

Optimal protein extraction methods from diverse sample types for protein profiling by using Two-Dimensional Electrophoresis (2DE)

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Abstract. There is a great diversity of protein samples types and origins, therefore the optimal procedure for each sample type must be determined empirically. In order to obtain a reproducible and complete sample presentation which view as many proteins as possible on the desired 2DE gel, it is critical to perform additional sample preparation steps to improve the quality of the final results, yet without selectively losing the proteins. To address this, we developed a general method that is suitable for diverse sample types based on phenol-chloroform extraction method (represented by TRI reagent). This method was found to yield good results when used to analyze human breast cancer cell line (MCF-7), *Vibrio cholerae*, *Cryptocaryon irritans* cyst and liver abscess fat tissue. These types represent cell line, bacteria, parasite cyst and pus respectively. For each type of samples, several attempts were made to methodically compare protein isolation methods using TRI-reagent Kit, EasyBlue Kit, PRO-PREP™ Protein Extraction Solution and lysis buffer. The most useful protocol allows the extraction and separation of a wide diversity of protein samples that is reproducible among repeated experiments. Our results demonstrated that the modified TRI-reagent Kit had the highest protein yield as well as the greatest number of total proteins spots count for all type of samples. Distinctive differences in spot patterns were also observed in the 2DE gel of different extraction methods used for each type of sample.

INTRODUCTION

2DE Gel-based proteomics analysis still remains the most effective way to resolve complex protein mixtures. In a 2DE system, proteins are separated by two distinct properties according to their net charge in the first dimension and molecular mass in the second dimension. One of the greatest strengths of 2DE is the ability to resolve proteins that have undergone some form of post-translational modification. For example, the phosphorylated and glycosylated form of a protein can be resolved from the non-phosphorylated and non-glycosylated form

by the 2DE method (Schulenberg *et al.*, 2003; Zhou *et al.*, 2007). In short, the 2DE method is able to detect different isoforms of proteins that arise from alternative mRNA splicing and proteolytic processing. Through this approach, real total protein expression would be qualitatively and quantitatively compared (Yan *et al.*, 1999).

In this study, four different kits or methods, including the phenol-chloroform extraction method represented by TRI-reagent and EasyBlue, PRO-PREP™ Protein Extraction Solution and lysis buffer were used and applied. Using a diverse collection of protein samples represented by the

human breast cancer cell line (MCF-7), liver abscess fat tissue/pus, *Vibrio cholerae*, and *Cryptocaryon irritans* cyst, each type of 2DE gel sample was compared in terms of their protein yield and spot pattern for each of the above test kits. Although many studies had reported on the technique used in the extraction of mammalian cell line (Wang *et al.*, 2004; Hardouin *et al.*, 2006), none of them have included the more complicated samples, such as pus and parasite cyst.

The presence of highly concentrated lipid, cholesterol, fibrin, extracellular fluid, mineral ions and actual necrotic tissues complicates proteomic analysis of liver pus samples extracted from liver abscess patients (Wells, 2009). Furthermore, *C. irritans*, a ciliated protozoan parasite which invades eyes, gills and skin of host fish and causes white spots disease in marine fish, (Brown, 1951) is always a challenge when it comes to protein extraction and separation. According to Herwig (1978), the parasite becomes encysted during the reproductive stage and resists chemical treatment (Matthews *et al.*, 1993; Kesintepe, 1995). Besides, *Vibrio cholerae*, a motile, gram negative bacterium causing cholera disease among humans is another interesting sample type to study. The bacteria possesses an outer membrane with strong polyanionic lipopolysaccharide and glycerophospholipids electrostatically linked by divalent cations that forms an effective barrier against antibiotics, detergents and chemicals (Nikaido & Vaara, 1985; Sukupolvi & Vaara, 1989; Vaara, 1992). Despite the recent advances in proteomic research, the analysis of *V. cholerae* and *C. irritans* cyst proteome still remains a considerable challenge due to their thick and chemical resistive wall.

To our knowledge, no study to date has addressed the proteome map for parasite cyst and pus samples due to difficulties in the protein extracting procedure. Although various methods are available for protein extraction, most of the methods require further optimization to extract proteins from samples. Therefore, our aim of this study is to develop and standardize a method to extract the total protein from different types

of samples effectively by generating highly-resolved 2DE protein profiles.

