54
Q9NFS5	Spermidine synthase (e.c. 2.5.1.16). *Plasmodium falciparum* (isolate 3d7)	6.98	36,596.47
Q9U5M1	Polyubiquitin. *Plasmodium falciparum* (isolate 3d7)	7.00	42,829.21
O96189	Syntaxin. *Plasmodium falciparum*	7.06	37,127.14
		Calculated pI	**Calculated M.W.**
	Region 4	4.89	25,430.00
		pI	**M.W.**
P19599	Merozoite surface antigen 2 precursor (msa-2) (merozoite 45 kDa surface antigen) (ag513).	4.85	22,942.90

Accession No.	Location/protein name	Calculated pI	Calculated M.W.
P50499	Merozoite surface antigen 2 precursor (msa-2).	4.85	23,000.91
Q9NI00	Translation elongation factor 1 beta. *Plasmodium falciparum*	4.94	32,026.79
Q99320	Merozoite surface antigen 2 precursor (msa-2) (allelic form 4).	4.99	23,224.99
		Calculated pI	**Calculated M.W.**
	Region 5	4.89	21,550.00
		pI	**M.W.**
Q9NLA8	Hypothetical 19.2 kDa protein. *Plasmodium falciparum* (isolate 3d7)	4.83	19,209.77
P19599	Merozoite surface antigen 2 precursor (msa-2) (merozoite 45 kDa surface antigen) (ag513).	4.85	22,942.90
P50499	Merozoite surface antigen 2 precursor (msa-2).	4.85	23,000.91
		Calculated pI	**Calculated M.W.**
	Region 6	6.57	14,560.00
		pI	**M.W.**
Q25997	Protein antigen. *Plasmodium falciparum* (isolate nf54)	6.58	16,852.29

6. Acknowledgements

The authors gratefully acknowledge Prof. Dr. Yongyuth Yuthavong and Dr. Sumalee Kamchonwongpaisan for helpful comments and advice. PP receives a scholarship from the National Science and Technology Development Agency.

7. References

[1] Ridley, R.G., Malaria: to Kill a Parasite, Nature, Vol.424, No.6951, pp.887-889, 2003.

[2] Florens, L., Washburn, M.P., Raine, J.D., Anthony, R.M., Grainger, M., Haynes, J.D., Moch, J.K., Muster, N., Sacci, J.B., Tabb, D.L., Witney, A.A., Wolters, D., Wu, Y., Gardner, M.J., Holder, A.A., Sinden, R.E., Yates, J.R. and Carucci, D.J., A Proteomic view of the *Plasmodium falciparum* life Cycle, Nature, Vol.419, No.6906, pp.520-526, 2002.

[3] Lasonder, E., Ishihama, Y., Andersen, J.S., Vermunt, A.M., Pain, A., Sauerwein, R.W., Eling, W.M., Hall, N., Waters, A.P., Stunnenberg, H.G. and Mann, M., Analysis of the *Plasmodium falciparum* Proteome by High-accuracy Mass Spectrometry, Nature, Vol.419, No.6906, pp.537-542, 2002.

[4] Barrett, J., Brophy, P.M. and Hamilton, J.V., Analysing Proteomic Data, Int. J. Parasitol., Vol.35, No.5, pp.543-553, 2005.

[5] Rabilloud, T., Blisnick, T., Heller, M., Luche, S., Aebersold, R., Lunardi, J. and Braun-Breton, C., Analysis of Membrane Proteins by Two-dimensional Electrophoresis: Comparison of the Proteins Extracted from Normal or *Plasmodium falciparum*-infected Erythrocyte Ghosts, Electrophoresis, Vol.20, No.18, pp.3603-3610, 1999.

[6] Cooper, R.A. and Carucci, D.J., Proteomic Approaches to Studying Drug Targets and Resistance in *Plasmodium*, Curr. Drug Targets Infect. Disord., Vol.4, No.1, pp.41-51, 2004.

[7] Gelhaus, C., Fritsch, J., Krause, E. and Leippe, M., Fractionation and Identification of Proteins by 2-DE and MS: Towards a Proteomic Analysis of *Plasmodium falciparum*, Proteomics, Vol.5, No.16, pp.4213-4222, 2005.

[8] Makanga, M., Bray, P.G., Horrocks, P. and Ward, S.A., Towards a Proteomic Definition of CoArtem Action in *Plasmodium Falciparum* Malaria,

Proteomics, Vol.5, No.7, pp.1849-1858, 2005.

[9] Dunn, M.J., Gel Electrophoresis: Proteins, Bios Scientific, Oxford, 1993.

[10] Weiss, W., Postel, W. and Gorg, A., Application of Sequential Extraction Procedures and Glycoprotein Blotting for the Characterization of the 2-D Polypeptide Patterns of Barley Seed Proteins, Electrophoresis, Vol.13, No.9-10, pp.770-773, 1992.

[11] Weiss, W., Vogelmeier, C. and Gorg, A., Electrophoretic Characterization of wheat Grain Allergens from Different Cultivars Involved in Bakers' Asthma, Electrophoresis, Vol.14, No.8, pp.805-816, 1993.

[12] Molloy, M.P., Herbert, B.R., Walsh, B.J., Tyler, M.I., Traini, M., Sanchez, J.C., Hochstrasser, D.F., Williams, K.L. and Gooley, A.A., Extraction of Membrane Proteins by Differential Solubilization for Separation using Two-dimensional Gel Electrophoresis, Electrophoresis, Vol.19, No.5, pp.837-844, 1998.

[13] Trager, W. and Jensen, J.B., Human Malaria Parasites in Continuous Culture, Science, Vol.193, No.4254, pp.673-675, 1976.

[14] Lambros, C. and Vanderberg, J.P., Synchronization of *Plasmodium falciparum* Erythrocytic Stages in Culture, J. Parasitol., Vol.65, No.3, pp.418-420, 1979.

[15] Wray, W., Boulikas, T., Wray, V.P. and Hancock, R., Silver Staining of Proteins in Polyacrylamide Gels, Anal. Biochem., Vol.118, No.1, pp.197-203, 1981.

[16] Ames, G.F. and Nikaido, K., Two-Dimensional Gel Electrophoresis of Membrane Proteins, Biochemistry, Vol.15, No.3, pp.616-623, 1976.

[17] Gorg, A., Weiss, W. and Dunn, M.J., Current Two-dimensional Electrophoresis Technology for Proteomics, Proteomics, Vol.4, No.12, pp.3665-3685, 2004.