MATERIALS AND METHODS

Sample Preparation

Cell culturing

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Bovine Serum (Invitrogen) and maintained in a 5% CO₂ atmosphere at 37°C. Cells were allowed to grow until 80-90% confluent and were subsequently harvested by using trypsin digestion. Approximately 5x10⁵ of cells were then spin down in a 1.5mL microcentrifuge tube by centrifugation (4°C, 10 minutes, 220g) before subjected to each extraction methods.

Harvesting of *Cryptocaryon irritans* cyst

The *C. irritans* population was maintained using the sea bass, *Lates calcarifer*, as a host in a tank containing sea water kept at 27±1°C. Glass slides were placed on the bottom of the tank for 15 hours to collect adhering *C. irritans* cysts. The collected cysts were then cleaned with sterile sea water and transferred to a 1.5mL microcentrifuge tube and centrifuged at 2000g for 5 minutes at 4°C to remove the sea water. The cysts were stored at -80°C until further processing.

Harvesting of *Vibrio cholerae*

Vibrio cholerae strain el tor was obtained from Microbiology & Parasitology Department, School of Medical Science, Universiti Sains Malaysia. The bacteria were grown in Luria Bertani (LB) broth at 37°C in a shaking incubator for 24 hours. Preceding each protein extraction method, cells were harvested from 10mL of culture broth by centrifugation (4°C, 15 minutes, 1000g).

Pus Sample Collection

Pus samples from liver abscess patients were collected from the Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. Approximately 1-3mL of liver pus was aseptically aspirated from the liver abscess under ultrasound guidance from each patient.

This study was conducted in accordance with the requirements of USM Human Ethics Committee.

Protein Extraction

TRI-reagent Kit

Protein extraction using TRI-Reagent kit (Molecular Research Center, Cincinnati, US) was done as recommended by the supplier. Briefly, 1mL of TRI-reagent and chloroform was added sequentially per 5×10^5 of cells / 0.05g of pus and centrifuged at 12 000g at 4°C for 15 minutes to separate into DNA, RNA and protein layers. Instead of following the manufacturer's protocol, we modified the protein precipitation method where the protein was precipitated by pre-chilled acetone at ice for 15 minutes.

EasyBlue Kit

Protein Extraction using EasyBLue Kit (Talron Biotech) was done as recommended by the supplier. Briefly, 1mL of EasyBlue reagent and chloroform was added sequentially per 5×10^5 of cells/ 0.05g of pus and centrifuged at 12 000g at 4°C for 15 minutes to separate into DNA, RNA and protein layers. Isopropanol was then added to precipitate the protein.

PRO-PREP™ Protein Extraction Solution

Protein Extraction using PRO-PREP™ Protein Extraction Solution (iNtRON BIOTECHNOLOGY) was performed as recommended by the supplier. Briefly, 5×10^5 of cells or 0.05g of pus tissue was suspended in 40µl or 1mL of PRO-PREP™ solution respectively and incubated in -20°C for 30 minutes. The resulting cell lysate was then centrifuged at 14 000g for 5 minutes at 4°C. The supernatant was collected and subjected to downstream processing.

Lysis Buffer

Harvested cells, bacteria, pus or cysts were disrupted with a cocktail of 8M urea (Bio-Rad), 2M thiourea (Bio-Rad), 4% (w/v) CHAPS (GE HealthCare), 20mM DTT, 2% Pharmalyte (GE healthcare) and protease inhibitor cocktail (GE HealthCare) and incubated for 30 minutes in 4°C. The resulting cell lysate was then centrifuged at 14 000g for 15

minutes at 4°C. The supernatant was collected and subjected to downstream processing.

Two-dimensional Electrophoresis

The extracted proteins was rehydrated in 150µl of rehydration buffer (8M urea, 2M thiourea, 4% CHAPS, 0.5% pharmalyte (GE Healthcare), 20mM DTT) overnight in 13cm precast immobilized dry strips pH 4-7 (GE Healthcare Bio-Sciences, Uppsala, Sweden). The strips were then subjected to isoelectric focusing (IEF) as previously described (Chen *et al.*, 2008). After the IEF, the strips were equilibrated and subjected to second dimensional separation at 16°C using the 8-18% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). All samples were analyzed in duplicate.

Silver staining

The 2D-PAGE gels were visualized by silver staining as described by Heukeshoven & Dernick (1985).

Image analysis

The LabScan image scanner (Version 5) was used to capture and store images of 2DE gels. The ImageMaster™ 2D Platinum Software (Version 5) was used to evaluate the protein profiles and present information obtained from the 2DE gels. Average number of proteins spot (n) including the unresolved peptides in each sample was tabulated.

RESULTS

Analysis of 2DE protein profiles

When these four types of samples were subjected to 2DE and silver staining under the resolving conditions adopted in the present study, most of the high abundance proteins were detected. When the 2DE experiments were performed on human breast cancer cell line (MCF-7) using four different kits or methods, comparable protein profiles or patterns were obtained for most of the resolved proteins (Figure 1). However, profiles of cell line treated with TRI-Reagent kit had shown distinctive protein spots with highest amount of protein spots (n=175)

shown as compared to other kits or methods used (Figure 1, Panel A). Panels B, C and D of Figure 1 demonstrate typical 2DE protein profiles of cell lines treated with EasyBlue Kit, PRO-PREP™ and lysis buffer respectively.

The average number of protein spots appeared fewer in the lysis buffer method (n=155), EasyBlue Kit (n=125) and finally PRO-PREP™ (n=71) (Table 1). Although a comparable protein content was obtained by using the lysis buffer method, the buffer was

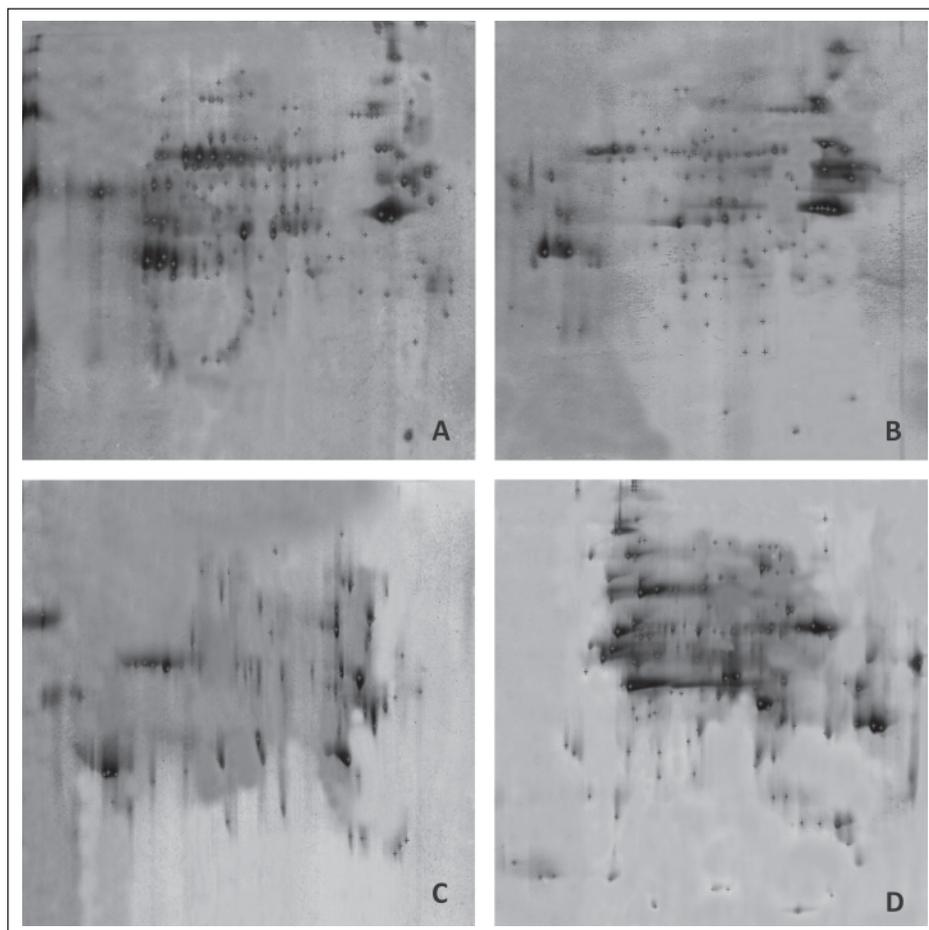


Figure 1. Representative 2DE protein profiles of the MCF-7 lysate extracted using (A) TRI-reagent, (B) EasyBlue reagent, (C) ProPrep™ solution and (D) lysis buffer. MCF-7 lysate extracted samples were treated with four different methods before subjected to 2DE and silver stained. Protein spots were compared and analysed using ImageMaster™ 2D Platinum Software Version 5

Table 1. Average number of protein spots visualized on 2DE protein profiles of MCF-7 cell line, *Vibrio cholerae*, *Cryptocaryon irritans* cyst and pus, which samples extracted using four different kits or methods. All samples were analyzed in duplicate

Type of samples	Average number of protein spots visualized on the 2DE protein profiles, (n)			
	TRI-reagent	EasyBlue reagent	ProPrep solution	Lysis Buffer
MCF-7 cell line	175	125	71	155
<i>Vibrio cholerae</i>	595	ns	ns	ns
<i>Cryptocaryon irritans</i> cyst	435	ns	ns	ns
Pus	306	ns	ns	ns

n.s. the protein spots was not detected accurately due to the heavy streaking on the 2DE gel

unable to eliminate the contaminated substance effectively thus causing slight streaking on the gel (Figure 1, Panel D). Of these four methods tested, ProPrep solution gave the lowest protein content extracted with major lines of streaking (Figure 1, Panel C). Even though the EasyBlue reagent contains the same key ingredients (phenol and guanidine thiocyanate) as TRI-reagent, less protein was recovered and minor streaking observed in samples treated with TRI-reagent (Figure 1, Panel B).

Similar analysis was performed on *C. irritans* cyst, *V. cholerae* and the liver abscess fat tissue/pus using four different extraction kits or methods. The results also revealed the highest expression of protein spots in those samples treated using TRI-Reagent kit. The average number of protein spots visualised and detected in *C. irritans* cyst (Figure 2), *V. cholerae* (Figure 3) and the liver abscess fat tissue/pus (Figure 4) was high at n= 435, 595 and 306, respectively. Again, of all kits or methods used, only those samples treated with TRI-reagent kit achieved the best separation and produced highly resolved gel. This had been shown in

Figure 1, 2, 3 and 4, where minimum streaking with reproducible results only observed in those samples treated with TRI-reagent kit. The other kits and methods were unable to produce usable images due to heavy streaking or low number of proteins spots counted (data not shown).

DISCUSSION

The key to obtain adequate results is in the way samples are treated. Ideally, the pattern of the 2DE gel should reflect the protein composition without any losses and modifications. The samples must not be contaminated with proteins and peptides not belonging to the sample. Furthermore, too much salt in the sample disturbs IEF and leads to streaking patterns. For example, amphoteric compounds in samples will buffer the gradient excessively in the areas of their pIs, which results in vertical narrow areas without protein spots. Therefore, the chemicals used must be of low salt content and of high purity. Finally, the time taken for the treatment must be kept to a minimum to

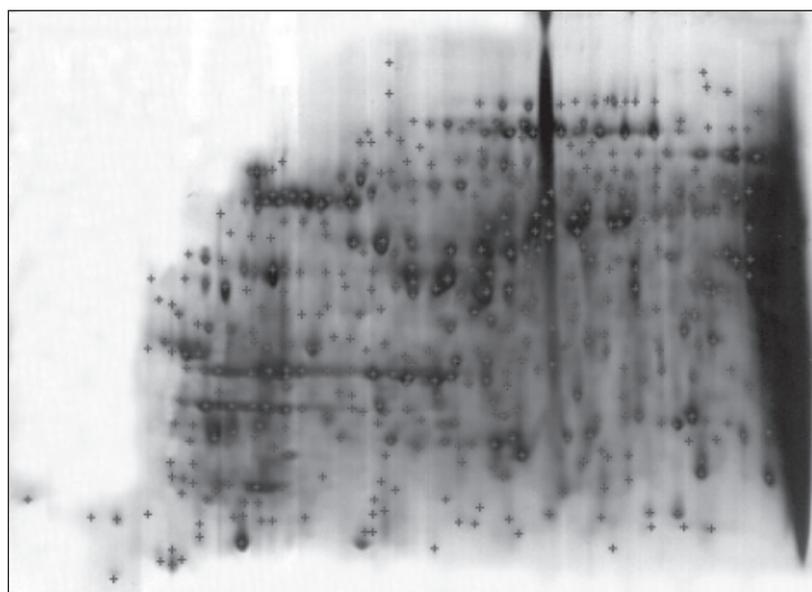


Figure 2. Representative 2DE protein profiles of the *C. irritans* cyst extracted using TRI-reagent. TRI-reagent treated *C. irritans* cyst samples were subjected to 2DE and silver stained. Protein spots were compared and analysed using ImageMaster™ 2D Platinum Software Version 5

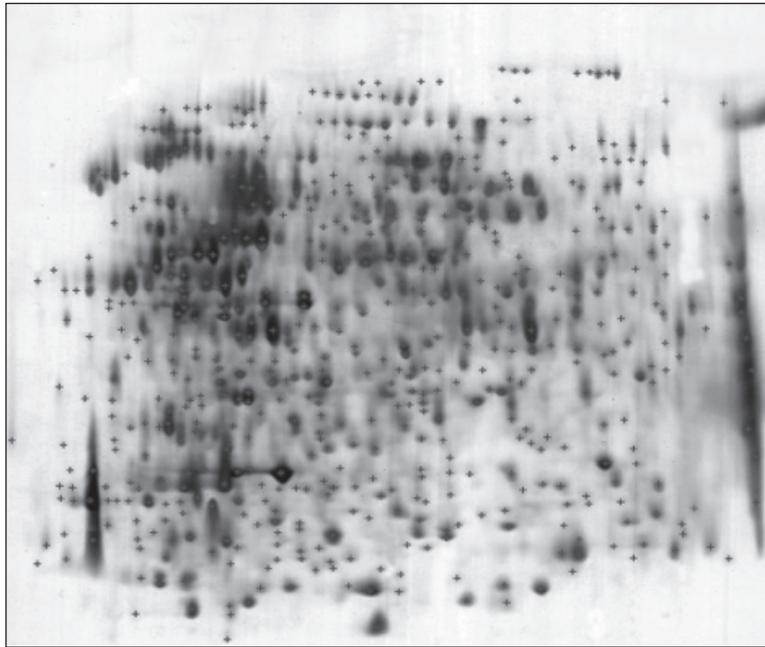


Figure 3. Representative 2DE protein profiles of the *V. cholerae* extracted using TRI-reagent. TRI-reagent treated *V. cholerae* samples were subjected to 2DE and silver stained. Protein spots were compared and analysed using ImageMaster™ 2D Platinum Software Version 5



Figure 4. Representative 2DE protein profiles of the pus from liver abscess extracted using TRI-reagent. TRI-reagent treated pus samples were subjected to 2DE and silver stained. Protein spots were compared and analysed using ImageMaster™ 2D Platinum Software Version 5

reduce the possibility of protein losses and modification.

We attempted to standardize protein preparation method for good reproducible 2DE protein profiles from diverse sample types, which was represented by human breast cancer cell line (MCF-7), *V. cholerae*, *C. irritans* cyst and liver abscess fat tissue in this study. We found that the most effective protein extraction methods for the 2DE analysis would be the phenol-chloroform extraction method by TRI-reagent kit. The kit allowed the visualization of maximum proteins spots while at the same time, produced highly resolved gels.

By using 2DE and image analysis, any methods or kits used generally demonstrated good expression of proteins for MCF7, mammalian cell lines. The sample preparation of lysates from cultured cells is relatively easy to prepare and stable. Unlike other sample types, cell lines do not have a protective mechanical barrier, thus the plasma membrane could be easily broken by any treatment to release the protein content. Among the four extraction methods tested in this study, TRI-reagent gave the best results with most protein spots visualized, minimum streaking and a highly resolved image. Although experiments performed using lysis buffer and EasyBlue extraction had shown almost comparable results as TRI-reagent, the protein expression had markedly less spots and more streaking lines. ProPrep kit was tested but found unsuitable for extracting protein from MCF7.

By evaluating the protein profiles of *V. cholerae* strain El Tor, it was found that the samples treated with TRI reagent showed visualization of most protein spots using silver staining. Also, in comparison to a previous proteome map of *V. cholerae* strain El Tor reported by Coelho *et al.* (2004), TRI-reagent kit was able to recover 56 more spots than reported. It was found that TRI-reagent is the most effective way to extract proteins from this bacteria cell without involving mechanical disruption (eg. Ultrasonication) to break the cell wall. Other kits or methods presented significant challenges in producing a presentable gel for *V. cholerae* sample preparation; hence, they are not

suitable for protein extraction without mechanical disruption.

Our group is the first to report the 2DE protein profile of parasite cyst of *C. irritans*. The TRI-reagent kit was able to extract protein effectively despite the tough cystic wall of the parasite. On the other hand, much streaking was observed for those samples treated with lysis buffer, EasyBlue and ProPrep kit. These kits created a major interference during the IEF. It was deduced that due to its chemical properties, the TRI-reagent not only lyses most of the cyst wall, furthermore it effectively removes salts and ions that can interfere with IEF in order to produce a good 2DE gel.

This study is also the first to isolate proteins from complicated and challenging pus samples using TRI reagent kit before subjecting to 2DE separation. In general, proteins extracted by most extraction methods affected the IEF and subsequently produced heavy streaking lines on the gel due to the interference of substances such as salt, lipid and ions in the pus. However, TRI reagent treated samples still rendered the best among the methods compared although minor streaking was observed. In short, with regards to 2DE profiling studies, TRI-reagent is still the best for extraction of protein from thick and chemically resistant cell wall samples coupled with the presence of interfering compounds such as DNA, RNA, carbohydrate, proteolytic enzymes and oxidative enzymes.

TRI-reagent mainly contains phenol, chloroform and a chaotropic denaturing solution (guanidine thiocyanate). Guanidine thiocyanate and phenol are strong denaturalization and lysis reagents that can simultaneously separate RNA, DNA and proteins from biological samples through centrifugation (Wu, 1995; Bracete *et al.*, 1999). The protein samples separate into the upper aqueous phase (RNA) and a lower organic phase (DNA & protein) (Chomczynski & Sacchi, 1987). The DNA of lower organic phase is further precipitated using ethanol, whilst precipitation of protein occurs in acetone. Chloroform together with acetone will facilitate the dissolution of lipid (Merrill & Fleisher, 1932). For this study, the

TRI reagent protein precipitation method was modified by using ice cold acetone instead of acetone kept in room temperature. This is in agreement to the study conducted by Askonas (1951) and Merrill & Fleisher (1932) that the precipitation of protein achieved the best result by minimizing the degradation of protein at low temperature (0-5°C) alone. In short, TRI-reagent has the ability to separate out RNA and DNA which effectively minimized the contaminant present and reduce the streaking on gel. Besides, guanidine thiocyanate denatures protease by preventing further protein degradation and subsequently enhances the protein recovery of samples. All these factors make TRI reagent an excellent kit to extract protein specifically when used with cold acetone.

Although EasyBlue also contains guanidine thiocyanate and phenol, the amount of protein extracted was still lesser than that treated with TRI-reagent. It demonstrated more streaking and interference. We attribute this to the isopropanol protein precipitation used in EasyBlue protocol. Comparable results with TRI-reagent treatment was obtained when the protocol was modified by replacing isopropanol precipitation solution with acetone. By acetone precipitation, we agreed that the protein concentration could be retrieved with an increase of 10-20% (Wu, 1995) and with reduced streaking on gel (result not shown).

ProPrep protein extraction solution is the fastest performing kit which requires less than 30 minutes to complete the extraction and contains 5 different kinds of protease inhibitors. However, unsatisfying results were obtained when compared to TRI-reagent, which lacks protease inhibitors. Due to the short incubation time of the samples using the ProPrep reagent, incomplete lysis might have caused production of low protein spots. The failure to remove interfering agents from the samples also contributed to major streaking lines in the gel.

Conventional lysis buffer was used as a control in this study. This buffer extracted proteins from MCF7 cell lines rather

efficiently. However, the streaking on those 2DE gels indicated that lysis buffer fails to eliminate the interfering substance effectively. A further step involving sample clean-up is required to eliminate the streaking lines. Another setback of the lysis buffer is its inefficiency to extract protein from challenging samples such as cyst and bacteria. Mechanical disruptions may be required to facilitate the extraction of protein from these samples.

Though TRI-reagent effectively extracted most of the protein in a sample, the running process is tedious and requires approximately 2-3 hours to complete. Furthermore, materials used in the kits (i.e. phenol and chloroform) are hazardous and a fume hood facility is required. Nonetheless, it is still the best method to extract protein from various challenging and difficult samples. Its compatibility with MS-MS (Xanthopoulou *et al.*, 2010) makes it a most ideal protein extraction method.

Appropriate sample preparation is crucial for the outcome of 2D electrophoresis (Fountoulakis, 2001). The choice of method used for sample preparation is the most critical step in any proteomics strategy as each step influences protein yield, biological activity and the structural integrity of the target protein. Among the four different methods tested, TRI-reagent delivered the highest protein recovery and resolution for 2DE proteome map as compared to others. Through the analysis of these 2DE protein profiles of diverse sample types involving human breast cancer cell line (MCF-7), *V. cholerae*, *C. irritans* cyst and liver abscess fat tissue which represented most cell lines, bacteria, cyst and pus, TRI reagent was found to be an ideal method to extract proteins from these challenging samples. Furthermore, it required little optimization for each sample type and is suitable for small laboratories with limited resources. It should be noted that we were only able to optimize the protein extraction method for the above samples. Further studies should be done on other challenging samples to confirm the effectiveness of TRI reagent.

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